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Research Article

The effects of ozone therapy on extremity ischemia-reperfusion injury in a rabbit model

Tavşan modelinde ekstremite iskemi-reperfüzyon hasarına ozon tedavisinin etkisi

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ABSTRACT

Introduction: Ischemia is reversible or irreversible cell/tissue damage that is secondary to insufficient blood flow to tissues or organs. Ischemia causes many metabolic and structural changes at the cellular level. Ozone therapy is often used as an antioxidant remedy. This study aimed to investigate the effects of ozone therapy on extremity ischemia-reperfusion (IR) injury.

Methods: Twenty-four New Zealand White (NZW) rabbits were randomly allocated into three groups. Each group consisted of eight rabbits. Group I was the control group, Group II was the ischemia group, and Group III, the ozone group. Femoral arteries of the right legs were dissected, and femoral arterial occlusion was performed in Group II and III. Hematological and histopathological evaluation was performed in all groups.

Results: The levels of total antioxidant status (TAS) after the surgical procedure was higher in the ozone group compared to the ischemia group (p=0.036). In addition, the levels of malondialdehyde (MDA) after the surgical procedure were lower in the ozone group compared to the ischemia group but not statistically significant (p=0.093). The control and ozone groups were histopathologically similar.

Conclusions: Ozone therapy may be used as an alternative treatment modality with its anti-inflammatory and antioxidant effects for the treatment of extremity IR injury.

Keywords: Rabbits, ozone, femoral artery, reperfusion injury, ischemia, antioxidants

ÖZ

Giriş: İskemi; organı veya dokuyu perfüze eden kan akımındaki yetersizliğe bağlı olarak gelişen geri dönüşümlü veya dönüşümsüz hücre/doku zedelenmesidir. İskemi sonrasında hücrelerde pek çok metabolik ve yapısal değişiklikler oluşmaktadır. Ozon (O3) çeşitli hastalıkları tedavisi için antioksidan olarak terapötik amaçlı kullanılmaktadır. Bu çalışmada, ozon tedavisinin ekstremite iskemi-reperfüzyon hasarı üzerine olan etkisinin incelenmesi amaçlanmıştır.

Yöntem: 24 adet New Zealand White (NZW) erişkin erkek tavşan rastgele 3 gruba ayrıldı.Grup1: Kontrol grubu (n:8) Grup II: İskemi grubu (n:8) ve Grup III: Ozon grubu (n:8). Deneklerin sağ bacaklarına femoral arter oklüzyonu uygulanıp Grup II ve Grup III de iskemi-reperfüzyon hasarı uygulandı. Kontrol grubunda disseksiyon yapılıp oklüzyon uygulanmadı. Deneklerin kan değerleri ve sakrifikasyon sonrası kas dokusu histopatolojik değerlendirmesi yapıldı.

Bulgular: İşlem sonrası iskemi grubuyla ozon tedavisi uygulanan gruplar karşılaştırıldığında; TAS (total antioksidan status) düzeyinin ozon tedavisi uygulanan grupta anlamlı düzeyde daha yüksek (p=0,036) ve MDA (malondialdehit) düzeyinin de ozon tedavisi uygulanan grupta daha düşük olduğu, ancak istatistiksel olarak anlamlılığa (p=0,093) ulaşmadığı görüldü. Histopatolojik açıdan da ozon grubu ile kontrol grubunun benzer olduğu görüldü.

Sonuç: Ozon tedavisi güçlü anti-inflamatuvar ve antioksidan etkinliği ile ekstermite iskemi-reperfüzyon (IR) hasarını azaltmada alternatif bir tedavi seçeneği olabilir.

Anahtar kelimeler: Tavşanlar, ozon, femoral arter, reperfüzyon hasarı, iskemi, antioksidanlar

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Introduction

Ischemia is a reversible or irreversible cell/tissue injury that develops due to insufficient blood perfusions to organs or tissues. Ischemia disrupts oxidative phosphorylation in the cell, leading to a decrease in intracellular adenosine triphosphate (ATP) and phosphocreatine synthesis. This, in turn, interrupts the ATP-dependent ionic pump function of the cell membrane, resulting in increased calcium, sodium, and water influx. Destruction of the adenine nucleotide during ischemia is also increased. This increases the intracellular accumulation of reactive oxygen species (ROS) precursor hypoxanthine. After ischemia, ROS rapidly develop with reperfusion and re-introduction of intracellular molecular oxygen. Ischemia also promotes the synthesis of some proinflammatory gene products (leukocyte adhesion molecules, cytokines, etc.) and bioactive compounds (endothelin, thromboxane A2, etc.) in endothelial cells, while suppressing some protective gene products (structural nitric oxide synthase, cyclooxygenase-2) and inhibiting the expression and synthesis of products of these enzymes [nitric oxide (NO), prostacyclin]. To prevent irreversible cell damage, blood flow to the organ/tissue must be restored. However, performing reperfusion may cause more damage in ischemic tissues than ischemia itself [1, 2].

Ozone (O3) is used as a therapeutic agent for the treatment of various diseases. Ozone stimulates endogenous antioxidant mechanisms and regulates redox status in the cells. Strong anti-apoptotic and anti-inflammatory properties of ozone have been demonstrated in several studies [2-4]. Ozone can be administered to the body by rectal insufflation, intra-articular injection, as well as major or minor auto-hemotherapy.

This study aimed to investigate the effects of ozone as an endogenous antioxidant on extremity ischemia-reperfusion injury using biochemical and histopathological approaches.

Methods

The study was initiated after ethical clearance from the Canakkale Onsekiz Mart University (ÇOMÜ) Ethics Committee for Animal Experiments (Date: 25.12.2014, number: 2014-166). Twenty-four New Zealand White (NZW) adult male rabbits (weight: 2.5–3 kg) were kept at appropriate feeding conditions and in experimental animal cages at the Canakkale Onsekiz Mart University Experimental Research Center during the study period. Temperature-controlled (2-25 ˚C) and alternating illumination (8 am – 8 pm: light, 8 pm – 8 am: darkness) conditions with food and water ad libitum were provided. The animals were randomly allocated into three groups.

Group 1: Control group (n=8)

Group II: Ischemia group (n=8)

Group III: Ozone group (n=8)

The Anesthesia Protocol: Before starting the study, 10 mg/kg ketamine was given intramuscularly to all animals for premedication. With this dose, rabbits had sufficient tranquilization and sedation after approximately 15 minutes. Injections were performed on the *Quadriceps femoris* muscle. If a single infusion over 2.5 ml was required, it was equally divided into the right and left *Quadriceps femoris* muscles. After this procedure, venous catheterization was performed from the ear veins.

Catheterization, Application of Ozone using Major Autohemotherapy, and Airway Placement: When the animals were adequately anesthetized, first a 24 G arterial catheter, then a 36 G venous catheter was inserted in the ear, and blood was taken for measuring blood parameters. In the ozone group, additional blood was withdrawn for ozone therapy. After 5 ml of blood was mixed with ozone at a ratio of 50 µg/ml, it was administered to the ozone group via the venous route within 5 minutes (major autohemotherapy). Total oxidant status (TOS), total antioxidant status TAS), malondialdehyde (MDA), superoxide dismutase (SOD), and ischemia-modified albumin (IMA) were measured. For the induction of anesthesia, 2 mg/kg ketamine, 1mcg/kg fentanyl, and 0.5 mg/kg rocuronium bromide were given. Following adequate muscle laxity, a V-GEL RABBIT was placed on all animals for airway safety. Pedal and palpebral reflexes were checked to ensure the effectiveness of anesthesia.

The Surgical Procedure: After the airway safety was ensured, the right thighs of the animals were shaved, sterilized with 10% polyvinylpyrrolidone iodine complex, and the femoral artery was dissected through an incision of approximately 3 cm. In the control group, femoral artery dissection was performed without performing an occlusion. In the ischemia group, occlusion was performed by a vessel clamp after femoral artery dissection. The same procedure was applied in the ozone group but with administering IV ozone treatment before the process. Additionally, 50 µg/mL ozone (20 mL) was given to the subjects in the ozone group by rectal insufflation within 5 days before the surgery. After approximately 120 minutes of ischemia, the clamp was opened and allowed for 60-minutes reperfusion (Figure 1). Again, blood was drawn from the animals, followed by sacrification. Besides, tissue samples (anterior compartment of the thigh, *Tibialis anterior*, and *Tibialis extensor* muscle mass) were taken from the extremities for histopathological examination.

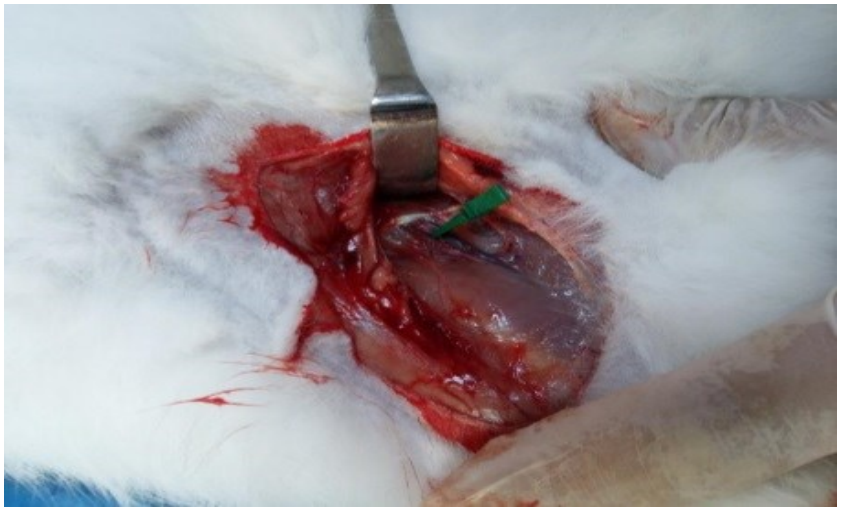

Figure 1. Femoral artery dissection and clamping of the artery

Histopathological Evaluation: Materials of three different groups were fixed with 10% formol. The tissues loaded into cassettes, and taken to a fully automatic tissue processor (Thermo Fisher Scientific, Waltham, MA, USA). Then, they were embedded in paraffin blocks after processing. Slices of 4-6 microns thick were taken with a microtome. All sections were stained with Hematoxylin-Eosin and evaluated under a light microscope. Histopathologically, loss of striation in the muscle cells, diameter differences, centralization, perivascular neutrophil infiltration, and necrosis were assessed as present or absent.

Statistical Analysis: The Kruskal Wallis test (SPSS 21.0, SPSS Inc., Chicago, IL, USA) was used for the evaluation of blood parameters and histological measurements, while the Mann-Whitney U test was used to determine the differences between the groups. A p-value<0.05 was considered statistically significant.

Results

Blood values (mean) of the three groups examined before and after the surgical procedure are summarized in Table1.

Table 1. Total antioxidant status (TAS), total oxidant status (TOS), ischemia modified albumin (IMA), malondialdehyde (MDA), and superoxide dismutase (SOD) values before and after the procedure

GROUPS	TAS	TOS	IMA	MDA	SOD
GROUP 1 (CONTROL)	O.68	8.54	0.27	6.21	97.52
PRE-OPERATIVE	(SD:0.22)	(SD:4.19)	(SD:0.04)	(SD:1.01)	(SD:1.38)
GROUP 1 (CONTROL)	0.52	9.82	0.34	7.43	96.97
POST-OPERATIVE	(SD:0.18)	(SD:4.27)	(SD:0.08)	(SD:2.35)	(SD:2.24)
GROUP 2 (ISCHEMIA)	0.61	6.65	0.25	6.02	96.55
PRE-OPERATIVE	(SD:0.13)	(SD:1.31)	(SD:0.03)	(SD:1.18)	(SD:1.63)
GROUP 2 (ISCHEMIA)	0.61	8.86	0.36	6.62	97.48
POST-OPERATIVE	(SD:0.13)	(SD:2.69)	(SD:0.04)	(SD:3.47)	(SD:0.66)
GROUP 3 (OZONE)	0.83	8.11	0.32	4.16	98.40
PRE-OPERATIVE	(SD:0.18)	(SD:2.97)	(SD:0.03)	(SD:0.78)	(SD:0.34)
GROUP 3 (OZONE)	0.81	10.32	0.37	4.54	97.75
POST-OPERATIVE	(SD:0.15)	(SD:6.62)	(SD:0.03)	(SD:0.52)	(SD:1.40)

SD; standard deviation

There was no significant difference between the groups regarding the blood measurements before surgery. When the ischemia and ozone-treated groups were compared post-procedure, TAS (total antioxidant status) levels were significantly higher in the ozone-treated group (p=0.036), and MDA (malondialdehyde) levels were lower in the ozone-treated group but did not reach statistical significance (p=0.093). No significant difference was found between the ischemia and ozone groups concerning the other variables.

The histopathological evaluation results are summarized in Table2.

Histopathological evaluation revealed rare nuclear centralization, slight diameter differences, and focal perivascular neutrophil infiltration in the ischemia group, whereas the ozone group demonstrated features almost similar to the control group (Figure 2).

Table 2. Histopathological evaluation results

	Striation loss	Centralization	Diameter difference	Neutrophil infiltration	Necrosis
GROUP 1	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
GROUP 2	0	0	1	0	0
	0	0	1	0	1
	1	1	1	1	0
	0	0	0	0	0
	1	1	1	1	0
	0	0	1	0	0
	1	1	1	1	0
	1	0	1	1	1
GROUP 3	0	0	1	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	1	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0
	0	0	0	0	0

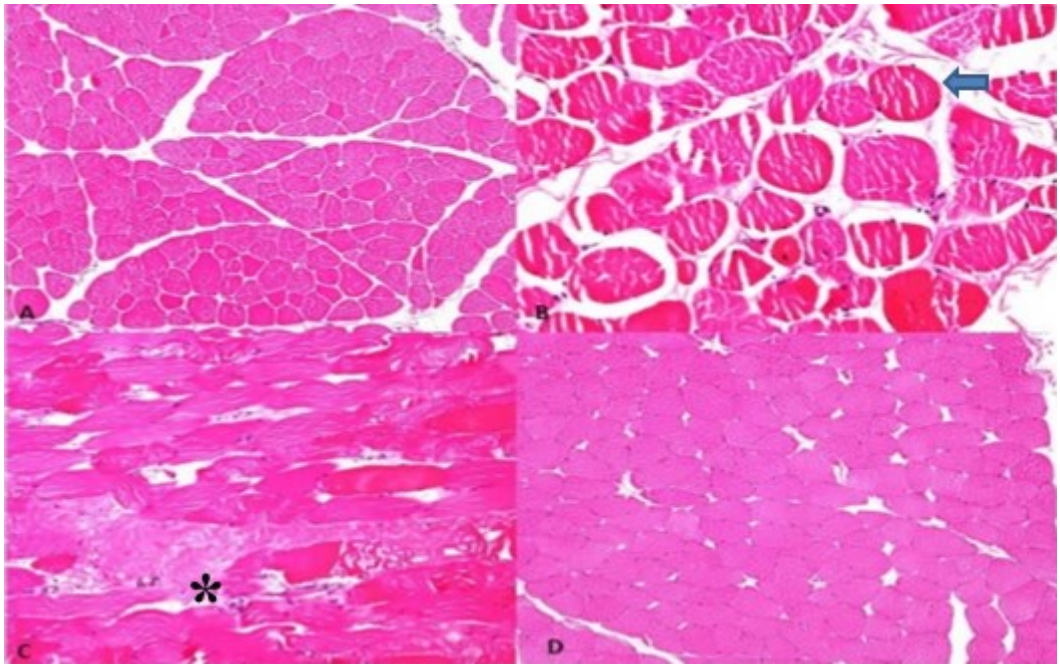

Figure 2. A. Regular-looking muscle cells (HEx100) in the control group; B-C: Intracellular edema, diameter differences (thick arrow), loss of striation, and central location of the nucleus (*) in the ischemia group (HEx100); D: Regular appearing muscle cells in the ozone group (HEx100)

Discussion

The leading cause of mortality in many diseases, such as myocardial infarction and stroke is tissue damage after ischemia. During the treatment of ischemic disorders, it was seen that the trauma was not only caused by ischemia, but reperfusion injury following blood supply was also a contributing factor. Ischemia-reperfusion (IR) injury can be seen in obesity, atherosclerosis, traumatic shock, hypertension, thrombosis, as well as after surgical intervention and organ transplantation [5-8].

IR damage is caused by excessive production of reactive oxygen species. Under normal conditions, the production and destruction process of reactive oxygen radicals in our body progresses in a balanced way. However, in pathological conditions, the production of reactive oxygen radicals increases, and antioxidant enzyme activity decreases. This leads to cell membrane damage and cell death because of lipid peroxidation reaction. Inflammatory mediators produced during IR injury lead to rolling, adhesion, and migration of the leukocytes. This leads to deterioration of vascular integrity, edema, thrombosis, and tissue necrosis [9, 10]. Experimental animal and clinical studies have shown that natural antioxidants such as Vit C, Vit E, and SOD may inhibit the release of free oxygen radicals and prevent some damage [11-13].

Ozone (O3) is used as a therapeutic agent for the treatment of various diseases. Ozone stimulates endogenous antioxidant mechanisms and regulates the redox status in cells. The strong anti-apoptotic and anti-inflammatory properties of ozone have been demonstrated in several studies [2-4]. Ozone can be administered to the body by rectal insufflation, intra-articular injection, as well as major or minor autohemotherapy. Studies have investigated the use of ozone therapy for the treatments of spinal cord injury, disc herniation, sudden hearing loss, or rheumatologic diseases; beneficial results have been reported [14-17].

Turel et al. showed that ozone treatment accelerates the spinal cord healing process, increases vascularity, and reduces neuronal damage in an experimental spinal cord injury model [14]. Taşdöven et al. showed that ozone application, in addition to steroid treatment, gave more successful results than steroids alone in sudden hearing loss. They have linked this to increased oxygen, glucose, and ATP transport to ischemic tissues, and the stimulation of angiogenesis [16]. Leon et al. have shown that ozone therapy increases the clinical response of methotrexate in patients with rheumatoid arthritis (RA) receiving methotrexate due to its anti-inflammatory efficacy. They suggested its use in combination with methotrexate in patients with RA [17]. In the literature, many studies were performed by taking advantage of the anti-inflammatory and antioxidant effects of ozone treatment. Still, to the best of our knowledge, no study has examined the effects of ozone therapy on limb ischemia-reperfusion injury. When the hematological markers were analyzed, TAS (total antioxidant status), showing the antioxidant status of the body, was significantly higher in the ozone-treated group compared to the ischemia group. MDA (malondialdehyde), a lipid peroxidation product, was also lower in the ozone group; however, it did not reach statistical significance (p=0.093). No significant difference was found between the ischemia and ozone groups regarding other parameters. Histopathological evaluation revealed rare nuclear centralization, slight diameter differences, and perivascular neutrophil infiltration in focal areas, whereas no significant findings were observed in the ozone group. Nevertheless, this was approximately the same as in the control group. Similar to other studies, our experiment investigating the biochemical and histopathological effects of ozone application demonstrated positive results concerning its antioxidant effects in a limb ischemia-reperfusion injury.

In this experiment, the efficacy of ozone in the ischemia-induced animal model was examined by both biochemical markers and histopathological evaluation. Conducting two assessments together and obtaining concordant results are the strengths of the study. However, having an experimental animal study, we cannot foresee the same efficacy in clinical practice. In addition, the small number of subjects can be considered as some limitations of this study.

Conclusion

The efficacy of ozone application in preventing extremity IR damage could be demonstrated both biochemically and histopathologically. Anti-inflammatory and antioxidant activity of ozone is thought to play a role in the emergence of this result. In light of these findings, ozone application can be considered as a method to reduce extremity IR damage. However, clinical studies are still needed to see more far-reaching effects.

Conflict of interest: None

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