

Ozone oxidative preconditioning inhibits inflammation and apoptosis in a rat model of renal ischemia/reperfusion injury

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Abstract

Ischemia/reperfusion injury, which is commonly seen in the field of renal surgery or transplantation, is a major cause of acute renal failure. Previous studies showed that ozone oxidative preconditioning (OzoneOP) attenuated renal ischemia/reperfusion injury. The objective of this study was to examine the role of the OzoneOP in modulating inflammation and apoptosis after renal ischemia/reperfusion injury. Rats were subjected to 45 min of renal ischemia, with or without treatment with OzoneOP (1 mg/kg). Renal function, inflammation and apoptosis were compared at 24 h after renal injury. OzoneOP improved the renal dysfunction and reduced inflammation and apoptosis after ischemia/reperfusion injury. In conclusion, OzoneOP has potent anti-apoptotic and anti-inflammatory properties. These findings may have major implications in the treatment of human ischemic acute renal failure.

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Keywords: Ozone oxidative preconditioning; Ischemia/reperfusion; Apoptosis; Inflammation

1. Introduction

Renal ischemia, whether caused by shock or during surgery or transplantation, is a major cause of acute renal failure. Although reperfusion is essential for the survival of ischemic tissue, reperfusion itself causes additional cellular injury. It is important to improve the ability of organs to tolerate ischemic injury.

The pathophysiology of ischemic acute renal failure is very complex but ultimately results from tubular destruction, obstructive cast formation, and widespread vascular damage. These are thought to occur secondary to an intense inflammatory response initiated by the infiltration of leukocytes and the production of pro-inflammatory cytokines (Bonventre and Weinberg, 2003). Recent studies demonstrated that tubular cell apoptosis emerged as a primary and major contributor to the

pathophysiology of renal ischemia/reperfusion and determined the outcome of renal damage (Bonegio and Lieberthal, 2002; Padanilam, 2003). Nevertheless, as both inflammation and apoptosis coexist in renal ischemia/reperfusion, the ideal preventative or therapeutic approach would indeed target both processes.

Ozone (O₃) has been used as a therapeutical agent for the treatment of different diseases (Hernandez et al., 1995; Martínez-Sánchez et al., 2005). Moreover, ozone could prepare the host to face physiopathological events mediated by reactive oxygen species (Bocci, 2004). It has been demonstrated that ozone induced protective effects in renal and hepatic ischemia/reperfusion injury (Barber et al., 1999; Peralta et al., 1999, 2000; Ajamieh et al., 2002, 2004, 2005). This phenomenon was called ozone oxidative preconditioning (OzoneOP). It is a simple and harmless method which provides a new tool to protect organ from ischemia/reperfusion injury. However, it remains to be determined whether the protective effects of OzoneOP are associated with modulation of apoptosis and/or inflammation. In this study, the major purposes were to

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determine whether OzoneOP decreased the inflammatory response and attenuated apoptosis after renal ischemia/reperfusion injury.

2. Materials and methods

2.1. Animal preparation and experimental design

Adult male Wistar rats (250–280 g) were from the Center of Experimental Animal, Wuhan University. This project was approved by the committee of experimental animals of Wuhan University, and the procedures were carried out according to the routine animal-care guidelines. All experimental procedures complied with the Guide for the Care and Use of Laboratory Animals. Briefly, rats were anesthetized with pentobarbital (45 mg/kg, i.p.) and placed on a homeothermic table to maintain core body temperature at 37 °C. The midline laparotomy was made and the left kidney was subjected to 45 min of ischemia followed by reperfusion after right nephrectomy.

Rats were randomly separated into the following experimental groups. Group 1. sham-operated control ($n=12$): rats were subjected to surgical manipulation, without the induction of renal ischemia. Group 2. sham-operated control+OzoneOP ($n=15$): Before the sham-operated procedure (as in group 1), rats were treated with ozone as previously described (Ajamieh et al., 2002, 2004, 2005). Briefly, rats received 15 ozone treatments by rectal insufflations 1 mg/kg, once a day, at an ozone concentration of 50 $\mu\text{g/ml}$. Group 3. ischemia/reperfusion (I/R) ($n=15$): rats were subjected to left renal ischemia for 45 min followed by reperfusion. Group 4 ($n=15$). OzoneOP+I/R ($n=15$): Before the ischemia/reperfusion procedure (as in group 3), rats were treated with ozone (as in group 2). The left renal artery and vein were occluded with a non-traumatic clamp. Rats were killed at 24 h after ischemia/reperfusion injury. Blood samples (1 mL) were taken from the inferior vena cava for the measurement of urea nitrogen and creatinine.

2.2. Preservation of kidney

The left kidney was removed under fully maintained anaesthesia. After removal, the kidney was fixed in 10% phosphate-buffered formalin or immediately frozen, and stored at $-80\text{ }^{\circ}\text{C}$ for different determinations.

2.3. Serum assays

To assess creatinine and urea nitrogen, blood samples were collected, centrifuged and kept at $-20\text{ }^{\circ}\text{C}$ until analyses, adopting standard techniques using an Olympus AU 2700 Analyzer (Olympus Optical Co. Ltd, Tokyo, Japan).

2.4. Histological examination

The kidney was fixed in 10% neutral-buffered formalin, paraffin embedded and sectioned at 4 μm thick according to the

standard procedure. The sections were deparaffinized and hydrated gradually, and examined by HE staining. Morphological assessment was performed by an experienced renal pathologist who was unaware of the treatment. An established grading scale (scores of 0 to 4) for assessment of necrotic injury to the proximal tubules was used for the histopathologic assessment of ischemia/reperfusion-induced damage, as outlined by Jablonski et al. (1983) (Table 1).

2.5. Determination of oxidative Stress

Renal cortical samples from 4 groups ($n=6$ for each group) were used for experiments. The malondialdehyde (MDA) concentration was measured by the thiobarbituric acid (TBA) method. The amounts of lipid peroxides (LPO) were measured as the production of MDA. Absorbance was measured at 532 nm using a spectrometer (Assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

All protein concentrations of renal tissue homogenate samples were determined with Coomassie blue method (Assay kit was purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.6. Renal cortical myeloperoxidase (MPO) assay

MPO is an enzyme present in leukocytes and is a marker of leukocytes infiltration into the renal parenchyma (Donna-hoo et al., 1999a). Accumulation of leukocytes in the kidney was evaluated by measuring renal MPO activity as described previously (Okusa et al., 2000). Renal cortical samples ($n=6$ for each group) were weighed and homogenized in ice-cold 50 mM potassium phosphate buffer. The homogenate was centrifuged at 15,000 g for 15 min at $4\text{ }^{\circ}\text{C}$, and the resultant supernatant fluid was discarded. The pellet was washed twice, resuspended in 50 mM potassium phosphate buffer with 0.5% hexadecyltrimethylammonium bromide, and sonicated. The suspension was subjected to three freeze–thaw cycles, sonicated for 10 s, and centrifuged at 15,000 g for 15 min at $4\text{ }^{\circ}\text{C}$. The supernatant fluid was added to an equal volume of a solution consisting of o-dianisidine (10 mg/ml), 0.3% H_2O_2 , and 45 mM potassium phosphate,

Table 1
Histopathological description and grading scale, developed by Jablonski et al (1983)

Grade	Description
0	Normal
1	Mitosis and necrosis of individual cells
2	Necrosis of all cells in adjacent proximal convoluted tubules, with survival of surrounding tubules
3	Necrosis confined to the distal third of the proximal convoluted tubule, with a band of necrosis extending across the inner cortex
4	Necrosis affecting all three segments of the proximal convoluted tubule

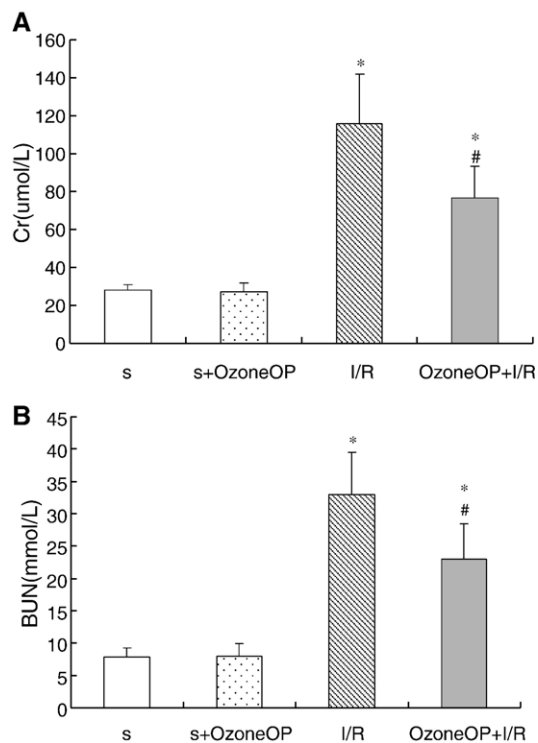


Fig. 1. Effect of OzoneOP on renal dysfunction caused by ischemia/reperfusion. OzoneOP treatment significantly improved renal dysfunction. (A) Effect of OzoneOP on the serum creatinine concentrations after 45 min of ischemia. (B) Effect of OzoneOP on the serum urea nitrogen concentrations after 45 min of ischemia. Bars represent means \pm S.E.M. ($n=12$); *, $P<0.05$ versus sham, #, $P<0.05$ versus ischemia/reperfusion. S: sham-operated rats; I/R: rats subjected to ischemia followed by reperfusion; OzoneOP+I/R: rats treated with OzoneOP before ischemia followed by reperfusion; S+OzoneOP: sham-operated rats treated with OzoneOP.

pH 6.0. Absorbance was measured at 460 nm over a period of 5 min.

2.7. RNA isolation and RT-PCR

Samples from 4 groups ($n=6$ for each group) were used for experiments. Total RNA (2 ug) was isolated by TRIzol reagent (Invitrogen) and reverse transcription was performed with the Revert Aid TMH Minus M-uLV Reverse Transcriptase kit (Fermentas Life Sciences) according to the manufacturer's instructions. PCR was performed with primers for Tumor necrosis factor- α (TNF- α) (F: CGAGTGACAAGCCCGTAG; R: GGATGAACACGCCAGTCG; 469 bp; GenBank accession no. NM012675), interleukin-1 β (IL-1 β) (F: CCAGGATGAGGACCCAAGCA; R: TCCCGACCATTGCTG TTTCC; 519 bp; GenBank accession no. NM031512), ICAM-1 (F: CGG TAG ACA CAA GCA AGAGA; R: GCAGGGATTGACCATAATTT; 517 bp; GenBank accession no. NM012967), and β -actin (F: TCATGAAGTGTGACGTTGACATCCGT; R: CCTAGAAGCATTGCGGTGCACGATG; 285 bp; GenBank accession no. NM031144). β -actin was used as an internal control for stable expression (housekeeping gene) in all experiments. PCR was performed by use of a Gene Cyclor (Bio-Rad). Initial denaturation was done at 94 °C for 5 min followed by 35 cycles of amplification. Amplification protocol was repeated cycles of denaturation (30 s, 94 °C), annealing (30 s; 56 °C), extension (1 min, 72 °C) and final extension (7 min, 72 °C). PCR products were electrophoresed through 2% agarose gels containing ethidium bromide (0.5 ug/ml). Gels were visualized under UV light, photographed and optical densities of the bands were analyzed using the Quantity One software (Bio-Rad).

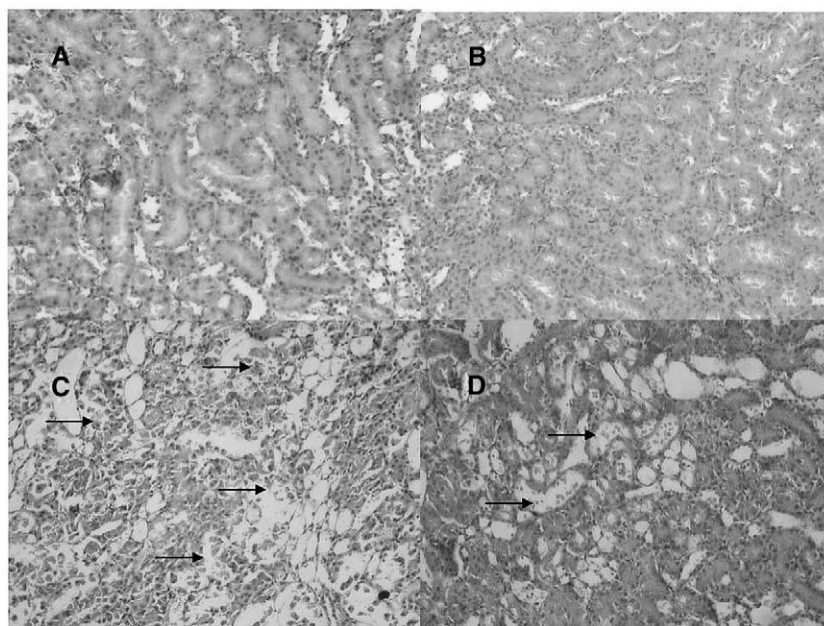


Fig. 2. Effect of OzoneOP on renal morphology after ischemia/reperfusion. A–D: representative HE-stained kidney sections after sham surgery or ischemia/reperfusion. (A) Section from sham-operated rat. (B) Section from sham-operated rat treated with OzoneOP. (C) Section from rat subjected to ischemia/reperfusion. (D) Section from rat subjected to ischemia/reperfusion and pretreated with OzoneOP. All Haematoxylin and Eosin $\times 200$. Arrows indicate necrotic cells in convoluted tubules.

2.8. DNA fragmentation analysis

Renal cortical samples from 4 groups ($n=4$ for each group) were used for experiments. Genomic DNA was extracted from samples using Easy DNA extraction kit (Fermentas Life Sciences). Ten micrograms of DNA were electrophoresed on a 2% agarose gel. Fragmented DNA was visualized by ethidium bromide under an UV light source.

2.9. Caspase-3 activity assay

Renal cortical samples from 4 groups ($n=6$ for each group) were used for experiments. Activities of caspase-3 were measured using commercialized caspase-3 activity kit (Beyotime Institute of Biotechnology, China). In brief, renal cortices were homogenized in lysis buffer. The lysate was centrifuged at 20,000 g for 10 min at 4 °C, and supernatants were incubated for 1 h at 37 °C with 10 μ l caspase-3 substrate (Ac-DEVDpNA) (2 mM). Substrate cleavage was measured with a spectrofluorometer at 405 nm.

2.10. Western blot analysis

The cytosolic/mitochondrial protein samples for Western blot analysis were prepared as described with some modifications (Matsumori et al., 2005). Whole-cell lysates were obtained by homogenizing the renal cortical samples ($n=6$ for each group) with a homogenizer in five volumes of buffer (20 mM Hepes, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 0.1 mM PMSF, 1 mM dithiothreitol (DTT) and proteinase inhibitor cocktail tablets; pH 7.9). Samples were further centrifuged at 750 g at 4 °C for 15 min to separate the sample into supernatant A and pellet A. Supernatant A, containing the cytosolic/mitochondrial protein, was further centrifuged at 16,000 g for 30 min at 4 °C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction and pellet B was used as the mitochondrial fraction after resuspension in buffer. The protein samples were separated on 10 or 12% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels (20–50 μ g/lane) and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk and then incubated with primary antibodies overnight at 4 °C. The primary antibodies and concentrations were as follows; Bcl-2, cytochrome c, and Bax (Santa Cruz Inc., 1:200), intercellular adhesion molecule (ICAM)-1 and caspase-3 (Santa Cruz Inc., 1:1000), β -actin and COX IV (Abcam Inc., 1:5000). After extensive rinsing, the membranes were incubated with secondary antibodies (Santa Cruz Inc., 1:2000) for 1 h at room temperature and then developed with the use of an enhanced chemiluminescence system (ECL kit, KPL Biotechnology Inc.).

2.11. Materials

Ozone (O_3) was generated by an OZOMED equipment manufactured by the Zhuhai Yike Medical Co., LTD (China). The ozone concentration was measured by spectrophotometer

set 254 nm (accuracy, 0.002A at 1 A, repeatability 0.001A and calibrated with internal standard). Ozone was obtained from medical grade oxygen and used immediately as generated.

2.12. Statistical analyses

All data are expressed as means \pm S.E.M. The Kolmogorov–Smirnov test was applied to test for a normal distribution. The means of the different groups were compared using one-way ANOVA Student–Newman–Keuls test. All statistical analyses were performed with the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL). Significant differences were accepted when P values were less than 0.05.

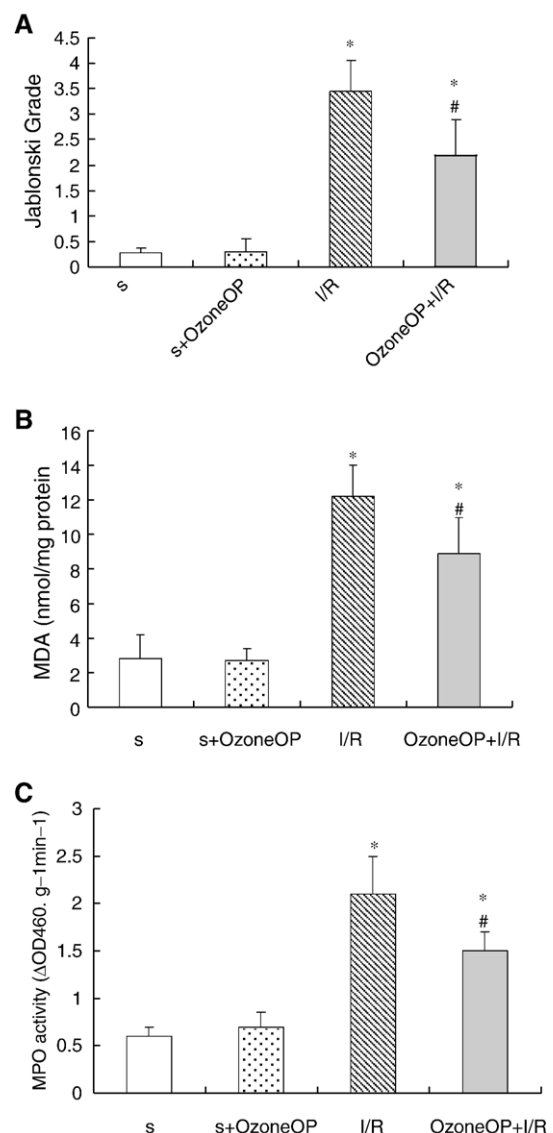


Fig. 3. (A) Effect of OzoneOP on the Jablonski grading scale scores at 24 h after reperfusion. Ischemia/reperfusion induced severe lesions in the kidney of rats and OzoneOP relieved these lesions. (B) Assay of MDA content in cortex. OzoneOP treatment inhibited the increase of MDA. (C) Assay of MPO activities in cortex. OzoneOP treatment reduced caspase-3 activity. Bars represent means \pm S.E.M. ($n=6$); *, $P < 0.05$ versus sham, #, $P < 0.05$ versus I/R.

3. Results

3.1. OzoneOP improves renal dysfunction

The renal functional parameters of rats were significantly different at 24 h after ischemia/reperfusion injury. Rats subjected to ischemia/reperfusion injury showed significant increases in urea nitrogen and creatinine compared with sham-operated rats. The renal function changes induced by ischemia/reperfusion were significantly improved by OzoneOP treatment (Figs. 1A, B).

3.2. OzoneOP improves the morphological features of injury

Renal ischemia/reperfusion resulted in significant renal injury as evidenced by tubular necrosis, medullary hemorrhage, congestion and development of proteinaceous casts. OzoneOP relieved these severe renal damages (Fig. 2). According to Jablonski scale histology grading scores, 45 min of renal ischemia followed by 24 h of reperfusion resulted in severe acute tubular necrosis. Quantitative analysis showed a dramatically decreased score in OzoneOP+I/R group compared with ischemia/reperfusion group (Fig. 3A).

3.3. OzoneOP attenuated oxidative stress

The MDA content in renal cortex as an index of lipid peroxidation was significantly higher in rats subjected to ischemia/reperfusion injury compared with sham-operated rats. This increase could be inhibited by OzoneOP treatment (Fig. 3B).

3.4. OzoneOP inhibits leukocytes accumulation

MPO activities in renal cortex were higher in ischemia/reperfusion group than in sham group. Treatment with OzoneOP significantly reduced MPO activities (Fig. 3C).

3.5. OzoneOP decreased ICAM-1, IL-1 β and TNF- α expression

To investigate the mRNA expression of ICAM-1, IL-1 β and TNF- α , we measured the levels of ICAM-1, IL-1 β and TNF- α by RT-PCR. The PCR products were separated on agarose-gel and the relative expression of ICAM-1, IL-1 β and TNF- α to β -actin are shown. The mRNA levels of ICAM-1, IL-1 β and TNF- α were significantly greater in ischemia/reperfusion group than in sham group. However, OzoneOP treatment inhibited the expression of ICAM-1, IL-1 β and TNF- α after renal ischemia/reperfusion (Fig. 4).

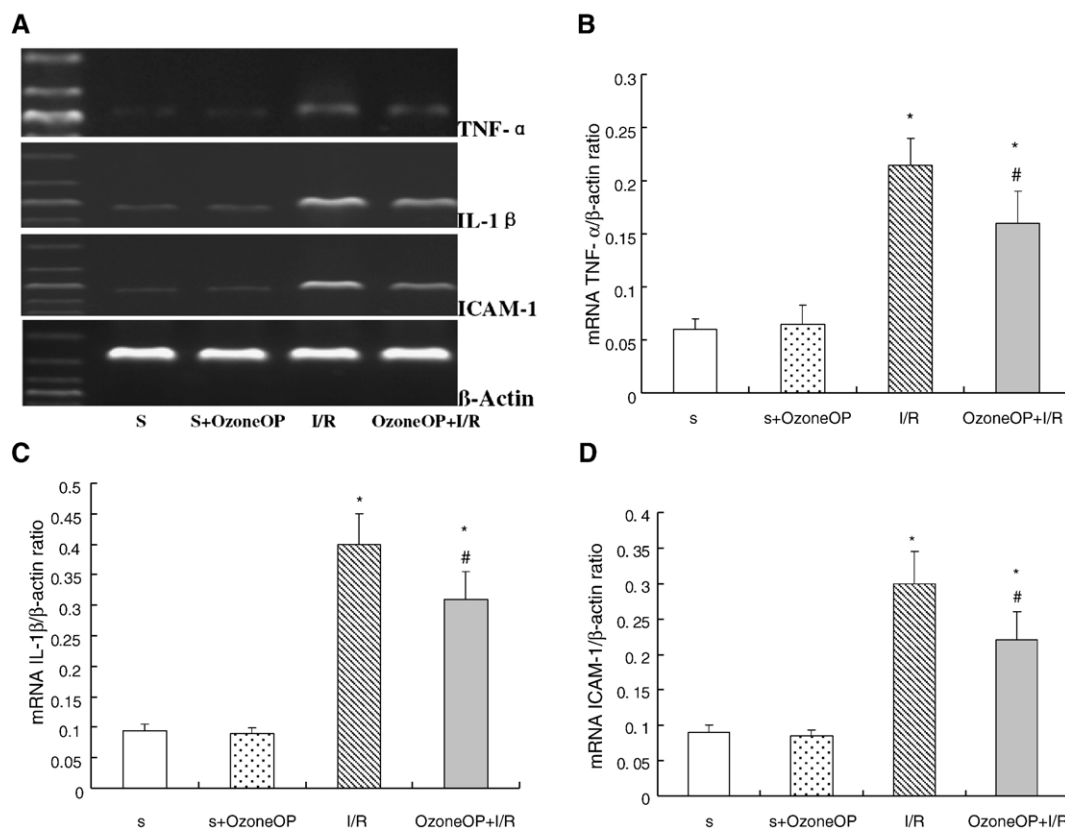


Fig. 4. Effect of OzoneOP on ICAM-1, IL-1 β and TNF- α mRNA expression in renal cortices at 24 h after reperfusion. (A) Representative RT-PCR results showing mRNA expression of TNF- α , IL-1 β , ICAM-1 and β -actin. (B) The relative band densities of TNF- α to the mean value of the control. (C) The relative band densities of IL-1 β to the mean value of the control. (D) The relative band densities of ICAM-1 to the mean value of the control. Bars represent means \pm S.E.M. ($n=6$); *, $P<0.05$ versus sham, #, $P<0.05$ versus I/R.

To determine whether OzoneOP inhibited ICAM-1 protein expression, we measured the level of ICAM-1 by Western blot analysis. Consistent with the RT-PCR analyses, renal ischemia/reperfusion increased ICAM-1 protein expression. This increase was suppressed by OzoneOP treatment (Fig. 7).

3.6. OzoneOP reduces cell apoptosis after renal ischemia/reperfusion

Apoptosis was evaluated by DNA fragmentation analysis and caspase-3 proteolytic activity. The typical DNA laddering pattern and increased caspase-3 activity were observed in ischemia/reperfusion group. OzoneOP treatment decreased ischemia/reperfusion-induced DNA fragmentation and caspase-3 activity (Fig. 5).

The mitochondrial and cytosolic expressions of cytochrome c were significantly decreased and increased, respectively, in ischemia/reperfusion group compared with the sham group. The cytochrome c release was significantly attenuated by OzoneOP

treatment (Fig. 6). Renal ischemia/reperfusion injury, also, decreased cytosolic Bax levels and enhanced mitochondrial Bax content compared with the sham group. Whereas the administration of OzoneOP reduced the ischemia/reperfusion-induced rise in mitochondrial Bax levels and restored cytosolic Bax levels (Fig. 6). In addition, the procaspases-3 and Bcl-2 levels were significantly reduced in ischemia/reperfusion group compared with the sham group. The administration of OzoneOP restored the levels of Bcl-2 and inhibited the decrease of procaspases-3 (Fig. 7).

4. Discussion

The major findings of this study were that the protective effects of OzoneOP in kidney were associated with inhibition of inflammation and apoptosis. The production of reactive oxygen species is a major pathophysiological component of renal ischemia/reperfusion injury. The generation of reactive oxygen species is also positively correlated with the degree of early apoptosis, inflammation, and necrosis in renal ischemia/reperfusion injury (Chien et al., 2001; Bonventre and Weinberg, 2003). Reactions of reactive oxygen species with biomolecules such as lipids can initiate chain reactions and lead to tissue damage. The optimal therapeutic strategy is to prevent the formation of reactive oxygen species as much as possible and neutralize the reactive oxygen species as early as possible. OzoneOP not only increases antioxidant endogenous systems but also regulates reactive oxygen species (Bocci, 2004). In previous study, Barber et al. (1999) demonstrated that OzoneOP attenuated oxidative stress and protected rat against renal ischemia/reperfusion injury, but a mechanism directly involving the kidney was not elucidated in this previous study. This study supported and extended previous findings (Barber et al., 1999), in that OzoneOP reduced urea nitrogen and creatinine, improved renal morphology and decreased MDA concentration. Moreover, we demonstrated for the first time that OzoneOP inhibited inflammation and apoptosis after renal ischemia/reperfusion injury in rat.

Inflammation is recognized as an important component of renal ischemia/reperfusion injury (Bonventre and Weinberg, 2003; Bonventre and Zuk, 2004). The key mediators that may fuel inflammatory reactions in renal ischemia/reperfusion injury are the leukocytes. Previous studies demonstrated that leukocytes infiltration into the kidney after ischemia/reperfusion injury participated in the pathogenesis of acute renal failure (Kelly et al., 1994, 1996; Heinzelmann et al., 1999). Leukocytes, including neutrophils, lymphocytes, and macrophages (Ysebaert et al., 2000), initiates a cascade of pro-inflammatory events involving cytokine/chemokine liberation and free radical-mediated tubular damage during reperfusion. Sublethally damaged proximal tubular epithelial cells release cytokines and up-regulate ICAM-1 to facilitate leukocytes infiltration and adhesion (Donnahoo et al., 1999a, b). Infiltrated cells were activated by various types of cytokines, including IL-1 β and TNF- α , and released lysosomal enzyme, prostaglandin and reactive oxygen species,

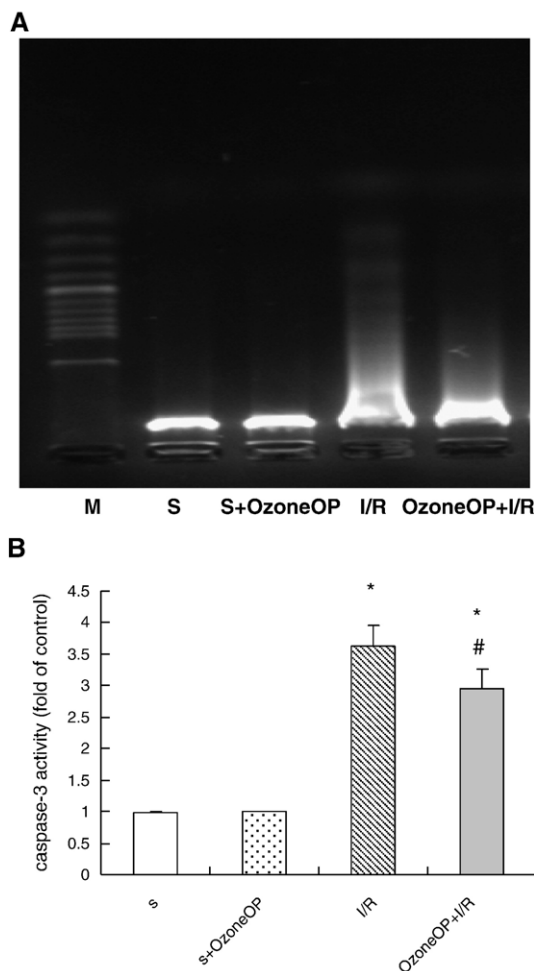


Fig. 5. (A) DNA fragmentation (laddering) in an ethidium bromide-stained agarose gel. Each lane represents DNA from an individual rat. A 100-bp DNA size marker (M) was loaded in the first lane. (B) Assay of caspase-3 activities in cortex. OzoneOP treatment reduced caspase-3 activity. Bars represent means \pm S.E.M. (n=6); *, $P < 0.05$ versus sham, #, $P < 0.05$ versus I/R.

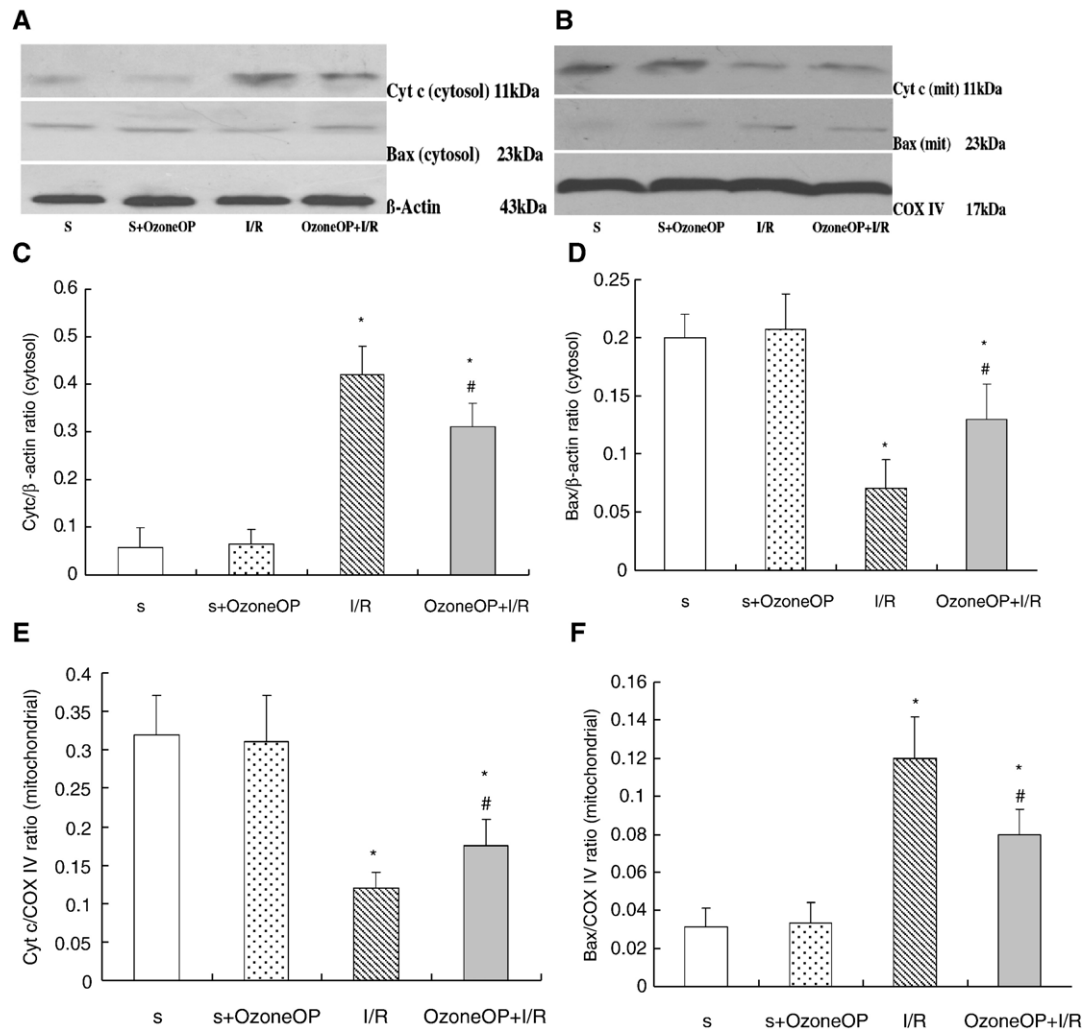


Fig. 6. Cytochrome c and Bax expressions at 24 h after reperfusion. OzoneOP treatment significantly reduced the accumulation of cytochrome c in the cytoplasm and the translocation of Bax to the mitochondria after ischemia/reperfusion injury. (A) Representative blots showing the effect of OzoneOP treatment on cytochrome c and Bax expressions in the cytoplasm. (B) Representative blots showing the effect of OzoneOP treatment on cytochrome c and Bax expressions in the mitochondria. (C) The relative band densities of cytosolic cytochrome c to the mean value of the control. (D) The relative band densities of cytosolic Bax to the mean value of the control. (E) The relative band densities of mitochondrial cytochrome c to the mean value of the control. (F) The relative band densities of mitochondrial Bax to the mean value of the control. Bars represent means \pm S.E.M. ($n=6$); *, $P<0.05$ versus sham, #, $P<0.05$ versus I/R.

which eventually induced renal tubular necrosis (Haq et al., 1998; Donnahoo et al., 1999b). On the other hand, IL-1 β and TNF- α play a key role in increasing the expression of ICAM-1 after injury (Collins et al., 1995; Donnahoo et al., 1999a). In this study, we observed that increased markers of inflammation (MPO activity and expression of IL-1 β , TNF- α and ICAM-1) were reduced by OzoneOP. Thus, our data indicated that OzoneOP reduced inflammatory responses after renal ischemia/reperfusion injury.

Renal apoptosis is an important factor in the development of acute renal failure after ischemia/reperfusion injury (Bonegio and Lieberthal, 2002; Padanilam, 2003). In response to oxidative load in the mitochondria, the outer membrane of mitochondria becomes permeabilized, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome c normally confined to the mitochondrial intermembrane space. Those proapoptotic proteins translocation is controlled by the Bcl-2 family

proteins (Antonsson et al., 1997; Yang et al., 1997; Jurgensmeier et al., 1998; Shimizu et al., 1999). Release of cytochrome c into the cytosol leads to the formation of the apoptosome, a complex comprised of apoptotic-protease-activating factor-1 (Apaf-1), procaspase-9 and ATP. The apoptosome permits the autoactivation of procaspase-9, which is followed by the activation of procaspase-3 (Li et al., 1997; Yang et al., 1997; Slee et al., 1999). Active caspase-3 activates the caspase activated DNAase, leading to DNA fragmentation. Our results showed that OzoneOP significantly inhibited apoptosis caused by renal ischemia/reperfusion injury, which was proved by DNA fragmentation and activated caspase-3. In order to further clarify the mechanism of OzoneOP protection, we investigated the expressions of key apoptotic related molecules. OzoneOP increased the levels of anti-apoptotic Bcl-2 protein and inhibited Bax translocation to the mitochondria and cytochrome c release from the mitochondria, thus attenuating

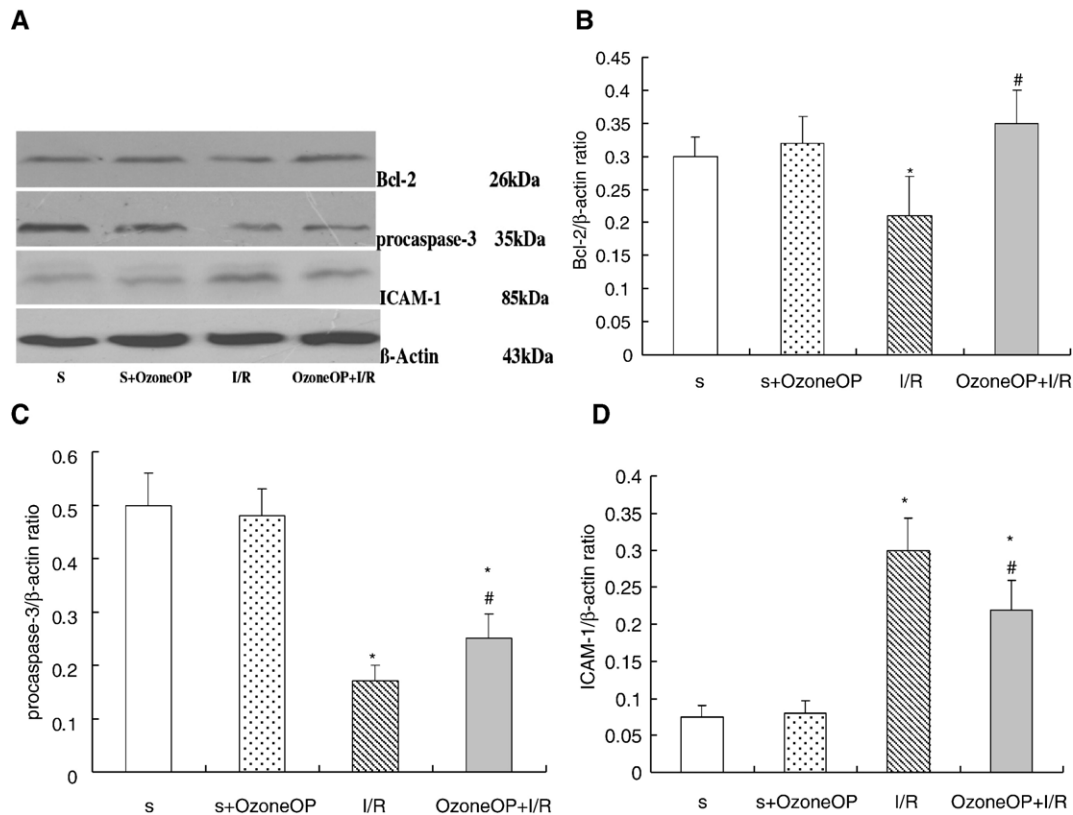


Fig. 7. Procaspases-3, Bcl-2 and ICAM-1 expressions at 24 h after reperfusion. Ischemia/reperfusion injury reduced procaspases-3 and Bcl-2 levels compared with the sham group. However, OzoneOP treatment restored the levels of Bcl-2 and inhibited the decrease of procaspases-3. In addition, the expression of ICAM-1 in ischemia/reperfusion group was greater than in sham group. OzoneOP treatment suppressed the increase of ICAM-1 after renal ischemia/reperfusion. (A) Representative blots showing the expression of procaspases-3, Bcl-2 and ICAM-1 in renal cortices. (B) The relative band densities of Bcl-2 to the mean value of the control. (C) The relative band densities of procaspases-3 to the mean value of the control. (D) The relative band densities of ICAM-1 to the mean value of the control. Bars represent means \pm S.E.M. ($n=6$); *, $P<0.05$ versus sham, #, $P<0.05$ versus I/R.

the downstream caspase activation. Our data suggested that the mitochondrial pathway was an important target for OzoneOP.

Apoptosis and inflammation are central mechanisms leading to organ damage in the course of renal ischemia/reperfusion (Daemen et al., 2002). Moreover, Daemen et al (1999, 2001, 2002) indicated that a significant component of inflammation is induced by apoptosis after renal ischemia/reperfusion injury. They also demonstrated that blockade of apoptosis prevented renal inflammation after ischemia/reperfusion. However, it was difficult to analyze whether decrease in apoptosis after renal ischemia/reperfusion injury was directly associated with inhibition of inflammation in this study.

In conclusion, we demonstrated that OzoneOP protected rats against ischemia/reperfusion-mediated renal injury. We further demonstrated that OzoneOP possessed both anti-apoptotic and anti-inflammatory properties after renal ischemia/reperfusion injury. These findings suggested the potential role of OzoneOP against renal failure during surgery or transplantation.

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