Effect of hyperbaric oxygen and ozone preconditioning on oxidative/nitrosative stress induced by tourniquet ischemia/reperfusion in rat skeletal muscle

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Objectives: The aim of the study was to investigate the effect of hyperbaric oxygen-preconditioning (HBO-PC) and ozone-preconditioning (O₃-PC) on oxidative/nitrosative stress induced by tourniquet ischemia/reperfusion (I/R) in rat skeletal muscle.

Methods: Thirty-two Wistar-Albino-type male rats included in the study were divided into four groups of equal number: 1) sham operation, 2) I/R, 3) I/R+HBO-PC, or 4) I/R+O₃-PC. One session of 3-4 L/min 100% oxygenation for 60 min at 3 absolute atmosphere (ATA) was defined as one dose of HBO; in total, 7 doses of HBO-PC were administered before ischemia. One dose of O₃ comprised 0.7 mg/kg ozone/oxygen mixture, administered intraperitoneally; a total of 4 doses of O₃-PC were administered before ischemia. The I/R model was performed in anesthetized rats by clipping right femoral artery to induce 2 h ischemia followed by 22 h of reperfusion. The right gastrocnemius muscle and venous blood samples were harvested. Tissue was assayed for levels of malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). Serum was assayed to measure the level of nitrite-nitrate (NOx).

Results: Tissue MDA level, SOD activity, and serum NOx level were increased, whereas GSH-Px was decreased in the I/R group. MDA and NOx levels were decreased, whereas GSH-Px activity was increased in both the I/R+HBO-PC and I/R+O₃-PC groups. SOD activity was increased in the I/R+O₃-PC group, but did not change significantly in the I/R+HBO-PC group. iNOS staining score and intensity were lower in the I/R+HBO-PC and I/R+O₃-PC groups than I/R group.

Conclusion: Both O₃-PC and HBO-PC reduced tissue lipid peroxidation, NOx levels, and iNOS staining scores in the experimental I/R model. Our data suggest that HBO-PC and O₃-PC protect against oxidative/nitrosative stress induced by I/R in rat skeletal muscle.

Key words: Ischemia-reperfusion injury; hyperbaric oxygenation; oxidative stress; ozone; rats.

Ischemia/reperfusion (I/R) injury is a serious problem found in cardinal states such as myocardial infarct, cerebrovascular occlusion, and organ transplantation. I/R injury can emerge in various cases of injury to the muscle and skeletal systems, such as trauma to the extremities, or be caused by an intraoperative tourniquet.[1] Reperfusion injury refers to tissue damage caused when blood supply returns to the tissue after a period of ischemia. The absence of oxygen and nutrients from blood creates a condition in which the restoration of circulation results in inflammation as well as oxidative and nitrosative damage through the induction of oxidative stress, rather than the restoration of normal function.[2] The most important factors in this process are reactive oxygen species (ROS) including superoxide anion

(O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), and reactive nitrogen species (RNS), including peroxynitrite ($ONOO^-$) anions. Under normal circumstances, these ROS are counteracted by antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).[3] However, the antioxidant enzyme system of the body is not sufficient to handle rapid-onset conditions such as I/R injury, in which high levels of ROS and RNS are produced.[4]

Numerous methods to reduce I/R injury, including hyperbaric oxygen (HBO) administration, have been investigated. Although the exact mechanism of the effect of HBO is not clear, HBO is known to increase oxygen concentration in all tissues, stimulate collateral angiogenesis, stimulate an adaptive increase in SOD, and aid in the treatment of infection by enhancing white blood cell action.[5] HBO preconditioning (HBO-PC) was found in various studies in I/R models to reduce local ischemic injury.[6] I/R studies conducted in different tissues showed that HBO-PC reduced apoptosis and ischemic injury.[7,8] The mechanism underlying the effect of HBO-PC in I/R could not be entirely illuminated, although the regulation of antioxidant enzymes and hypoxia-inducible factor-1 alpha in the ischemic area are thought to be involved.[6,9,10]

Ozone (O_3) was first used as a treatment for infection during World War I, with successful results.[11] Ozone was also successful in treating ischemia, inflammation, and infection.[12] The efficacy of ozone in treatment is thought to arise from its powerful oxidant capacity. In addition, O_3 preconditioning (O_3-PC) has been shown to attenuate I/R injury, similar to ischemic-preconditioning (IPC).[13]

Studies investigating HBO and O_3 preconditioning against I/R injury in skeletal muscle are limited in number. Therefore, in this study, we aimed to investigate the effect of HBO-PC and O_3-PC on tourniquet-induced I/R injury in rat skeletal muscle (frequently encountered in orthopedic interventions) in terms of oxidative and nitrosative stresses.

Materials and methods

Animals

The Experimentation Ethics Committee of our institution approved the experimental procedures of the study (GATA Ethics Committee). Thirty-two adult male Wistar Albino rats weighing 280-340 g were obtained from the GATA Research Center and housed in separate cages in standard conditions. The animals were fed with a standard diet and maintained in a 12-h light-dark cycle. The animals were divided into four groups: sham operation (n=8), I/R (n=8), I/R+HBO-PC (n=8), and I/R+O_3-PC (n=8).

HBO preconditioning

A special animal hyperbaric chamber (Etimesgut Military Equipment Factory, Ankara, Turkey) was used for HBO administration. One session of 3-4 L/min 100% oxygenation for 60 min at 3 ATA was defined as one dose of HBO. The first dose of HBO was administered 72 hours before ischemia. Doses of HBO were administered two times a day, at 08:00 am and 08:00 pm, until ischemia was achieved. In total, 7 doses of HBO-PC were administered (Table 1). Compression and decompression of the chamber were completed gradually, over a 5-10 min time period.

Table 1

iNOS immunostaining values [median (range)]

Groups	Staining intensity	Staining width	Staining score
Sham	0.5 (0-2)	0.5 (0-1)	0.5 (0-2)
IR	2 (2-3)[*]	3 (2-4)[*]	6 (4-9)[*]
IR+O_3-PC	1 (1-2)[†]	2 (2-3)	3 (2-6)[†]
IR+HBO-PC	1 (1-3)	2 (2-3)	3 (2-6)[†]

[*]p<0.01 for the I/R group vs. sham group, [†]p<0.01 for the I/R+HBO-PC and I/R+O_3-PC vs. the I/R group.

Ozone preconditioning

Administration of 0.7 mg/kg ozone/oxygen mixture intraperitoneally (IP) was defined as one dose of ozone. The volume of gaseous mixture administered IP to each animal was approximately 2.3-3.0 mL (60 μg/mL). The first dose of ozone was administered 72 hours before ischemia. All doses were started at 08:00 am and continued until ischemia was achieved. In total, 4 doses of O_3-PC were administered. Ozone was generated from medical-grade oxygen (O_2) using electrical corona arc discharge and the O_3 generator (model OZONOSAN Photonik 1014, Hansler GmbH, Nordring 8, Iffezheim, Germany), which allows the gas flow rate and ozone concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International Ozone Association. The ozone flow-rate was kept constant at 3 L/min in all the experiments and represented only approximately 3% of the gas (O_2+O_3) mixture. Tygon polymer tubing and single-use silicon-treated polypropylene syringes (ozone-resistant) were used throughout the reaction to ensure O_3 containment and concentration consistency.

The ischemia-reperfusion model

All rats weere anesthetized using an intramuscular injection of a rodent anesthetic mixture (ketamine and xylazine, 5:1 mg/mL) at a dose of 50 mg/kg ketamine and 10 mg/kg xylazine. Anesthesia was maintained by the administration of additional doses of the mixture during the procedure. The surgical region was shaved, and the skin cleaned with a 10% solution of povidone-iodine (Betadine, Purdue Products, Stamford, CT, USA). Body temperature was monitored using a rectal probe and maintained at approximately 36.5-37.5°C with a heating pad. The common iliac artery was found through a high right inguinal incision. The common iliac artery was clamped, and collateral blood flow was occluded using a rubber arterial tourniquet at the proximal third of the leg. After 2 h, the clamp and rubber tourniquet were removed, and the hind limb was allowed to reperfuse for 22 h. The entire procedure was performed in the sham operation group, but ischemia was not applied.

Tissue sampling

At the end of the IR period (2 h ischemia and 22 h reperfusion), the animals were sacrificed, and their leg was opened by an incision to right cruris region. Next, the soleus muscle was removed immediately, washed with physiologic serum (PS) to remove residual blood, placed into tubes, frozen with liquid nitrogen and stored at -70°C. Histopathologic tissue samples were preserved in 10% formalin solution.

Tissue preparation

The frozen tissues were weighed, placed into a plastic tube, and homogenized at a concentration of 100 mg tissue per mL of 25 mM phosphate buffer (pH 7.4) on ice using a homogenizer (Heidolph Diax 900, Heidolph Elektro GmbH, Kelheim, Germany) at a setting of 8 (out of 10) for 30-s bursts. The homogenates were centrifuged for 10 min at 2500 g, and the pellet (cellular debris) was discarded. The supernatant was allocated into 2-3 separate tubes and used for biochemical assays.

Biochemical analysis

Tissue protein assay

First, the protein content of the tissue homogenates was measured using Lowry's method,[14] with bovine serum albumin as the standard. Tissue lipid peroxidation (MDA) levels were measured with the thiobarbituric acid (TBA) reaction using Ohkawa's method.[15] This method was used to obtain a spectrophotometric measurement of the color produced during the reaction TBA with MDA at 535 nm. For this purpose, 2.5 mL of 100 g/L trichloroacetic acid solution was added to 0.5 mL homogenate in each centrifuge tube and placed in a boiling water bath for 15 min. The mixture was cooled and centrifuged at 1000 g for 10 min. Next, 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled, and its absorbance was measured with a spectrophotometer (Helios Epsilon, Thermo Electron Corporation, Pittsford, NY, USA) at 535 nm. MDA levels are expressed as mmol/g protein.

Tissue superoxide dismutase (SOD)

SOD activity was assayed using the nitroblue tetrazolium (NBT) method proposed by Sun et al.[16] NBT was reduced to blue formazan by superoxide, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity is expressed as SOD (U)/protein (g).

Tissue glutathione peroxidase (GSH-Px)

GSH-Px activity was measured using the method described by Paglia and Valentine[17] in which GSH-Px activity was coupled with the oxidation of NADPH by glutathione reductase. The oxidation of NADPH was spectrophotometrically determined at 340 nm at 37°C. The absorbance at 340 nm was recorded for 5 min. The activity was the slope of the lines (mmol) of oxidized NADPH/min. GSH-Px activity is presented as GSH-Px (U)/protein (g).

Serum nitrite-nitrate (NOx)

Serum samples were passed through 0.45-µm pore membrane nitrocellulose filters before NOx analysis. The NOx levels were detected by means of an ion chromatograph (Dionex ICS-1000, Sunnyvale, CA, USA). Anion and guard columns (AS-9HC/AG-9HC, CS12A/CG12A, Sunnyvale, CA, USA) and automated suppression were used. NOx levels were quantified using separate standard solutions for each ion.[18]

Histopathology

Inducible nitric oxide synthase (iNOS) immunostaining

Tissues were fixed in 10% formaldehyde for 24 h and embedded in paraffin. Five-micrometer sections were cut, and sections were immunohistochemically stained with iNOS. The staining procedure was the same as that described by Özcan et al.[19] To remove the variability in staining intensity caused by technical problems, an automatic stainer (autostainer-480-LV1, Labvision, Freemont, CA, USA) was used; all sections were stained in duplicate. Samples were evaluated using a light microscope (Nikon E-600, Tokyo, Japan). Staining scores were calculated by multiplying staining intensity with width. Staining intensity was scored as follows: 0 (no staining), 1+ (weak), 2+ (moderate) or 3+ (strong). Staining width was scored as follows: 1 (1-25%), 2+ (25-50%), 3+ (50-75%) or 4+ (75-100%). Therefore, the minimum staining score was 0, and the maximum value was 12.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, version 11.0, Chicago, IL, USA). All numerical data were analyzed first using the Kruskal-Wallis test to iden-tify differences between the groups; the Mann-Whitney U-test was used to analyze two groups consecutively. Statistical significance was accepted at a value of $p < 0.01$.

Results

Tissue lipid peroxidation

The level of MDA was increased in the I/R group as compared to the sham operation group. MDA was significantly decreased in the HBO+I/R and O_3+I/R groups as compared with the I/R group (Fig. 1)

Antioxidant enzymes (SOD, GSH-Px)

SOD activity was increased in the I/R group as compared to the sham operation group ($p < 0.01$). O_3-PC significantly increased SOD activity, but HBO-PC had no noticeable effect on SOD activity. Unlike that of SOD, GSH activity was decreased in the I/R group as compared to the sham operation group ($p < 0.01$). O_3-PC and HBO-PC significantly increased the level of GSH-Px activity (Fig. 1).

Serum nitrite-nitrate

I/R injury resulted in a significant increase in the serum level of NOx, as compared to the sham operation group ($p < 0.01$). O_3-PC and HBO-PC decreased the level of NOx (Fig. 1).

iNOS immunostaining

The mean values of iNOS staining scores, width and intensity were higher in the I/R group than in the others. No staining was observed in the sham operation group. O_3-PC and HBO-PC decreased the staining scores and intensity, but had no effect on width of the stain (Table 1 and Fig. 2).

Discussion

The present study evaluated the effects of both O_3-PC and HBO-PC on oxidative/nitrosative stress induced by tourniquet I/R in rat skeletal muscle. The results showed that O_3-PC and HBO-PC reduced oxidative/nitrosative status and iNOS immunostaining scores in skeletal muscle. Therefore, they may reduce the extent of tissue injury mediated by the oxidative/nitrosative stress induced by I/R. Prior to injury, O_3-PC and HBO-PC prepare the antioxidant system by inducing oxidative/nitrosative stress and by reducing the potential induction of iNOS expression in skeletal muscle subjected to I/R injury.
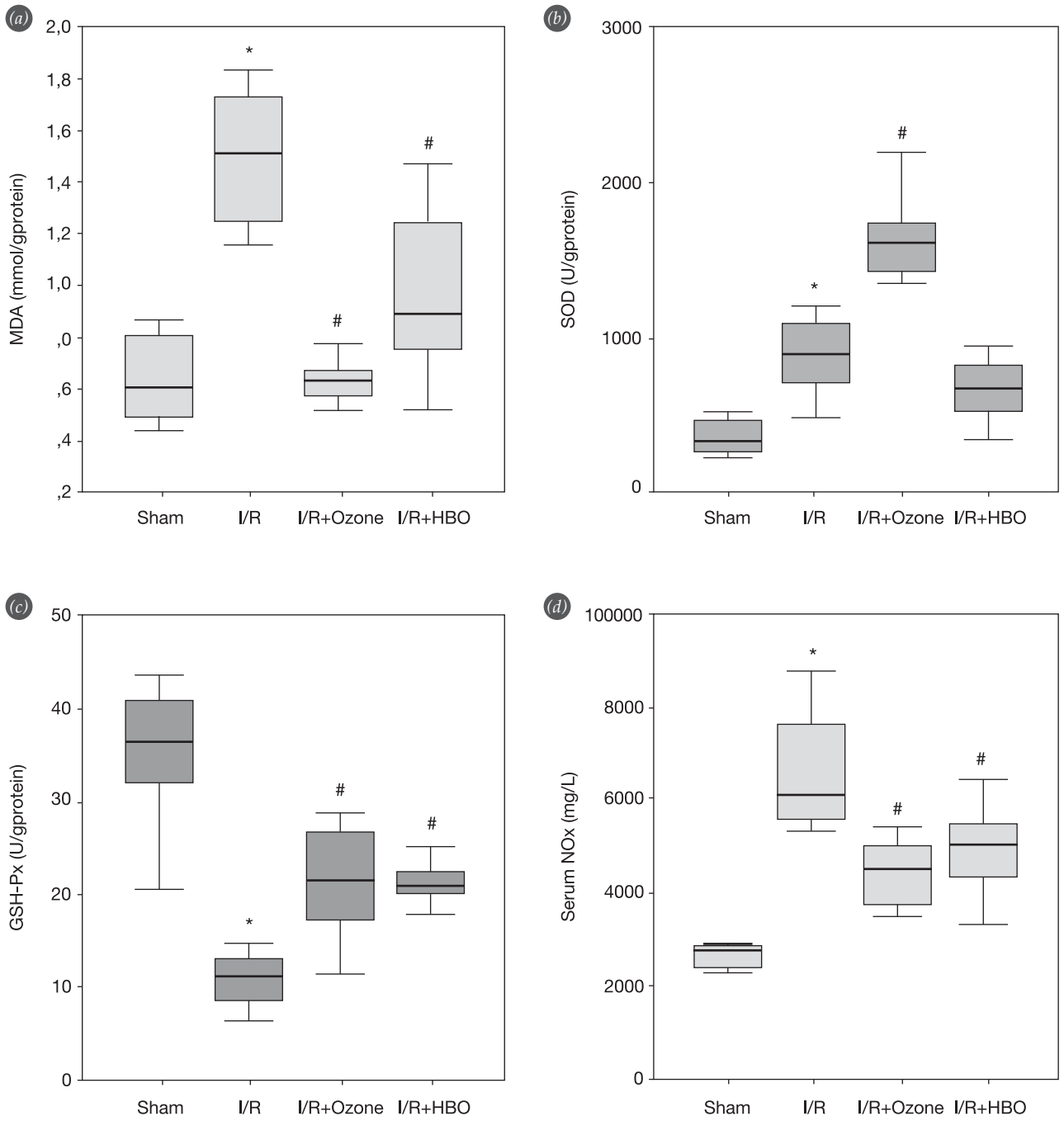
Fig. 1. Tissue **(a)** MDA, **(b)** SOD, **(c)** GSH-Px, and **(d)** serum NOX levels in all groups (median±standard deviation). *$p < 0.01$ for I/R group vs. the sham group, #$p < 0.01$ for I/R+HBO-PC and I/R+O$_3$-PC groups vs. the I/R group.

We found that lipid peroxidation (MDA), which is an oxidative stress marker, and serum NOx levels, which are a nitrosative stress marker, were increased in the I/R-induced rats. Antioxidant enzyme (SOD and GSH-Px) activities were also increased in the same group. In addition, iNOS staining scores, width

and intensity were higher in the I/R group than in the others. These results are similar to those reported previously.[20,21]

HBO administration has many beneficial pharmacological effects, such as edema reduction, impairment of leukocyte adhesion, enhancement of
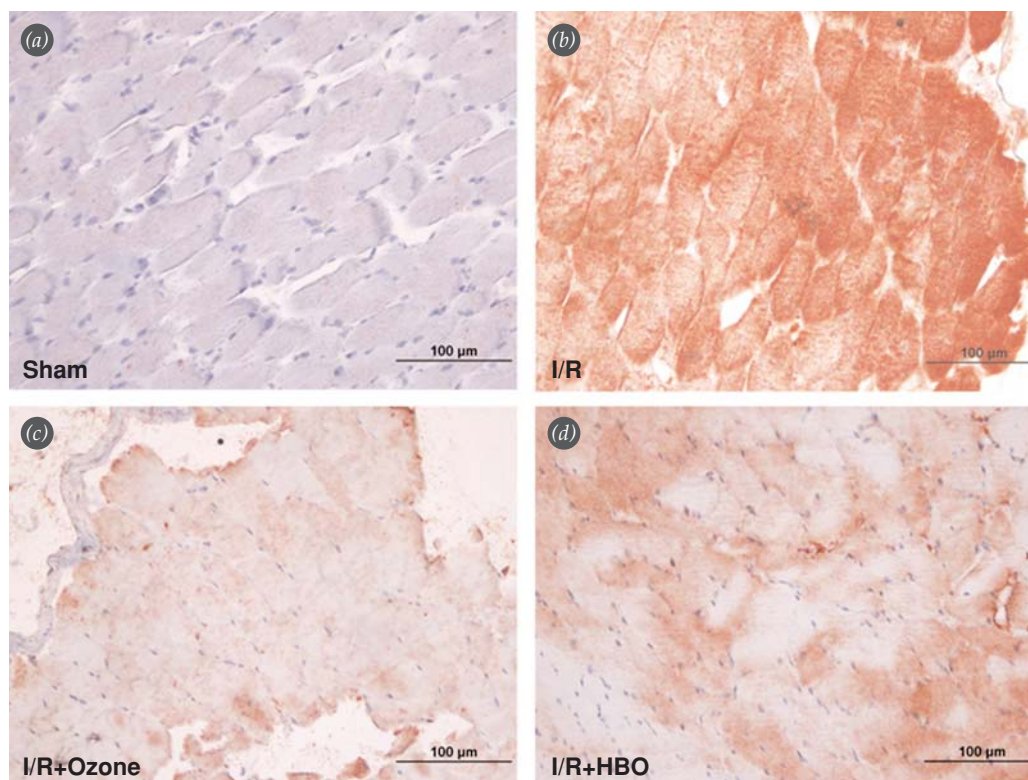
Fig. 2. Immunostaining images of skeletal muscle tissue from **(a)** sham, **(b)** I/R, **(c)** I/R+O$_3$, and **(d)** I/R+HBO groups.

antibacterial mechanisms, and stimulation of fibroblast proliferation, and neo-vascularization.[5] HBO-PC increases ROS production, similar to ischemic preconditioning.[22] Increased ROS production provides important signaling molecules that facilitate host defense system activity.[23] Furthermore, some of HBO's beneficial effects may be mediated by reactive molecules, in particular, hydrogen peroxide (H$_2$O$_2$).[24] HBO administration can either increase or decrease levels of oxidative stress, depending on the cellular environment.[25] However, none of the basic mechanisms of these benefits have been established conclusively. HBO administration has been used in various pathological conditions caused by oxidative stress including cystitis, sepsis, nephritis, and colitis. All of these studies have shown that HBO suppresses increased oxidative stress.[26-29] Moreover, it has been shown that HBO has ameliorative effects in the treatment of acute pancreatitis, mediated by decreases in oxidative stress.[30] Li et al.[9] reported that HBO-PC increases the levels of catalase (CAT) and SOD activity, but decreases the level of MDA in I/R injury of brain tissue. Busco et al.[31] reported that

CAT- and MDA-induced skeletal I/R were decreased by HBO-PC. Akgül et al.[32] reported that HBO elevated the levels of asymmetric dimethylarginine (ADMA) and decreased NOx levels through the inhibition of nitric oxide synthase (NOS) in brain. In the present study, MDA and serum NOx levels were decreased and GSH-Px enzyme activity was increased significantly by HBO-PC. The activity of SOD, however, was not altered significantly by HBO-PC. These results indicated that HBO-PC decreased level of MDA and NOx, similar to published reports. Activity levels of antioxidant enzymes, however, were different than those reported in the literature. These discrepancies may result from the dose and frequency of HBO-PC administration as well as the duration of the ischemia-reperfusion period.

Notably, O$_3$ dissolves in biological fluids such as plasma, lymph. and urine; and reacts immediately with polyunsaturated fatty acids, antioxidants, reduced glutathione, and albumin. All of these compounds act as electron donors and undergo oxidation, resulting in the formation of H$_2$O$_2$ and lipid oxi-

dation products (LOPs). H_2O_2, an essential ROS molecule, is able to act as an ozone messenger to elicit several biological and therapeutic effects.[33,34] In contrast to the conventional idea that H_2O_2 is harmful, it has been widely accepted that H_2O_2 acts as a regulator of signal transduction, and is an important mediator of host defense and immune responses.[9,35] While H_2O_2 acts immediately and then disappears (an early and fast-acting messenger), LOPs distribute throughout the tissues via the circulation, and become late and long-lasting messengers.[30] This process stimulates the innate immune system and helps the cell to survive when an injury occurs. In addition, it has been demonstrated that O_3-PC supports cellular antioxidant systems involving glutathione, SOD, and catalase and enzymatic reactions, preparing the host to face pathophysiological conditions mediated by oxidative/nitrosative stress and septic shock.[36,37] It has also been shown that O_3-PC has beneficial effects against I/R mediated injury in liver and kidney tissues.[32,38] In the present study, O_3-PC decreased levels of MDA and NOx and increased levels of SOD and GSH-Px. It also reduced iNOS immunostaining scores, width, and intensity. These findings are similar to those reported by other authors.

In conclusion, both O_3-PC and HBO-PC reduced tissue lipid peroxidation (which reflects oxidative stress), NOx levels (which reflect nitrosative stress), and iNOS staining scores in a model of I/R. The effects of O_3-PC and HBO-PC were not significantly different. Our data suggest that both O_3-PC and HBO-PC protect against antioxidative and nitrosative stress induced by I/R in rat skeletal muscle.

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