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Original article

Ozone protects rat heart against ischemia-reperfusion injury: A role for oxidative preconditioning in attenuating mitochondrial injury

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ABSTRACT

Ischemia-reperfusion injury (IRI) is a major cause of cardiac dysfunction during cardiovascular surgery, heart transplantation and cardiopulmonary bypass procedures. The purpose of the present study was to explore, firstly, whether ozone induces oxidative preconditioning by activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and, secondly, whether ozone oxidative preconditioning (OzoneOP) can protect the heart against IRI by attenuating mitochondrial damage. Rats were subjected to 30 min of cardiac ischemia followed by 2 h of reperfusion, with or without prior OzoneOP (100 μg/kg/day) for 5 days. Antioxidant capacity, myocardial apoptosis and mitochondrial damage were evaluated and compared at the end of reperfusion. OzoneOP was found to increase antioxidant capacity and to protect the myocardium against IRI by attenuating mitochondrial damage and myocardial apoptosis. The study suggests a potential role for OzoneOP in protecting the heart against IRI during cardiovascular surgery, cardiopulmonary bypass procedures or transplantation.

1. Introduction

The imbalance in myocardial blood demand and supply caused by coronary artery disease results in injury to the ischemic myocardium that is exacerbated by subsequent reperfusion of the ischemic area. Myocardial ischemia-reperfusion injury (IRI) is a pathophysiological process that commonly occurs during coronary artery bypass grafting and cardiopulmonary bypass procedures. Unfortunately, at the present time, pharmacological agents capable of effectively limiting the ultimate size of myocardial infarcts associated with IRI are unavailable. Therefore, many recent studies have focused on the adaptive and endogenous mechanisms protecting the heart against IRI.

A phenomenon referred to as ischemic preconditioning (IP) has been observed in several animal experiments. The ability of repetitive and short-term coronary artery occlusion to protect against subsequent long-term ischemia was first demonstrated by Murry et al. in dogs [1]. Altered energy metabolism, stress proteins, ATP-sensitive potassium channels and adenosine have all been proposed to play a role in the protection against myocardial infarction afforded by IP [2,3]. IP confers two distinct “protective windows”, an acute window that occurs immediately after brief repetitive episodes of ischemia and a delayed window that occurs 24 h after preconditioning [4]. Elevation of myocardial antioxidant activity and increased levels of antioxidant enzymes are involved in the delayed phase of myocardial protection against IRI. Although both the acute and delayed protective windows play pivotal roles in resistance to subsequent severe IRI, the delayed protective window has more persistent beneficial effects on ischemic tissue. For both practical and ethical reasons, it is difficult to extend the use of IP to clinical situations and a more effective approach may be to elicit an antioxidant response that would confer resistance to IRI.

Ozone, which has been suggested to play a role in inflammation as an immune effector [5] and to function as a signaling molecule in physiological processes [6], has been used to treat many diseases [7]. As with IP, repetitive pretreatment with small doses of ozone, referred to as ozone oxidative preconditioning (OzoneOP), has been demonstrated to protect against IRI in rat models of renal, hepatic and cardiac ischemia [8–10]. The specific mechanisms by

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which OzoneOP prevents or attenuates IRI are, however, unclear. In the present study, we aimed to address whether mild oxidative stress induced by ozone might protect the myocardium against IRI by increasing local antioxidant capacity.

2. Material and methods

2.1. Animal preparation and experimental design

Adult male Sprague-Dawley rats (200–250 g) were used for the OzoneOP and cardiac IRI models. Rats were acclimated in a quiet environment and fed a standard diet before the experiments. Ozone was generated using an ozone therapy device (HUMARES, Bruchsal, Germany). The study was approved by the Animal Care and Use Committee of Harbin Medical University.

2.1.1. Experiment 1

Rats were randomly divided into two groups (n = 6/group): an oxygen (control) group and an ozone (test) group. Oxygen (2 ml once daily for 5 days) was administered intraperitoneally (i.p.) to the control group and an oxygen/ozone mixture (2 ml once daily for 5 days) was administered i.p. to the test group. (Fig. 1a and b). The dose of ozone administered to the test group was 100 μg/kg/day. On day 6, the rats were sacrificed and the left ventricles (LVs) were harvested and stored in liquid nitrogen for further tests.

2.1.2. Experiment 2

Rats were randomly divided into three groups (n = 6/group): a sham group that were treated i.p. with oxygen (2 ml once daily for 5 days) and underwent left thoracotomy without additional procedures on day 6 (Fig. 1c), an I/R group that were treated i.p. with oxygen (2 ml once daily for 5 days) and were subjected to surgical cardiac ischemia-reperfusion (I/R) on day 6 (Fig. 1d) and an I/R + Ozone group that were treated i.p. with an oxygen/ozone mixture (2 ml, containing 100 μg/kg ozone, once daily for 5 days) and were subjected to cardiac I/R on day 6 (Fig. 1e). After surgery on day 6, all rats were sacrificed and areas of heart compromised by I/R insult were removed to evaluate mitochondrial injury and its effects on morphology and protein and mRNA levels.

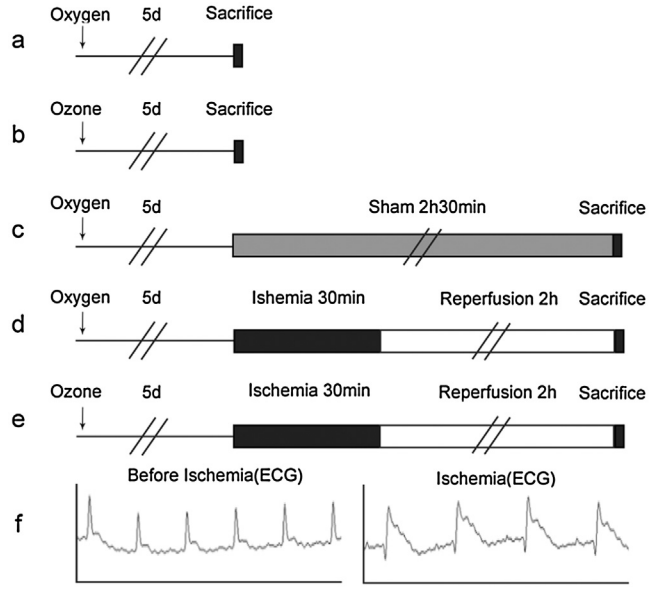

Fig. 1. Treatment groups used in this study and electrocardiograms (ECG) before and after ligation of left anterior descending coronary artery (LAD). (a) Control group in Experiment 1; (b); Ozone group in Experiment 1; (c) Sham group in Experiment 2; (d) I/R group in Experiment 2; (e) I/R + Ozone group in Experiment 2; (f) ECG results before and after ligation of LAD.

2.1.3. Surgical preparation

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed on a surgical table in a supine position. Electrocardiograms (ECGs) were continuously monitored and recorded. A tracheotomy was carried out, followed by endotracheal intubation with a 16-g needle connected to a small animal ventilator. Left thoracotomy was performed at the third or fourth intercostal space and the pericardium was opened to expose the heart. After exposure of the proximal left anterior descending coronary artery (LAD) adjacent to the pulmonary trunk and left atrial appendage, a single pledgeted 5-0 polypropylene suture was passed around the LAD, followed the path of the needle which penetrated the pledget and was secured by a snare to occlude the LAD. Fastening and loosening the snare produced ischemia and reperfusion of the heart. The duration of cardiac ischemia was 30 min and the duration of reperfusion was 2 h. Successful generation of cardiac ischemia was demonstrated by a marked ST segment elevation in the ECG immediately after ligation of the LAD (Fig. 1f).

2.2. Nuclear and mitochondrial isolation

LVs were homogenized using a Dounce homogenizer (Kimble Chase, Vineland, NJ, USA), followed by isolation of the nuclei (for Experiment 1) and mitochondria (for Experiment 2). The nuclear extraction kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) and mitochondrial isolation kit (Thermo Fisher Scientific, Inc.) were both used according to the manufacturer's instructions.

2.3. Morphology under electron microscope

LV biopsies (not exceeding 1 mm^3) from rats in Experiment 2 were removed for examination by electron microscopy. The samples were pre-fixed in 3% glutaraldehyde (4 °C) and 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide, dehydrated using an ethanol gradient and embedded in epon resin. Ultrathin sections (80 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined under an H-7650 electron microscope (Hitachi-Science & Technology, Berkshire, United Kingdom).

2.4. Quantitative real-time PCR

Total RNA was extracted from LVs using Trizol Reagent (Thermo Fisher Scientific, Inc.) and Direct-zol™ RNA kits (Zymo Research, Irvine, CA, USA), according to the manufacturers' instructions. The levels of purification and concentration of the RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was produced from total RNA using a PrimeScript™ RT reagent kit (Takara Biomedical Technology, Beijing, China). Quantitative real-time PCR was performed using an AccuPower® 2X Greenstar qPCR Master Mix kit (Bioneer, Daejeon, Republic of Korea). The sequences of primers for nuclear factor (erythroid-derived 2)-like 2 (Nrf2), glutamate-cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM) and β-actin are shown in Table 1. β-Actin was used as an endogenous control. Nrf2, GCLC and GCLM mRNA levels were determined using a LightCycler 480 System (Roche Diagnostics, Indianapolis, IN, USA).

2.5. Western blot

Levels of TATA-binding protein (TBP), cytochrome c oxidase subunit IV (COX4), Nrf2, microtubule-associated protein 1 light

Table 1
Sequences of primers used in RT-PCR studies.

No.	Sequence	Primer name
1	TGCCATTAGTCAGTCGCTCTC	Forward Nrf2
2	ACCGTGCCTTCAGTGTGC	Reverse Nrf2
3	TTCATTTCCCAGGCTAGG	Forward GCLC
4	TGGCACATTGATGACAAC	Reverse GCLC
5	TGGTCAGGGAGTTTCCAGATG	Forward GCLM
6	CGATCCTACAATGAACAGTTTAGC	Reverse GCLM
7	GAGGGAAATCGTGCGTGAC	Forward β-actin
8	GCATCGGAACCGCTCATT	Reverse β-actin

Abbreviations: GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

chain 3 (LC3B), PTEN-induced putative kinase 1 (PINK1), Parkin, cytochrome c, cleaved caspase 3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined by western blot. Briefly, myocardium from rats subjected to IRI was dissected and homogenized. Cytoplasmic, nuclear, mitochondrial and total protein samples were separated using 12% SDS-PAGE. After electrophoresis, samples were transferred to PVDF membranes and incubated with antibodies against TBP (Abcam, Cambridge, MA, USA), COX4 (Bioworld Technology, Nanjing, China), Nrf2 (Abcam), LC3B (Cell Signaling Technology, Danvers, MA, USA), PINK1 (Abcam), Parkin (Abcam), cytochrome c (Abcam), cleaved caspase 3 (Cell Signaling Technology) and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were subsequently incubated with peroxidase-conjugated secondary antibody (ZSGB-BIO, Beijing, China). After rinsing, the blots were developed using the enhanced chemiluminescence method and scanned using an ImageQuant LAS 4000 mini system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The amount of protein was assessed by quantitative densitometry analysis, using a digital image analyzing system (ImageJ, Version 1.51 g, NIH, Bethesda, ML, USA).

2.6. TUNEL analysis

Myocardial apoptosis was quantified using a terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) kit (Roche Diagnostics), using the manufacturer's protocol. The sections were stained with 4′,6-diamidino-2-phenylindole (DAPI) for examination under a fluorescence microscope. The total numbers of TUNEL-positive cells were counted in five different fields for each section in a blinded manner using light microscopy at ×400 magnification.

2.7. Statistical analysis

Statistical analysis of RT-PCR, western blots and apoptotic index results were performed using Student's *t*-test and one-way analysis of variance (ANOVA), in conjunction with Scheffe's post hoc test. Values are expressed as mean ± S.E.M. (n = 6 per group), with $p < 0.05$ considered to be statistically significant.

3. Results

3.1. Validation of nuclear extracts and mitochondrial fractions from LV

TBP is a general transcription factor located in the nucleus that can be used as a marker to test the purity of nuclear extracts. The expression of TBP in cytoplasmic extracts was less than in nuclear extracts for the oxygen (control) and ozone (test) groups in Experiment 1, indicating high quality purification (Supplementary file, a). A highly specific antibody against COX4, a marker for mitochondria-enriched fractions, was used to confirm by western blot that the mitochondrial fraction had been successfully separated from the cytoplasmic fraction. In Experiment 2, expression of COX4 was virtually undetectable in the LV cytoplasmic fraction but strongly positive in the mitochondrial fraction from the sham, I/R and I/R + Ozone groups (Supplementary file, b).

3.2. Ozone initiates oxidative preconditioning by activating Nrf2 and increasing expression of downstream antioxidant proteins

Levels of both Nrf2 mRNA (Table 2) and cytoplasmic Nrf2 protein (Fig. 2a) were increased in ozone-treated rats compared with control rats. The level of translocation of Nrf2 from the cytoplasm into the nucleus was also higher in the ozone-treated group, indicating activation of Nrf2 (Fig. 2b). Nrf2 regulates the expression of many antioxidant enzymes, including glutamate-cysteine ligase (GCL), which comprises a catalytic subunit (GCLC) and modifier subunit (GCLM) [11]. Activation of Nrf2 by ozone-mediated oxidative preconditioning increased expression of mRNA for GCLC and GCLM and also increased expression of mRNA for superoxide dismutases (SODs) (Table 2).

3.3. Antioxidant activities were compromised after I/R

IRI markedly suppressed expression of mRNA for antioxidant enzymes in the I/R model. This suppression was prevented by ozone pretreatment, analogous to the OzoneOP-induced enhancement of antioxidant activities described above (Table 3). Ischemic reperfusion preconditioning has also been shown to prevent decreases in SOD1, SOD2, SOD3, and GPx mRNA expression following I/R in mice [12].

3.4. Transmission electron microscopy of cardiomyocytes

Intracellular architecture was intact and morphology was normal in the sham group (Fig. 3a), whereas sarcomere hypercontraction, a characteristic manifestation of IRI, was observed in the I/R group (Fig. 3b). Mitochondrial swelling, with decreased matrix density and separation of cristae, intermyofibrillar edema and rupture of sarcomeres were also seen in the I/R group (Fig. 3c). Irregular sacomeres and mitochondria were seen in the I/R + Ozone group, indicating relatively mild mitochondrial injury compared with the I/R group (Fig. 3d). Autophagosome with a double membrane containing intact mitochondria were seen in the I/R group, possibly indicating the start of mitophagy (Fig. 3e). Mitochondrial debris was seen in autolysosomes with a single membrane in the I/R group (Fig. 3f).

Table 2
Expression levels of mRNA for Nrf2, GCLC, GCLM, SOD1 and SOD2 in LV from control and ozone groups.

	Fold vs. Normal	
	Normal	Ozone
Nrf2	1.00 ± 0.07	7.07 ± 0.98[a]
GCLC	1.01 ± 0.09	4.94 ± 0.63[a]
GCLM	1.00 ± 0.03	4.22 ± 0.52[a]
SOD1	1.00 ± 0.07	1.32 ± 0.06[a]
SOD2	0.94 ± 0.08	1.33 ± 0.09[a]

Abbreviations: GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; LV, left ventricle

[a] $p < 0.05$ versus control group.

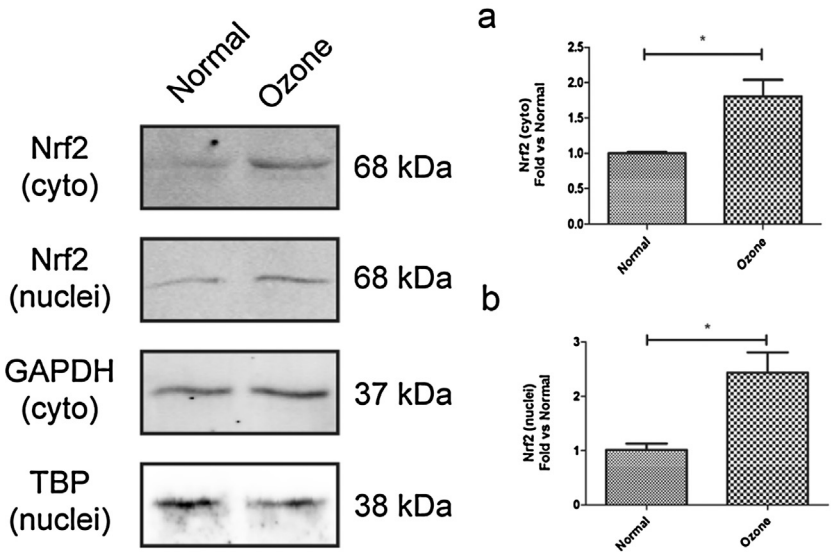

Fig. 2. Ozone activates Nrf2 and initiates expression of downstream modulators GCLC and GCLM in ozone treated rats. Western blots showing increased levels of Nrf2 in (a) cytoplasm and (b) nuclear extract in ozone-treated rats. Abbreviations: Nrf2, nuclear factor (erythroid-derived 2)-like 2; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TBP, TATA-binding protein. $^{*}p < 0.05$ versus control group.

Table 3
Expression levels of mRNA for Nrf2, GCLC, GCLM, SOD1 and SOD2 in LV from sham, I/R and I/R + Ozone groups.

	Sham	Fold vs. sham I/R	I/R + Ozone
Nrf2	1.00 ± 0.09	0.38 ± 0.02[a]	0.89 ± 0.04[b]
GCLC	1.01 ± 0.07	0.22 ± 0.07[a]	0.49 ± 0.09[a]
GCLM	1.01 ± 0.11	0.34 ± 0.08[a]	0.69 ± 0.13
SOD1	1.00 ± 0.08	0.43 ± 0.01[a]	1.30 ± 0.11[b]
SOD2	1.00 ± 0.03	0.32 ± 0.03[a]	1.02 ± 0.08[b]

Abbreviations: I/R, ischemic reperfusion; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; Nrf2, nuclear factor (erythroid-derived 2)-like 2; SOD1, superoxide Dismutase 1; SOD2, superoxide dismutase 1; LV, left ventricle.
[a] $p < 0.05$ versus sham group.
[b] $p < 0.05$ versus I/R group.

3.5. Effects of ozone pretreatment on mitochondrial injury

Ozone pretreatment significantly decreased the ratio of LC3B-II to LC3B-I (Fig. 4a). Levels of PINK1 protein were significantly reduced in the I/R + Ozone group compared with the I/R group (Fig. 4b). More Parkin proteins were translocated into the mitochondria in the I/R group than in the I/R + Ozone group (Fig. 4c). The ratio of Cyt-C in cytoplasm to Cyt-C in mitochondria, used to demonstrate the extent of Cyt-C release from the mitochondria into the cytoplasm, was significantly higher in the I/R group (Fig. 4d). Cleaved caspase 3, the degradation product of caspase 3, was significantly increased in the I/R group (Fig. 4e).

3.6. TUNEL assay

TUNEL staining was used to identify apoptosis in each group. Apoptotic cells, which are TUNEL-positive, were labeled with green fluorescence. DAPI was used as a nuclear marker for all cells. The apoptotic index was expressed as the percentage of TUNEL-DAPI double-labled nuclei over the total number of nuclei labeled by DAPI. The apoptotic index of the I/R + Ozone group (19.86 ± 2.16%) was significantly increased ($p = 0.000$, $p < 0.05$) compared with the sham group (0.77 ± 0.21%) and decreased ($p = 0.013$, $p < 0.05$) compared with the I/R group (29.12 ± 2.04%) (Fig. 5).

4. Discussion

Nrf2 is an important transcription factor that controls expression of antioxidant enzymes that protect against oxidative damage caused by injury and inflammation [13]. After activation of Nrf2, many beneficial downstream proteins, including NAD(P)H quinone oxidoreductase 1 (Nqo1) [14], glutamate-cysteine ligase [11], heme oxygenase-1 (HO-1) [15] and glutathione S-transferase (GST) family members [16], are induced.

The first objective of the present study was to determine whether multiple episodes of pretreatment with low-dose ozone might initiate OzoneOP *via* activation of Nrf2. In Experiment 1, we found increased expression of Nrf2 mRNA and protein in the hearts of rats treated with ozone and increased translocation of Nrf2 into the nucleus, which is indicative of activation of Nrf2. In Experiment 2, we found that expression of mRNA for antioxidant enzymes was markedly suppressed in the I/R group, whereas the effect was less pronounced in rats pretreated with ozone. This suggests that ozone might cause local activation of Nrf2 in the rats' hearts. Increased antioxidant capacity may be a pivotal mechanism for reducing oxidative stress and protecting against IRI in the I/R + Ozone group. Downregulation of antioxidant enzymes caused by the insult of IRI *per se* [17,18] could be responsible for suppression of antioxidant systems. A recent study has shown that treatment of blood with ozone before reinfusion in human autohemotherapy triggers activation of Nrf2 in circulating mononuclear cells [19]. Valacchi et al. have demonstrated that ozone exposure can also elicit a significant transient induction of Nrf2 in keratinocytes [20].

Ozone-induced biochemical effects are mediated by products formed from reactions of ozone with hydrosoluble antioxidants, especially polyunsaturated fatty acids (PUFA) bound to albumin [21]. Lipid oxidation products and hydrogen peroxide produced from interactions of ozone with PUFA [22] act as messengers that mediate many biochemical and therapeutic effects. Bell et al. reported that mild oxidative stress, caused by sub-toxic levels of hydrogen peroxide, is able to activate Nrf2 in astrocytes, thereby contributing to neuroprotective ischemic preconditioning [23]. We used Nrf2 as a marker of OzoneOP to indirectly indicate the relationship between antioxidant capacity and oxidative stress.

The therapeutically effective, non-toxic, concentration range of ozone is defined as the "Therapeutic Window". This has been

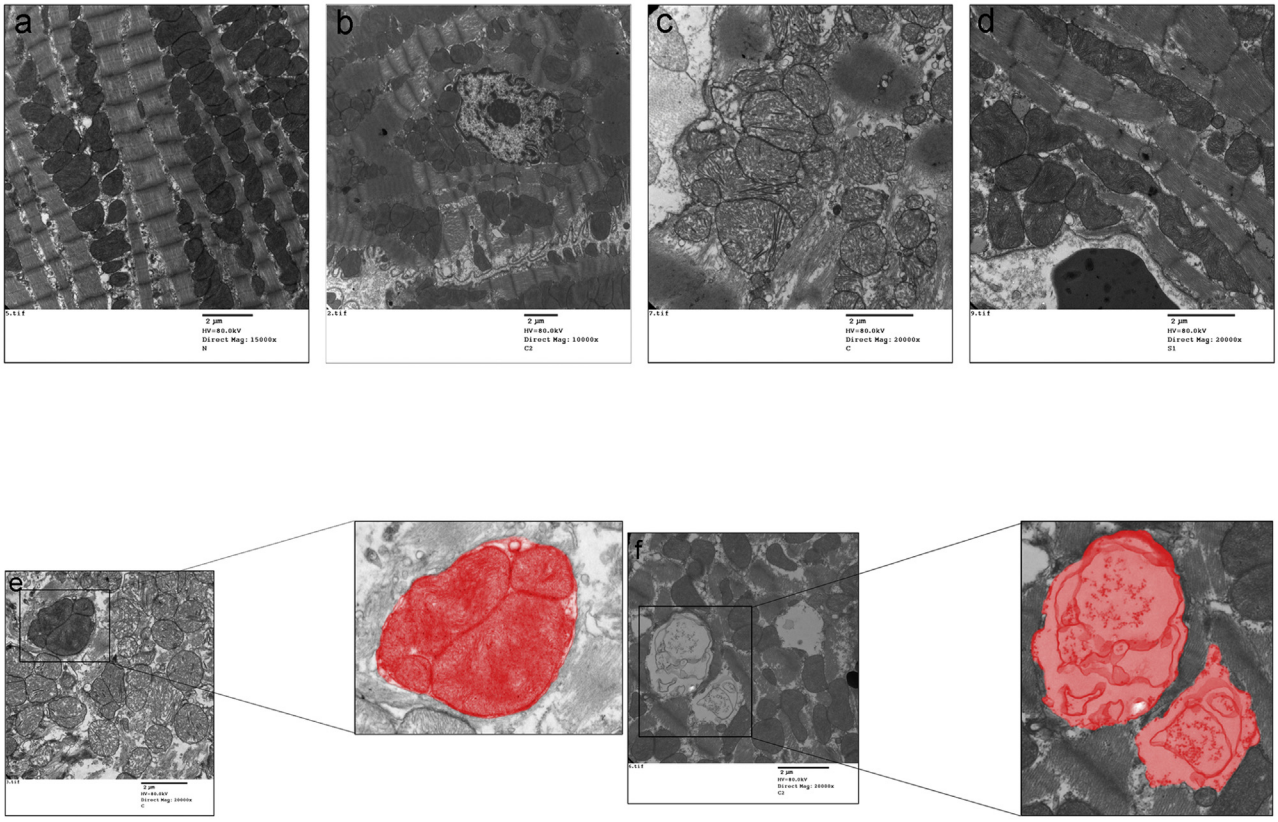

Fig. 3. Electron micrographs showing ultrastructures of sham group, I/R group and I/R + Ozone group. (a) Normal sacomeres and mitochondria in sham group. Bar = 2 μm, original magnfication = × 15,000. (b) Hypercontractive sarcomeres caused by IRI in I/R group. Bar = 2 μm, original magnfication = × 10,000. (c) Mitochondrial edema with decreased matrix density and separation of cristae in I/R group. Bar = 2 μm, original magnfication = × 20,000. Irregular sacomeres and mitochondria in I/R + Ozone group. Bar = 2 μm, original magnfication = × 20,000. (e, f) Autophagosomes in I/R group. Bar = 2 μm, original magnfication = × 20,000. Abbreviation: IRI, ischemic reperfusion injury.

carefully calculated and found to be 10–80 μg/ml of ozone in blood [7]. Typical regimens for clinical application in autohemotherapy use 200 ml of oxygen/ozone mixed with 180 ml of blood and 20 ml of anticoagulant [21]. Allowing for a body weight of ∼250 g and a blood volume of ∼14 ml [24] in Sprague-Dawley rats, which equates to 0.3% of that of adult humans, we selected an ozone dose for this study of 100 μg/kg since this is within the "Therapeutic Windows" defined for humans. We showed that one of the mechanisms by which this small, calculated, dose of ozone triggered mild oxidative stress (i.e. OzoneOP) in rats was increasing the activity of Nrf2.

The second objective of the study was to explore whether the beneficial effects of OzoneOP on IR-compromised myocardium mediate protection against mitochondrial injury. It is well established that I/R insult initiates myocardial apoptosis and necrosis. We found that levels of cleaved caspase 3, an activated form of caspase 3, were lower in the I/R + Ozone group than in the I/R group. Analysis of apoptosis, using a TUNEL assay and western blot, suggested that treatment with ozone before IR attenuated myocardial injury by inhibiting activation of apoptosis. Caspases 3 is an effector caspase activated by both extrinsic and intrinsic pathways [25,26] that proteolytically degrades many intracellular proteins involved in programmed cell death. We detected myocardial apoptosis attributable to the intrinsic apoptotic pathway (also referred to as the mitochondrial pathway), confirming that mild myocardial apoptosis is a result of the ability of OzoneOP to reduce mitochondrial injury. We also observed less release of Cyt-C from mitochondria into cytoplasm in the I/R + Ozone group than in the I/R group. Cardiolipin-bound Cyt-C anchored to the outer surface of the inner mitochondrial membrane is a component of the electron transport chain in mitochondria that controls electron transport and apoptosis. Upon insult by reactive oxygen species, Cyt-C functions as a peroxidase that catalyzes peroxidation of cardiolipin, leading to the release of pro-apoptotic factors, including Cyt-C [27], and triggering the caspase cascade. Release of Cyt-C was thus used as a marker for mitochondrial injury in this study.

As well as promoting release of Cyt-C from mitochondria, we found that OzoneOP decreases the ratio of LC3B-II to LC3B-I, indicating that mitophagy (the selective degradation of mitochondria by autophagy) might be involved in OzoneOP. LC3B-II, a lipid-conjugated form of LC3B, is commonly used as a marker of autophagy [28] since conversion of LC3B-I to LC3B-II indicates formation of autophagosomes. We found that both levels of PINK1 and accumulation of Parkin in mitochondria were reduced by OzoneOP. Mitophagy is the process of selective removal of damaged mitochondria by autophagosomes (Fig. 3ef), followed by catabolism by lysosomes. PINK1 is degraded by proteases within the mitochondria under normal conditions, leaving a low concentration of PINK1 for checking aberrant mitochondria. When mitochondria are damaged, however, cleavage of PINK1 is blocked and PINK1 accumulates in unhealthy mitochondria [29], with subsequent activation of Parkin, a type of E3 ubiquitin ligase. Recruitment of Parkin to mitochondria activates the ubiquitin-proteasome system, which is required for the widespread degradation of outer membrane proteins preceding mitophagy [30], and mediates engulfment of damaged mitochondria by autophagosomes [31]. The autophagy adaptor protein p62/

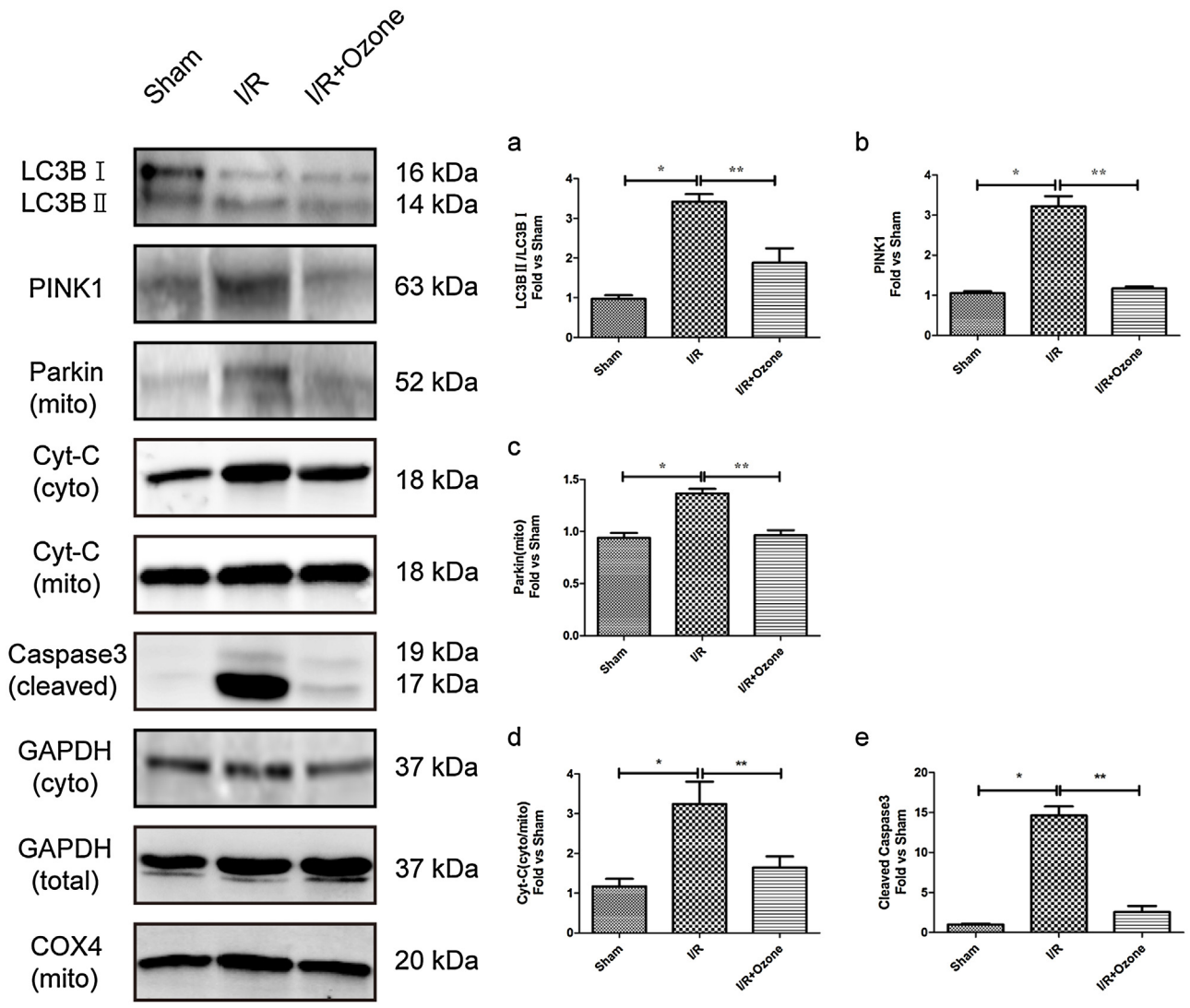

Fig. 4. Effect of ozone pretreatment on mitochondrial injury. IRI significantly increased the ratio of LC3B-II to LC3B-I (a) and PINK1 protein levels (b), compared with ozone pretreatment. A trend towards elevation was seen in the mitochondrial fraction of the I/R group compared with the ozone pretreatment group (c), but this did not reach statistical significance. The ratio of Cyt-C in cytoplasm to Cyt-C in mitochondria (d) and levels of cleaved caspase 3 (e) significantly increased in the I/R group compared with the I/R + Ozone group. Abbreviations: COX4, cytochrome c oxidase subunit IV; Cyto, cytoplasm; Cyt-C, cytochrome c; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRI, ischemic reperfusion injury; LC3B, microtubule-associated protein 1 light chain 3(MAP1LC3B); Mito, mitochondria; PINK1, PTEN-induced putative kinase 1. $^{*}p < 0.05$ vesus sham group; $^{**}p < 0.05$ versus I/R group.

SQSTM1 bridges damaged mitochondria and autophagosomes by binding to ubiquitinated proteins on mitochondria and to LC3 on phagophores [32,33], culminating in fusion of autophagosomes with lysosomes and degradation of damaged mitochondria by lysosomal enzymes [34]. A recent study found that autophagy was activated 30 min after coronary ligation in mice [35].

Matsui et al. showed that autophagy is initially induced by ischemia and further enhanced by reperfusion in mice [36]. They also found that autophagy may be protective during ischemia, whereas it may be harmful during reperfusion. It was hypothesized that severe stress, such as ischemia and subsequent reperfusion, results in excessive upregulation of autophagy (mitophagy) and causes cell death by excessive self-digestion of essential organelles such as mitochondria and proteins. It is very possible that both duration and extent of mitophagy are important in determining whether mitophagy is beneficial or detrimental to cardiomyocytes during reperfusion. It was inferred that more severely damaged mitochondria were present in the I/R group, in which more mitophagy occurred.

Mitophagy is a complex process involving a series of signal molecules and numerous modulators. The process of mitophagy comprises autophagosome synthesis, delivery of autophagic substrates (mitochondria) to the lysosome and degradation of autophagic substrates within the lysosome. The term “autophagic flux” is used to denote this dynamic process [37]. Our study demonstrated significant changes in some important proteins associated with mitophagy, which may mirror the extent of mitochondrial damage, but the occurrence of mitophagy should be further confirmed in future studies using specific agonists or antagonists to monitor mitophagy.

Because the heart has a high energy requirement, cardiomyocytes need many mitochondria to maintain normal heart function, rendering the heart especially vulnerable to mitochondrial injury. OzoneOP activates Nrf2 in rat hearts, leading to enhanced antioxidant capacity that protects the myocardium against IRI.

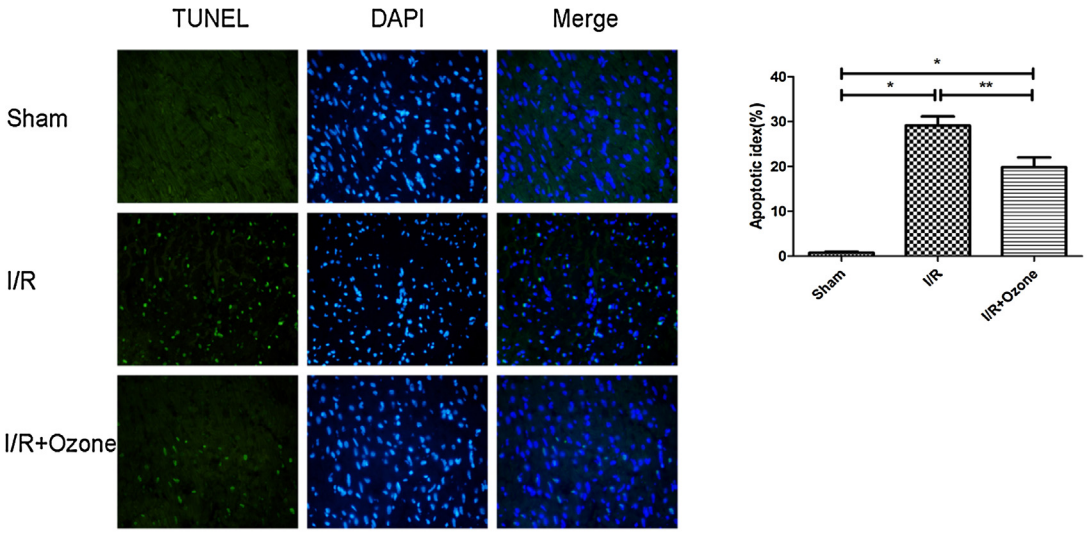

Fig. 5. TUNEL staining of sham, I/R and I/R + Ozone groups. TUNEL-positive (apoptotic) cells show green fluorescence and nuclei stained with DAPI show blue fluorescence under a fluorescence microscope (×200). The apoptotic index of the I/R + Ozone group (19.86 ± 2.16%) was significantly higher than that of the sham group (0.77 ± 0.21%) and lower than that of the I/R group (29.12 ± 2.04%). Abbreviations: TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.$^{*}p < 0.05$ versus sham group; $^{**}p < 0.05$ versus I/R group.

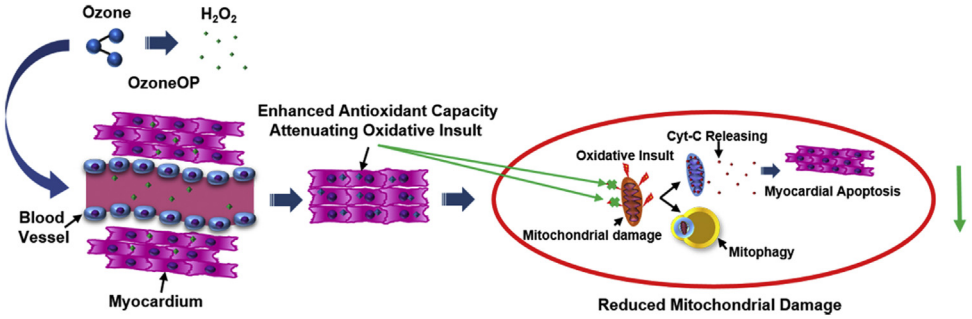

Fig. 6. Mechnisms of OzoneOP Reducing Mitochondrial Damage.
Abbreviations: OzoneOP, Ozone oxidative precondtioning; Cyt-C, cytochrome C.

OzoneOP does not protect against type I programmed cell death (apoptotic cell death) but rather protects against type II programmed cell death (autophagic cell death) as a result of reduced mitochondrial damage (Fig. 6). Our preliminary findings suggest a cardioprotective role for OzoneOP against IRI during cardiovascular surgery, cardiopulmonary bypass procedures or transplantation.

Author declaration

The authors declare that there are no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2017.01.151.

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