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ORIGINAL RESEARCH

Ozone Administration Reduces Myocardial Ischemia Reperfusion Injury in Streptozotocin Induced Diabetes Mellitus Rat Model

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Objective: This study aimed to demonstrate whether ozone has cardioprotective effects on the myocardial ischemia-reperfusion injury (IRI) in rats with streptozotocin(STZ)-induced diabetes.

Methods: A total of 38 male Wistar Albino rats were divided into five groups as follows: control group (group C,*n*=6), diabetic group (group D,*n*=6), diabetic ozone group (group DO,n=6), diabetic-ischemia/reperfusion (group DIR,*n*=6), diabetic-ischemia/reperfusion-ozone (group DIRO,*n*=6). Six rats died during this period and two died because of surgical complications. A myocardial ischemia-reperfusion model was created using a thoracotomy incision from 4th intercostal space. The LAD was ligated using an 8–0 prolene suture for 30min. Ozone was administered intraperitoneally(1mg/kg) 5min before reperfusion. The reperfusion time was 120 min. At the end of the reperfusion procedure, myocardial tissue histopathological examinations, and serum biochemical analyses were performed.

Results: The percentage of TUNEL(+) cardiomyocytes/HPF was significantly higher in the DIR group than in the C, D, and DO groups. Conversely, TUNEL positivity was significantly lower in the DIRO group than in the DIR group. The IRI score was significantly higher in the DIR and DIRO groups than that in the C, D, and DO groups. In contrast, the IRI damage score in the DIRO group was significantly lower than that in the DIR group. Serum MDA levels were significantly higher in the DIR group than in the C, D, and DO groups. Similarly, MDA levels were significantly higher in the DIRO group than in the C and D groups. CAT activity was significantly higher in the DIR group than in the C and D groups. SOD activity was significantly higher in the DIR group than in the C and DO groups.

Conclusion: Our study showed that ozone exerts cardioprotective effects in STZ-induced diabetic rats through its antioxidant role against oxidative stress. Both biochemical and histological analyses clearly revealed that ozone has beneficial effects against IRI in the diabetic rat myocardium.

Keywords: diabetes mellitus, ozone, myocard, SOD, MDA, ischemia-reperfusion

Introduction

Cardiovascular disease (CVD) is the most common cause of death worldwide.[1] Coronary heart disease (CHD) is the leading cause of this statistic in terms of both singularity and CVD.[1] Myocardial infarction (MI) occurs almost every 40 min in America.[1] In contrast, revascularization techniques for CHD have gradually developed into both interventional and surgical techniques. This situation makes ischemia and reperfusion a major topic in the modern world.

Ischemia and reperfusion (IR) indicate that sudden re-oxygenation of the organ interrupts blood supply.[2] Re-oxygenation, because of the sudden restoration of blood flow, often aggravates tissue injury and may cause a new inflammatory response.[2]

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Myocardial ischemic injury is usually caused by a severe reduction in blood supply.[3] After reperfusion, several complex mechanisms, including oxygen radicals, inflammation, and mitochondrial pathways, are triggered.[3] In this regard, many new drugs and chemicals have been investigated to reduce ischemia and reperfusion injury.

Diabetes mellitus is another important point in CVD. Approximately 40% of the population has diabetes, including prediabetes.[1] Diabetes mellitus (DM) is a major risk factor for CVD.[1] Even if it seems to decrease, patients with DM have higher CVD mortality rates than those without DM.[4] Furthermore, CVD is the leading cause of death in patients with DM, especially in those with DM for more than 10 years.[5]

Ozone was discovered as a disinfectant in 1834.[6] During World War 1, ozone was used to treat gangrene.[6] It has been reported to exert cardioprotective effects.[7] Afterwards, experimental animal studies supported this idea.[8,9] First, it is believed that the mechanism of action of ozone occurs through oxygen radicals.[7] Subsequently, the new mechanisms of action are discussed. Endothelial nitric oxide synthase (eNOS) activity is one such type of activity.[10] Finally, new pathways for the mechanism of ozone.[11,12] To date, there has been a clear scientific page on ozone studies.

This study aims to discuss ozone's positive effects on myocardial IRI, if exist. Also, as a genuine approach, we aim to observe ozone effects on the diabetic rat heart model. Because, to the best of our knowledge, diabetes is the one of the most important contributors of the coronary artery disease. So, this experimental model has provided realistic patient population excellently who benefits from coronary artery by-pass graft (CABG). As cardiac surgeons, we always face with this patient population. To best of our knowledge is CABG is superior to percutaneous intervention in diabetic population.[13] Thus, we are trying to reduce myocardial IRI in diabetic coronary artery disease. Because of this, we choose ozone for potential benefits against myocardial IR. We tested ozone in diabetic coronary artery IRI those as potential candidates for CABG.

Material and Methods

This experimental study was conducted at the Gazi University Laboratory Animal Breeding and Experimental Research Center (GÜDAM) in accordance with the ARRIVE guidelines. The study protocol was approved by the Gazi University Animal Experiments Local Ethics Committee (approval number G.Ü.ET-23.065 dated June 21, 2023), 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.

Induction of Diabetes

To induce diabetes, the rats received a single intraperitoneal (IP) injection of 65 mg/kg of streptozotocin (STZ dissolved in citrate buffer (pH 4.4)) (Sigma Chemical, St. Louis, MO, USA). The rats whose fasting blood glucose (FBG) (Standard GlucoNavii GGh, Korea) exceeded 200 mg/dL by measuring blood glucose levels 72 h after STZ administration were considered diabetic, and animals with FBGs >200 mg/dL were assigned to the diabetic groups (group D, DO, DIR, DIRO). The rats were kept alive for four weeks after STZ injection, resulting in the development of chronic diabetes. Insulin (1–3 U/day) was given to prevent weight loss and ketoacidosis over the next four weeks.

Surgical Procedure

Anesthesia was induced by an intramuscular injection of ketamine hydrochloride (50 mg/kg; Ketalar® 1 mL = 50 mg; Pfizer, Istanbul, Turkey). The procedure was performed with the rats in a supine position under a heat lamp. After placing the rats in the supine position and shaving the surgical areas, a vertical incision of approximately 1 cm was made at the midline of the neck. Blunt dissection was performed to reach the trachea and tracheostomy was performed using a 16 G intra-catheter (Medipro Nova Cath®, Istanbul, Turkey). The rats were connected to a mechanical ventilator (Harvard Apparatus Rodent Model Ventilator, Inspiraasv, Hollistone, USA) and provided ventilation support with 100% oxygen, a tidal volume of 10–15 mL/kg, and a respiratory rate of 65–80/min throughout the procedure. A thoracotomy was performed through an incision in the left 4th intercostal space. The pericardial sac was dissected, and ischemia was induced in the LAD area using 8/0 Prolene.

Experimental Model

In this study, 38 adult Wistar-Albino rats, with an average weight of 250 g, were used. Animals were randomly divided into five groups. The Control Group in the study included six subjects (C, n=6), where only the myocardium, blood, and other tissues were collected without the application of STZ and myocardial ischemia-reperfusion. Total 32 rats received 65 mg/kg STZ, and a week after administration, diabetes was confirmed by a blood glucose test. They were fed ad libitum for four weeks to investigate the macrovascular complications of diabetes. Then, 32 rats were randomly divided into four groups; however, six died during the long wait and two died during the surgical procedure. The myocardium and blood were for immunohistochemical and biochemical analysis.

In the absence of IRI, thoracotomy was performed after tracheostomy, and the rats were sacrificed under intramuscular administration of ketamine (50 mg/kg) anesthesia after a 2.5-hour wait. In the Diabetic Ozone group (DO, n=6), rats underwent thoracotomy after tracheostomy without ischemia, followed by intraperitoneal administration of ozone at a dose of 1 mg/kg at the 25th min. A medical ozone generator was used; the mixed gas concentration (oxygen/ ozone) was controlled with an ultraviolet spectrophotometer at 254 nm. To ensure the retention of O3, disposable silicone-treated polypropylene syringes (ozone resistant) were used in the experiment. After a 2-hour and 5-minute wait, the rats were sacrificed under intramuscular administration of ketamine (50 mg/kg) anesthesia. In the diabetic ischemia reperfusion group (DIR, n=6), the rats underwent thoracotomy after tracheostomy, followed by 30 min of left anterior descending (LAD) ischemia. After reperfusion ended at the 120th minute, the rats were sacrificed intramuscular administration of ketamine (50 mg/kg) under anesthesia. In the diabetic ischemia-reperfusion ozone group (DIRO, n=6), rats underwent thoracotomy after tracheostomy, followed by LAD ischemia. Ozone was then administered at a dose of 1 mg/kg intraperitoneally at the 25th minute. After a 5-minute wait, reperfusion was initiated. Rats were sacrificed under intramuscular administration of ketamine (50 mg/kg) anesthesia after a 120-minute reperfusion period (Figures 1 and 2).

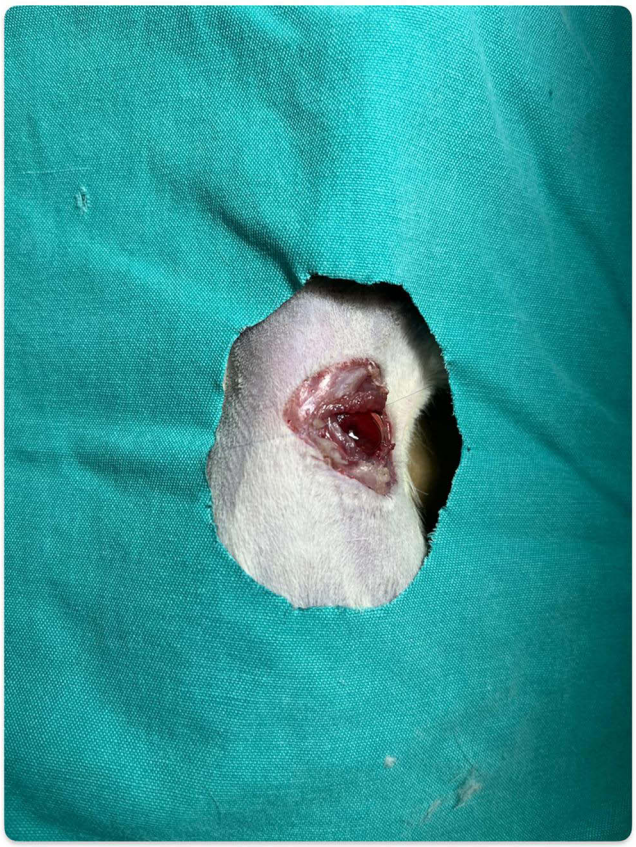

Figure 1 Color change in the myocardium of a rat subjected to myocardial ischemia-reperfusion.

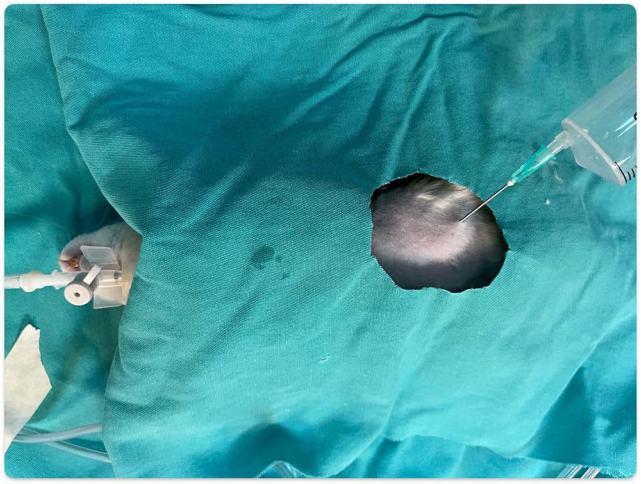

Figure 2 Photograph demonstrating the intraperitoneal administration of ozone.

Histopathological Analysis

After sacrification, the rat heart tissue samples were immersed in 10% buffered formalin for fixation. Afterward, the tissue samples were dehydrated using an increasing grade of ethyl alcohol series as 60%, 70%, 80%, 90%, 96%, and 100%. Subsequently, dehydrated tissue samples were cleared in xylene, infiltrated with liquid paraffin at 59°C in an oven, and embedded in paraffin. Transverse heart sections of 5 µm-thickness in the apex-base direction were cut from paraffin tissue blocks using a microtome (HistoCore MULTICUT, Leica, Germany). Following deparaffinization and hydration, sections were either stained with hematoxylin and eosin (H&E) for histopathological evaluation or used for the TUNEL (terminal deoxynucleotidyl transferase dUTP Nick End Labeling, in situ DNA End Labeling) assay to assess apoptosis in cardiomyocytes.

H&E-stained heart sections were examined under a computer-assisted light microscope (Leica DM 4000 B, Germany) at 200× and 400× magnifications, and images of the sections were captured using Leica LAS V4.12 software. In H&E-stained heart tissue samples, ischemia-reperfusion damage was evaluated using a semi-quantitative scoring system, considering interstitial edema, myocardial cell edema and necrosis, neutrophil infiltration, and hemorrhage. Sections were scored as 0 (no damage) when no damage was observed, 1 (mild damage) when interstitial edema and focal cardiomyocyte edema or necrosis were observed, 2 (moderate damage) when cardiomyocyte necrosis with remarkable neutrophil infiltration was observed, and 3 (severe damage) when cardiomyocyte necrosis and hemorrhage were present with widespread neutrophil infiltration.[14,15] The mean damage scores at the end of the evaluation were compared between the groups.

TUNEL Assay of Heart Sections

Apoptosis in cardiomyocytes was assessed using the TUNEL assay (In Situ Cell Death Detection Kit, POD; Sigma, 11684817910) in heart paraffin sections. The assay was performed according to the manufacturer's instructions. Briefly, 5µm-thick heart sections were incubated with TUNEL reaction mixture at 37°C for 60 min. The sections were then washed with PBS and incubated with Converter-POD solution at 37°C for 30 min. The sections were then rinsed in PBS and treated with DAB (3,3'-diaminobenzidine) substrate at room temperature for 3 min. The sections were rinsed with distilled water, dehydrated through an increasing grade alcohol series, and immersed in xylene before being mounted with entellan. The prepared samples were observed under a light microscope (Leica DM 4000 B, Germany), and images were captured using the software LAS V4.12 (Leica, Germany). To evaluate the apoptotic cardiomyocytes, six non-overlapping fields were captured from each sample at 400 × magnification. The number of TUNEL-positive apoptotic cardiomyocytes and the total number of cardiomyocytes in each field were determined

using the ImageJ software (1.53a; National Institutes of Health).[16] The percentage of TUNEL-positive cells in each high-power field (HPF) was calculated by dividing the number of TUNEL-positive cells by the total number of cells and expressed as "TUNEL (+) % cells per HPF".[17] The average values were compared between the groups.

Biochemical Analysis

At the end of the study, serum and tissue samples were frozen in liquid nitrogen and stored at −80°C. At the end of the study, the levels of catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), and Paraoxonase-1 (PON1) were measured in serum and tissue samples. Total Oxidative Status (TOS) and Total Antioxidant Status (TAS) were measured and the Oxidative Stress Index (OSI) was assessed.

Statistical Analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) 22. The normal distribution of variables was examined visually (histograms and probability plots) and analytically (Kolmogorov–Smirnov and Shapiro–Wilk tests). The results are presented as mean ± standard error (SE). Data were evaluated using Kruskal–Wallis variance analysis. Significant variables were assessed using the Bonferroni-corrected Mann–Whitney *U*-test. A total Type I error level of 5% was used for statistical significance, and statistical significance was set at $p<0.05$.

Results

Histopathological Assessment

Cardiomyocytes exhibiting a normal microscopic appearance were present in the heart sections of rats in group C. However, focal myocardial tissue regions with enlarged interstitial spaces between the cardiomyocytes with cytoplasmic hypereosinophilia and condensed nuclei were observed among the groups of normal cardiomyocytes in group D. Tissue foci consisting of cardiomyocytes with hypereosinophilia and condensed nuclei, and also involving expanded intercellular space, were more widespread in the DO group than in Group D (Figure 3). Unlike the heart sections of the C, D, and DO groups, in which an ischemia reperfusion model was not carried out, hemorrhage and neutrophil infiltration, besides the notable necrosis in the myocardial cells in the affected regions, were prominent in the DIR group. However, hemorrhage, neutrophil infiltration, and myocardial damage were milder in the DIRO group than in the DIR group (Figure 3). When statistically compared, there was a significant difference in the ischemia-reperfusion damage score between the groups ($p=0.001$). The ischemia-reperfusion damage score was significantly higher in the DIR group than that in the C, D, and DO groups (all $p<0.001$). Similarly, the ischemia-reperfusion damage score in the DIRO group was significantly higher than those in the C, D, and DO groups ($p<0.001$, $p=0.002$, and $p=0.008$, respectively). In contrast, the ischemia-reperfusion damage score in the DIRO group was significantly lower than that in the DIR group ($p=0.024$) (Table 1).

Evaluation of the Apoptosis

Statistically significant differences were observed between the groups in the apoptotic, TUNEL (+) cardiomyocyte percentages in each high-power field (HPF, 400× magnification) ($p < 0.001$). The percentage of TUNEL (+) cardiomyocytes/HPF was significantly higher in the DIR group than in the C, D, and DO groups ($P < 0.001$, $P = 0.001$, and $P < 0.001$, respectively). Conversely, TUNEL positivity was significantly lower in the DIRO group than that in the DIR group ($p = 0.005$) (Table 1, Figure 4).

Biochemical Findings

When comparing the groups based on serum MDA levels, a significant difference was observed ($p = 0.005$). MDA levels were significantly higher in the DIR group than in the C, D, and DO groups ($P = 0.011$, 0.006, and 0.045, respectively). Similarly, MDA levels were significantly higher in the DIRO group than in the C and D groups ($p = 0.025$ and 0.015, respectively). A significant difference was observed between the groups based on serum

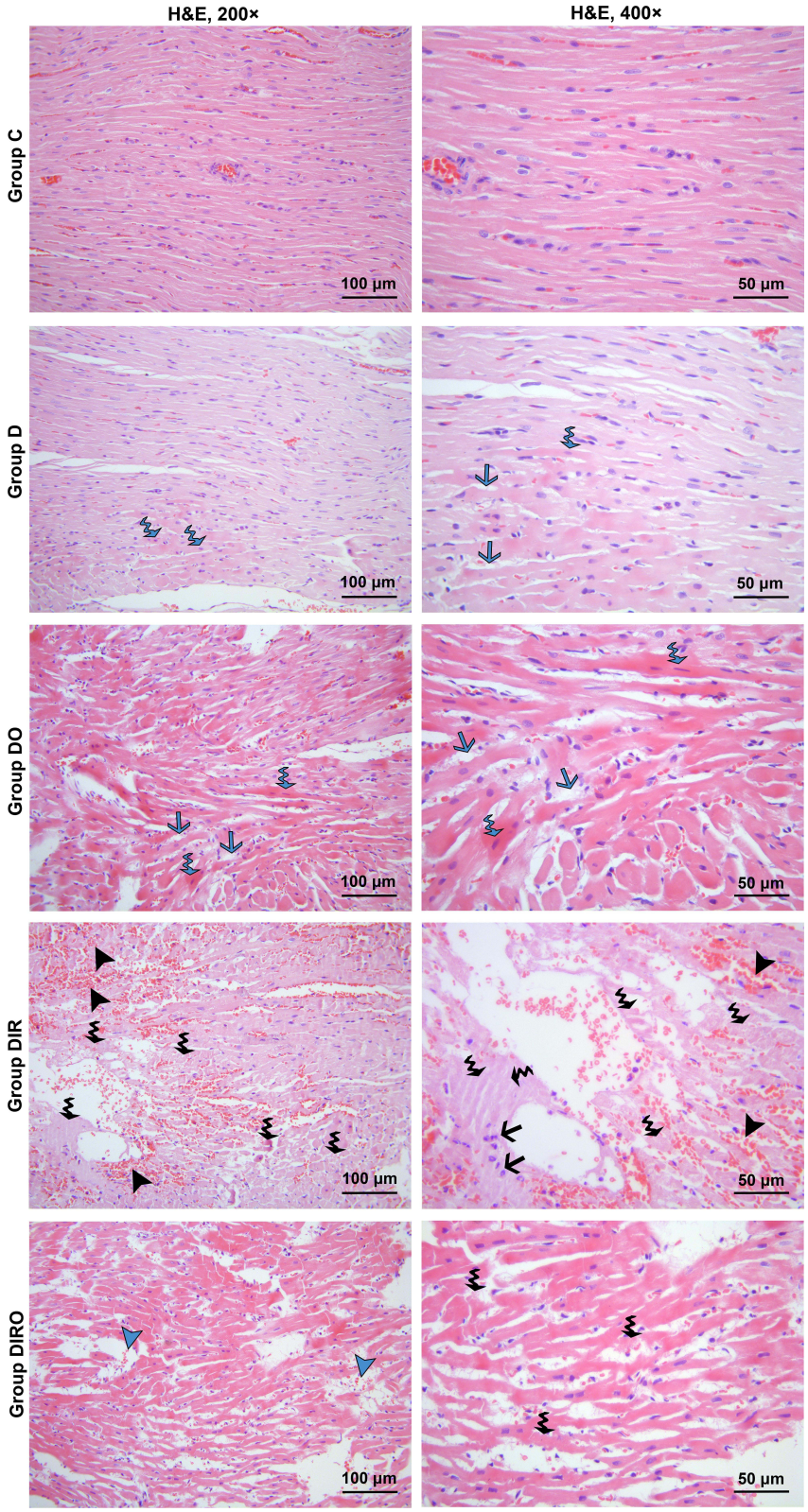

Figure 3 Micrographs representing the H&E stained heart sections. Blue wavy arrows indicate cardiomyocytes with nuclear condensation and increased cytoplasmic eosinophilia; blue arrows represent interstitial edema; black arrowheads indicate hemorrhage; black wavy arrows show areas of myocardial cell necrosis; black arrows denote neutrophil infiltration; blue arrowheads depict marked interstitial edema accompanied by erythrocyte extravasation. Group C, control group; Group D, diabetic group; Group DO, diabetic group treated with ozone; Group DIR, diabetic group subjected to ischemia reperfusion; Group DIRO, diabetic group subjected to ischemia reperfusion and ozone application. H&E, hematoxylin and eosin; 200× and 400× magnifications. Scale bars, 100 µm and 50 µm.

Table 1 Myocardial Ischemia-Reperfusion Damage Scores, and the Percentage of TUNEL (+) Cardiomyocytes in the HPF [Mean ± SE]

	Group C (n=6)	Group D (n=6)	Group DO (n=6)	Group DIR (n=6)	Group DIRO (n=6)	P**
Myocardial ischemia-reperfusion damage **score**	0.33±0.21	0.83±0.17	1.00±0.36	2.83±0.17*,and,+	2.00±0.26*,and,+,?	<0.001
TUNEL (+) % cardiomyocytes /HPF	6.43±5.56	16.20±6.14	11.08±5.52	54.21±7.67*,and,+	22.92±10.19 ?	<0.001

Notes: p**: significance level with Kruskal–Wallis test; p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group D; +p<0.05: Compared to Group DO; p<0.05: Compared to Group DIR. Group C, control group; Group D, diabetic group; Group DO, diabetic group treated with ozone; Group DIR, diabetic group subjected to ischemia reperfusion; Group DIRO, diabetic group subjected to ischemia reperfusion and ozone treatment.
Abbreviations: TUNEL, Terminal deoxynucleotidyl transferase dUTP Nick End Labeling; HPF, high-power field.

CAT enzyme activity (p=0.025). CAT activity was significantly higher in the DIR group than in the C and D groups (P = 0.002 and P = 0.015, respectively). A significant difference was observed between the groups based on serum SOD enzyme activity (p = 0.044). SOD activity was significantly higher in the DIR group than in the C and DO groups (P =0.007 and P =0.029, respectively). Similarly, SOD activity was significantly higher in the DIRO group than in the C group (p=0.025). There was a significant difference between the groups in terms of serum PON1 enzyme activity (p=0.006). PON1 enzyme activity was significantly lower in the DIR group than in the K and D groups (p<0.001 and p=0.017, respectively). Similarly, PON1 enzyme activity was significantly lower in the DIRO group than in the K group (P =0.014). Similarly, there was a significant difference in serum TAS levels between the groups (p=0.026). TAS levels were significantly higher in the DIR group than in the C, D, and DO groups (P =0.013, P =0.005, and P =0.005, respectively) (Table 2).

When comparing the groups in terms of the heart tissue, the results were similar. There was a significant difference in the MDA levels between the groups (p<0.001). MDA levels were significantly higher in the DIR group than in the C, D, and DO groups (p<0.001, p<0.001, and p=0.003, respectively). MDA levels were significantly higher in the DIRO group than in the C and D groups (p=0.010 and p=0.028, respectively). Hence, MDA levels were significantly lower in the DIRO group than in the DIR group (p=0.030) (Table 3). There was a significant difference (p=0.047) between the groups based on heart tissue TAS levels. TAS levels were significantly lower in all groups than in the C group (P =0.003, P =0.001, P =0.002, and P <0.001, respectively). There was a significant difference between the groups in terms of the heart tissue OSI (p=0.041). OSI was significantly higher in the DIR and DIRO groups than in the C group (p=0.015 and p=0.033, respectively) (Table 3).

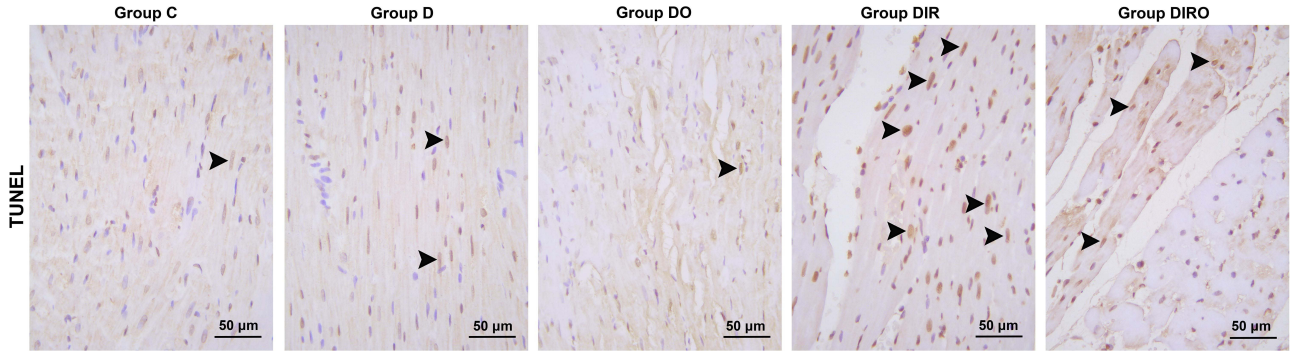

Figure 4 Micrographs representing the heart sections labelled through TUNEL assay. Arrowheads indicate the nuclei of TUNEL (+) cardiomyocytes. Group C, control group; Group D, diabetic group; Group DO, diabetic group treated with ozone; Group DIR, diabetic group subjected to ischemia reperfusion; Group DIRO, diabetic group subjected to ischemia reperfusion and ozone application. TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling). Scale bar, 50 µm.

Table 2 Oxidation Parameters of Serum [Mean ± SE]

	Group C (n=6)	Group D (n=6)	Group DO (n=6)	Group DIR (n=6)	Group DIRO (n=6)	P**
TAS (mmol/L)	1.20±0.07	1.08±0.08	1.10±0.06	1.93±0.38*,and,+	1.41±0.17	0.026
TOS (μmol/L)	9.62±1.38	12.05±0.80	12.72±0.53	15.39±5.14	19.05±4.6	0.243
OSI	0.85±0.16	1.16±0.14	1.19±0.10	1.11±0.33	1.00±0.15	0.649
PON-1 (U/L)	223.04±14.12	192.93±18.48	182.33±6.92	146.31±11.50*,and	174.97±9.60*	0.006
SOD (U/mL)	173.34±6.85	191.57±6.28	181.17±8.43	208.75±14.49*,+	199.80±3.51*	0.044
CAT (U/L)	372.39±89.19	500.92±86.73	610.23±128.50	897.56±118.84*,and	656.54±112.80	0.025
MDA (nmol/mL)	5.38±0.78	5.27±0.39	8.08±0.55	14.79±9.30*,and,+	11.70±1.81*,and	0.008

Notes: p**: significance level with Kruskal–Wallis test; p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group D; +p<0.05: Compared to Group DO; p<0.05: Compared to Group DIR. Group C, control group; Group D, diabetic group; Group DO, diabetic group treated with ozone; Group DIR, diabetic group subjected to ischemia reperfusion; Group DIRO, diabetic group subjected to ischemia reperfusion and ozone treatment.

Abbreviations: TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative stress index; PON-1: paraoxonase-1; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde.

Table 3 Oxidative Status Parameters of Cardiac Tissue [Mean ± SE]

	Group C (n=6)	Group D (n=6)	Group DO (n=6)	Group DIR (n=6)	Group DIRO (n=6)	P**
TAS (mmol/L)	0.72±0.09	0.43±0.02*	0.39±0.02*	0.41±0.08*	0.34±0.06*	0.047
TOS (μmol/L)	10.86±0.97	14.72±3.06	15.55±1.85	15.28±1.96	12.28±0.93	0.186
OSI	1.80±0.47	3.34±0.58	4.07±0.53	4.66±1.17*	4.26±0.85*	0.041
PON-1 (U/L)	24.03±2.02	19.83±2.07	15.76±2.24	17.37±2.56	20.04±1.17	0.091
SOD (U/mL)	212.74±20.48	161.39±20.49	173.10±33.02	161.25±18.24	174.88±27.54	0.533
CAT (U/L)	1355.71±154.37	1440.83±80.71	1692.60±94.68	1571.50±121.44	1511.40±83.09	0.319
MDA (nmol/Ll)	19.08±2.16	20.15±0.83	23.33±1.80	31.56±3.06*,and,+	25.76±0.86*,and,?	<0.001

Notes: p**: significance level with Kruskal–Wallis test; p< 0.05. *p<0.05: Compared to Group C; and p<0.05: Compared to Group D; +p<0.05: Compared to Group DO; p<0.05: Compared to Group DIR. Group C, control group; Group D, diabetic group; Group DO, diabetic group treated with ozone; Group DIR, diabetic group subjected to ischemia reperfusion; Group DIRO, diabetic group subjected to ischemia reperfusion and ozone treatment.

Abbreviations: TAS: total antioxidant status; TOS: total oxidant status; OSI: oxidative stress index; PON-1: paraoxonase-1; SOD: superoxide dismutase; CAT: catalase; MDA: malondialdehyde.

Discussion

Myocardial ischemic injury (MII) occurs after severely inadequate blood supply due to thrombosis or acute exacerbation of coronary atherosclerotic plaques.[3] Patients with diabetes have more atheroma and intraplaque thrombi with elevated inflammation and increased macrophage activity.[18] This increases the risk of myocardial ischemic injury. The initial reason for MII is the consumption of Adenosine Triphosphate (ATP) due to a lack of oxygen supply.[3] Anaerobic glycolysis dominates ATP production and increased hydrogen and lactate levels result in acidosis.[3] In addition, Na + K/+ ATPase was inhibited and the cells became swollen.[3] Thus, contraction rigor occurs.[19] With reperfusion, oxidative phosphorylation returns and intracellular acidosis rapidly regresses.[19] H is exchanged with Na; therefore, the 2Na/Ca exchanger is activated by the high Na levels.[19] Elevated Ca levels and oxidative stress trigger the mitochondrial permeability transition pore (PTP), which plays a key role in reperfusion injury.[19] Furthermore, reactive oxygen species (ROS) activate pro-inflammatory pathways and directly target cellular DNA.[19] As a result, the production of tumor

necrosis factor alpha (TNF-α) increases, triggering inflammation.[19] Taken together, these results indicate that PTP opens, ROS production increases, and inflammation begins. These factors can lead to cell death. However, ozone may heal this deadlock via the ferroptosis pathway.[12] Programmed cell death (PCD) plays a crucial role in CVD pathophysiology, and apoptosis is the first described type of PCD.[20] Therefore, new PCD models have been developed for pyroptosis, necroptosis, and ferroptosis.[20] Decreased glutathione levels downregulate glutathione peroxidase 4 (GPX4), and intracellular iron accumulation.[20] This causes an increase in lipid-originated ROS, which results in liposome peroxidation and cell death.[20] Ferroptosis is completely different from apoptosis in terms of its morphological, biochemical, and genetic mechanisms.[21] It is driven by iron-dependent lethal ROS activity.[21] The protective effects of ozone have been demonstrated through the Nrf-Slc7a11-GPX4 pathway, which is the main signaling pathway of ferroptosis.[12] It has been suggested that ozone exerts antioxidant effects through Nrf2, which increases the antioxidant capacity and reduces oxidative stress.[22] In addition, this is not the only cardioprotective mechanism of ozone. Jak2-Stat3 pathway which is related to cell survival, proliferation, and angiogenesis, is triggered by ozone.[11] Those activate thorough heat shock protein 70.[11] Difilippo et al[10] also suggested that the effects of ozone were related to elevated local e-NOS activity. There is a lot of studies which assert ozone has cardioprotective effects in the literature.

Condello et al[23] administered O3-AHT solution to patients during cardiac surgery via a venous reservoir and reported no adverse outcomes in their adventurous study. The researchers believe that their study could have a reducing effect on the inflammatory response.[23] We believe that this procedure will require further investigation.

In a study conducted on 50 male Wistar Albino rats in Egypt, two separate groups were formed based on increasing doses of ozone, and low and high doses were established.[24] A myocardial infarction model was created. The results of the study showed that rats exposed to high doses of the O3-O2 mixture had the least damage to the myocardium, followed by the group receiving low doses of the O3-O2 mixture.[24] The group receiving high doses of ozone was the most protective in terms of intracellular acidosis, energy production, oxidative stress, leukocyte infiltration, and NO production.[24] Researchers have suggested that these findings contradict the general assumption that low doses of ozone are more effective in protecting the myocardium.[24]

A recent study conducted in Havana used 50 male Sprague-Dawley rats to investigate the effects of ozone on oxidative stress in the blood and pancreas of STZ-induced diabetic rats.[25] Unlike our study, this study aimed to explore the effect of ozone on oxidative stress in the blood and pancreas of rats with established diabetes.[25] Researchers found that ozone application reduced pancreatic damage in rats with STZ-induced diabetes and decreases oxidative stress in the blood.[25] They also suggested that their method could contribute to the literature on diabetes and its complications, offering an alternative approach to managing the disease.[25] Similar studies conducted in Italy showed that ozone treatment in diabetic rats led to better glycemic control, increased pancreatic cell lifespan, and enhanced insulin and leptin levels.[26] Researchers have suggested that these studies can reduce the damage to distant organs associated with diabetes.[26] The focus of our study differs from these two studies because it does not solely involve blood glucose measurements to confirm diabetes in rats. Instead, we used diabetes to create a more realistic CAD model for coronary artery disease, setting our study apart from other ozone-induced IR studies.

In addition, Kocyigit et al.[27] Distant organ damage was investigated by administering intraperitoneal ozone injections at a dose of 1 mg/kg/day for 10 days. Researchers have reported lower SOD and CAT enzyme activities, lower troponin 1 levels, and decreased histopathological changes in the ozone-treated group.[27] This can be interpreted as a cardioprotective effect of ozone in terms of distant organ damage in the infrarenal aortic I/R model. However, we focused on the effects of ozone on myocardial ischemia and reperfusion damage using a direct myocardial ischemia model.

Di Filippo et al[8] conducted a study on Sprague-Dawley rats using an experimental myocardial ischemia-reperfusion method following a surgical approach similar to ours. After tracheostomy, a thoracotomy was performed through the 4th or 5th intercostal space to access the heart.[8] They performed their experiment by placing a vascular access on the jugular vein and achieved LAD ligation using silk sutures.[8] Catheter placement into blood vessels was considered unnecessary. We performed LAD ligation using an 8–0 prolene suture, which is more secure in diabetic animal models. There were also differences in the ozone application between the two studies. Researchers applied three separate doses of ozone 1 h before IR, started reperfusion at 25 min, and sacrificed the animals 2 h after reperfusion (136). We applied 1 mg/kg ozone 5 min before the start of reperfusion, after 25 min. Our study, similar to that of Di Filippo et al,[8] showed that ozone has cardioprotective effects

that favor IR. The same researcher observed the effect of the O2-O3 mixture on eNOS through eNOS inhibition in the experimental group without a cardioprotective effect, as seen in a study where eNOS was inhibited.[10]

Merin et al[9] administered an O3-O2 mixture during reperfusion, and the results favored cardioprotective effects in the ozone group. In our study, we administered the O3-O2 mixture intraperitoneally 5 minutes before reperfusion. Taken together, whether administered 1 hour before without inducing IR,[8] administered daily with increased dosage,[24,27] administered like us 5 minutes before reperfusion, or administered during reperfusion,[9] it has been showed that ozone has cardioprotective effects.

In studies involving ozone, it has been shown to have protective effects against the negative effects of myocardial ischemia-reperfusion injury along with many other mechanisms.[10–12,22] Studies on ozone and diabetes,[25,26] as well as those examining the relationship between ozone and myocardial ischemia-reperfusion, have yielded positive results.[8–10,24,27] However, studies on the effects of ozone on myocardial I/R injury in a diabetic population are not available in the literature. Our study was unique in this regard. Even though it was not statistically significant, the mild negative effects of ozone in the group without ischemia and its clear positive effect in the DIRO group were striking.

Ischemia and reperfusion injury are among the main topics of study for cardiologists and cardiac surgeons. This gray area still needs to be investigated for new and creative medicines and solutions. Based on these findings, it can be concluded that ozone administered significantly reduces the adverse effects of myocardial tissue by regulating lipid peroxidation levels and antioxidative enzyme activity in diabetic rats. These results will become even more important in diabetics who are exposed to chronic oxidative stress. Ozone, will continue to be the subject of many studies in the future on IRI and on preventing the negative effects of IRI.

Data Sharing Statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare no conflicts of interest in this work.

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