Combined Ischemic Postconditioning and Ozone Postconditioning Provides Synergistic Protection Against Renal Ischemia and Reperfusion Injury Through Inhibiting Pyroptosis

Lei Wang, Zhiyuan Chen, Xiaodong Weng, Min Wang, Yang Du, and Xiuheng Liu

OBJECTIVE To investigate whether ischemic postconditioning (IPO) and ozone postconditioning (OP) could synergistically attenuate renal ischemia-reperfusion (I/R) injury and its possible mechanism.

MATERIALS AND METHODS An in vivo rat model of renal I/R injury was established, and the serum and kidneys were harvested after reperfusion to assess renal function and histologic changes. For the in vitro study, the cultured NRK-52E cells were subjected to 3 hours of hypoxia (5% CO₂, 1% O₂, and 94% N₂) followed by 24 hours of reoxygenation (5% CO₂, 21% O₂, and 74% N₂). The mRNA expression levels were analyzed by real-time polymerase chain reaction, and the protein expression levels were analyzed by using Western blot, immunofluorescence staining and enzyme-linked immunosorbent assay.

RESULTS Kidneys undergone I/R showed characteristic renal dysfunction and pyroptosis. IPO or OP could prevent the elevated blood urea nitrogen and creatinine, renal damage, as well as pyroptosis, however, the combined application of them had more obvious protection. Oxidative stress and pyroptosis were increased in hypoxia and reoxygenation (H/R) model using NRK-52E cells. The combination of hypoxic postconditioning and OP had more protective effects on oxidative abnormalities and pyroptosis compared with the single application of hypoxic postconditioning or OP.

CONCLUSION Our in vivo and in vitro studies show the combination of IPO and OP synergistically protected the kidney from I/R by attenuating pyroptosis in kidney cells.

Renal ischemia-reperfusion injury (I/R) is a major cause of acute kidney injury (AKI) which often arises from hypovolemic conditions, septic shock, surgery, and transplantation. Although AKI contributes to high morbidity and mortality, to date there is no practical and effective treatment. I/R injury causes structural and functional damage of renal tubules by directly inducing death of tubular cells, and these dying cells may trigger damaged responses. As the importance of renal I/R is becoming increasingly evident, it is essential to develop new therapies to prevent renal damage caused by I/R.

Ischemic postconditioning (IPO) involves the application of a series of brief and rapid intervene at the beginning of reperfusion in ischemic tissue or organ. Compared with ischemic preconditioning, IPO is more clinically applicable and attracts greater attention. Medical ozone has been shown to have therapeutic effects in different diseases. Numerous studies have indicated that ozone postconditioning (OP) is able to attenuate organic I/R and is a relatively simple and harmless treatment compared with other therapies. Our previous report found that IPO could protect kidney from I/R injury through the inhibition of apoptosis. Also, we reported that OP could prevent oxidative stress and apoptosis caused by renal I/R. However, as far as we know, the combined application of IPO and OP on renal I/R injury has yet to be reported.

Pyroptosis is a proinflammatory programmed cell death. It has the morphologic characteristics of necrosis and apoptosis, but unlike apoptosis or necrosis (Nitro-oleic acid attenuates OGD/R-triggered apoptosis in renal tubular cells
via inhibition of Bax mitochondrial translocation in a PPAR-gamma-dependent manner. Cell Physiol Biochem], pyroptosis results in the release of cytokines that activate proinflammatory mediators. During pyroptosis, caspase-1 is activated by the pyroptosome and subsequently activates inflammatory cytokines interleukin (IL)-1β and IL-18 to trigger inflammatory responses. Thus, pyroptosis may not only lead to cell death but also activate the cascade of reactions that lead to damaged tissues. In the present study, we investigated the combined application of IPO and OP on renal I/R injury, both in vivo and in vitro. We also investigated the changes of pyroptosis related to these processes to determine whether and how IPO and OP provide synergistic protection against renal I/R injury.

**MATERIAL AND METHODS**

**Animal Preparation**

All adult Sprague Dawley rats (male, 220-250 g) were provided by the center of experimental animals in the medical college of our university. This project was approved by the committee of experimental animals of our university, and the procedures were carried out in accordance with routine animal-care guidelines. All procedures complied with the Guidelines for the Care and Use of Laboratory Animals. Before surgery procedures, rats were anesthetized with pentobarbital (45 mg/kg) and then placed on a homeothermic table to maintain core body temperature at 37°C. All rats then underwent a midline laparotomy followed by a right nephrectomy. Next, the left kidney was subjected to 45 minutes of ischemia with a non-trauma vascular clamp followed by 24 hours of unclamping.

All animals were divided into 5 different groups: sham-operated (sham) group, I/R group, I/R + IPO group, I/R + OP group, I/R + IPO + OP group. Eight rats were allocated to each group. In the sham group, only the right kidneys were removed. In the I/R group, the left kidney vessels were occluded using a clamp for 45 minutes; reperfusion was then permitted for 24 hours by unclamping. In I/R + IPO group, kidneys were subjected to 6 cycles of 10 seconds of reperfusion followed by 10 seconds ischemia immediately after 45 minutes of ischemia, then reperfusion 24 hours. In I/R + OP groups, after ischemic period, the rats administered with OP treatments (2 mg/kg), as previously described.9 In I/R + IPO + OP group, after ischemia, kidneys were subjected to 6 cycles of IPO, then followed by OP.

Then, the left kidneys were removed under fully maintained anesthesia and fixed in 10% phosphate-buffered formalin or immediately stored at −80°C to subsequent analysis. Blood samples were also collected for the detection of blood urea nitrogen (BUN) and creatinine (Cr) levels.

**Serum Assays**

Assays were carried out using commercial kits. All kits were used in accordance with the manufacturer’s instructions (Nanjing Jiancheng Co., China). Levels of BUN and Cr were calculated using measurements acquired from a spectrophotometer.

**Superoxide Dismutase (SOD) and Malondialdehyde (MDA) Measurement**

Commercial kits were used in accordance with the manufacturer’s instructions (Nanjing Jiancheng Co., China) to measure SOD activity (xanthineoxidase method, catalog# A001-3) and MDA concentration (thiobarbituric acid method, catalog# A003-1).

**Histologic Examinations**

Kidneys were first fixed and embedded, and then used to prepare 4-μm thick sections. Sections were then gradually deparaffinized and hydrated and stained with hematoxylin and eosin staining. Morphologic assessments were observed by 2 experienced renal pathologists who were unaware of the treatments. An established grading scale of 0-4, outlined by Jablonski et al, was used for the histopathologic assessment of I/R-induced damage.

**RT-PCR**

RNA was extracted from renal tissue or cultured cells with Trizol, and subsequently isolated through the use of a RNeasy Mini Kit (Qiagen). RNA (1.0 μg) was transcribed into cDNA, and real-time polymerase chain reaction (PCR) was performed using a Quantitative SYBR Green PCR Kit (Qiagen) and a 7500 Fast Real Time PCR System (Applied Biosystems). IL-1β primers included: forward 5′-ACTATGGCCAACGTCCCTGAAC′ and reverse 5′-GTGCTTGGGTCCCTCATCTTG-3′. IL-18 primers included: forward 5′-ACAACGCCGATATAGGAGCAGCA-3′ and reverse 5′-TGTGTCTGTGTTGACAGGTGTC-3′. Actin primers included: forward 5′-TGCTATGTGTGCCCAGACTTCG-3′ and reverse 5′-GTGCGATAGAGGTCTTACGG-3′. IL-1β and IL-18 transcript levels were normalized to actin transcript levels, with mean values ± SD reported for each group.

**Western Blotting**

Proteins from renal tissue or cell cultures were extracted and quantified using the bicinchoninic acid method. Then, equivalent concentrations of protein (40 μg/lane) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred to a nitrocellulose membrane. The dilution of primary antibodies against Caspase-1 (ab1872), Caspase-11 (sc-56038) and β-Actin (sc-47778) were 1:1000. β-Actin was used as a loading control to ensure equal loading. Subsequently, membranes were washed twice with phosphate-buffered saline (PBS) and then incubated with secondary antibody at room temperature for 1 hour. Specific bands were then visualized using Immobilon Western Chemiluminescence horseradish peroxidase (HRP) substrate (Merck Millipore, Darmstadt, Germany). Optical densities were detected using Quantity One software (Bio-Rad, Hercules, CA).

**Hypoxia and Reoxygenation (H/R) Model**

The renal tubular epithelial cell line, NRK-52E, was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Prior to the H/R protocol, all cells in every group were cultured in 2 mL of...
serum-free medium for 24 hours in order to be synchronized. The H/R injury protocol was then performed as previously described. Cells were then exposed to hypoxic (O2 fraction below 1%) condition for 3 hours followed by normal conditions (95% air/5% CO2) for 24 hours.

**Experimental Groups**

All cells were divided into 5 groups. The control group was just washed with PBS. The H/R group was subjected to hypoxia 3 hours, followed by normoxia 24 hours. After hypoxia 3 hours, the H/R + hypoxic postconditioning (HPO) group were placed 3 cycles of normal conditions 10 minutes and followed by ischemic conditions for 10 minutes, as previously described. The cells in H/R + OP group were incubated with ozonic medium for 2 hours after hypoxia 3 hours, as previously described. The cells in H/R + HPO + OP group were administrated with 3 cycles of HPO after hypoxia 3 hours, followed by incubation with ozonic medium for 2 hours. Then all cells were cultured under normal conditions for 24 hours.

**Cell Viability Assay**

After cultured under normal conditions for 24 hours, NRK-52E cells were treated with CCK8 (10 μL/well, Sigma) for an additional 2 hours, then we recorded the absorbance at 450 nm using a microplate absorbance reader.

**ELISA**

We tested IL-1β and IL-18 concentrations using an ELISA kit according to the manufacturer’s instructions (CUSABIO, Wuhan, China).

**Immunofluorescence Staining**

After treatment, slides were washed twice with PBS and fixed in 4% paraformaldehyde for 30 minutes. Blocking solution was used at room temperature for 2 hours. Primary antibodies against caspase-1 diluted in PBS were added to the fixed samples overnight at 4°C, after which secondary antibody was incubated with NRK-52E cells for 1 hour at room temperature. The nuclei were stained using 4’, 6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) for 20 minutes at room temperature. Immunofluorescence was examined under a fluorescence microscope (Olympus, Tokyo, Japan).

**Statistical Analysis**

Data are presented as mean ± standard error of the mean. The means of the different groups were compared using one-way analysis of variance and the Student-Newman-Keuls test. Differences were considered to be statistically significant when P<.05.

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**RESULTS**

**The Combination of IPO and OP Synergistically Protected the Kidney From I/R**

Sprague Dawley rats were subjected to sham operation, or 45 minutes of ischemia followed by 24-hour reperfusion with or without IPO or OP either, or both. I/R induced renal dysfunction with a significant increase in the levels of Cr and BUN (P<.05). The single application of IPO or OP could obviously ameliorated the increased BUN and Cr level. Furthermore, the combination of IPO and OP significantly prevented renal I/R, as demonstrated by a significant decrease in the levels of BUN and Cr compared with the I/R + IPO group or I/R + OP group (P<.05, Fig. 1A and B).

Renal I/R significantly increased MDA content and significantly decreased SOD activity (P<.05, Fig. 1C and D). Oxidative abnormalities were clearly ameliorated by treatment with IPO or OP either (P<.05, Fig. 1C and D). However, the combination of IPO and OP had more reductive effect on elevated levels of oxidative stress compared with I/R + IPO group or I/R + OP (P<.05, Fig. 1C and D).

Renal tissue morphology is shown in Fig. 1E and F. Renal tissues were normal in the sham. Kidneys in I/R group exhibited acute tubular damage in the proximal tubules, including tubular dilatation and loss of the brush border. IPO or OP could protect the tubular epithelium from swelling and from loss of the brush border, but the combined application of them had more protective effects.

**The Combined Application of IPO and OP Synergistically Inhibited Pyroptosis Induced by Renal I/R**

Western blot analysis and qPCR revealed that the protein and mRNA levels of caspase-1 and caspase-11 were markedly elevated after renal I/R (Fig. 2A-C), and similar changes in IL-1β and IL-18 expression were consistently observed (Fig. 2D-G). IPO or OP could prevent the elevated expression of them, but the combined application of them had more obvious reduction. These results suggest that I/R induced renal pyroptosis and the combination of IPO and OP could synergistically prevent it.

**The Combination of IPO and OP Synergistically Protected NRK-52E Cells Against Damage Induced by H/R**

HPO exhibited a protective effect on cell viability after H/R and similar change in H/R + OP group were also observed (Fig. 3A). However, the combination of HPO and OP had more protective effects (Fig. 3A). As shown in Figure 3B and C, the MDA content in H/R + HPO group or in H/R + OP group was higher than those in H/R + HPO + OP group (P<.05) and SOD activity was significantly lower in H/R + HPO or in H/R + OP group compared with H/R + HPO + OP group (P<.05), which indicated that the combination of HPO and OP had more protective effects on oxidative abnormalities compared with the single application of HPO or OP.

**The Combined Application of HPO and OP Synergistically Inhibited Pyroptosis Induced by H/R in NRK-52E Cells**

Immunofluorescence results of NRK-52E cells (Fig. 3D) showed that caspase-1 expression was remarkably increased after H/R treatment. In H/R + HPO + OP group, its expression had more reduction than those in H/R + HPO group or H/R + OP group.
Consistent with these findings, the protein and mRNA levels of caspase-1, caspase-11, IL-1β, and IL-18 were significantly increased in the H/R group (Fig. 4A-G). HPO or OP could prevent the increased expression of them, but the combined application had synergistic effect.

DISCUSSION
Renal ischemia-reperfusion injury (I/R) is a major cause of AKI which often arises from hypovolemic conditions, septic shock, surgery, and transplantation. I/R injury causes structural and functional damage of renal tubules by directly inducing death of tubular cells, and these dying cells may trigger damaged responses. In the past decades, therapeutic approaches to render organs more resistant to ischemia have been studied, including ischemic preconditioning. However, as the beginning of tissue ischemia is uncertain, the clinical application of preconditioning still remains limited. In our previous study, we found that IPO or OP could prevent renal I/R injury. And we still wonder whether the combination of them could have synergistical protection. This study
aimed to provide evidence that IPO and OP can synergistically alleviate renal I/R injury and suggest possible mechanisms that mediate this effect. We evaluated the effect of the combined application of IPO and OP using a classical animal model of I/R. Rat kidneys that had undergone IPO or OP either showed protective morphologic changes and the combination of them had synergistic protection. Furthermore, levels of BUN and Cr in I/R + IPO group or I/R + OP group were clearly higher than the combination group. The administration of IPO combined with OP could synergistically improve these renal morphologic and functional changes.

Renal tubular cell death induced by I/R is the main cause of the development and progression of AKI. Previous studies indicated that apoptosis and necrosis were the main process that leads to tubular cell death caused by I/R. Pyroptosis, characterized by rapid plasma membrane rupture and the release of proinflammatory intracellular contents, is morphologically and mechanistically distinct from other forms of cell death, including necrosis and apoptosis. Caspase-1, the definitive feature of pyroptosis, is the primary enzyme that mediates this process. Caspase-1 was first recognized as a protease that activated IL-1β and IL-18 and was initially called IL-1β-converting enzyme. The activation of caspase-1 could induce pore formation on the cell membrane, which resulted in the generation and release of inflammatory factors that subsequently contributed to pyroptosis. Our in vivo study has shown that pyroptosis is characterized by increased caspase-1, caspase-11, IL-1β and IL-18 expression after I/R. The altered levels were correlated with structural and functional changes that occurred in these renal tissues. Further, our in vitro study also indicated that H/R caused elevated expression of aspase-1, caspase-11, IL-1β and IL-18 in NRK-52E cells, which was accompanied by reductive cell viability and SOD activity as well as increased MDA content. These results together suggest that pyroptosis are closely associated with the development and progression of I/R in renal tubular cells.

Ischemic preconditioning has been demonstrated to protect organs against the tissue damage induced by I/R. However, its clinical application is often restricted since the onset of ischemic injury is difficult to predict. IPO was first reported by Zhao et al. as an effective strategy against cardiac I/R injury. Our previous study revealed that IPO attenuated oxidative stress and protected rats against renal I/R injury. Also in our

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Figure 2. The combination of ischemic postconditioning and ozone postconditioning reduced pyroptosis and inflammation induced by ischemia-reperfusion. Spague Dawley rats were administered with IPO and OP either, or both. Kidneys were collected for immunohistochemistry and western blotting after 24 hours of reperfusion. (A-C) Representative blotting and quantification of protein levels are shown. (D-G) IL-1β and IL-18 protein levels in serum concentrations and mRNA expression levels in renal tissues. Data are presented as mean ± SEM. *P<.05 vs sham group; # P<.05 vs I/R group; ΔP<.05 vs I/R + IPO group or I/R + OP group. HPO, hypoxic postconditioning; IPO, ischemic postconditioning; I/R, ischemia-reperfusion; OP, ozone postconditioning; SEM, standard error of the mean.
previous study, an in vitro HPO model was established to effectively simulate the process of IPO against I/R through reduction cell apoptosis. Some researchers have been engaged in investigating the possible mechanisms of action triggered by ozone, which is briefly mixed with human serum. Just like oxygen, ozone dissolves in the human serum and reacts rapidly with many substrates, generating with numerous products such as H₂O₂, MDA, and so on. They act as cellular signals and trigger a variety of biological effects. Our previous study investigated the role of OP on I/R-induced oxidative stress and apoptosis, both in vivo and in vitro and found that OP exerts a significant renal protective effect against I/R injury by attenuating oxidant stress and apoptosis in kidney cells.

In the present study, we first found that the combination of IPO and OP synergistically protected the kidney from I/R. The combination of IPO and OP significantly prevented renal I/R, as demonstrated by a significant decrease in the levels of BUN and Cr compared with I/R + IPO or I/R + OP. Also, the combined application had more reductive effect on elevated levels of oxidative stress compared with IPO group or OP either. Similar changes were also observed in the expression of caspase-1, caspase-11, IL-1β and IL-18 after I/R, which indicated that the combination of IPO and OP synergistically inhibited pyroptosis induced by renal I/R. Consistent with in vivo findings, our in vitro finding indicated that the combination of IPO and OP synergistically protected NRK-52E cells against damage.

Figure 3. H/R induces NRK-52E cell pyroptosis. After H/R treatment, cell viability (A), SOD activity (B) and MDA content (C) of the NRK-52E cells were analyzed. (D) Immunofluorescence results (magnification × 400) indicate the expression of caspase-1 in H/R-treated NRK-52E cells with HPO and OP either, or both. Blue: nuclear staining (DAPI); red: caspase-1 staining (scale bar: 20 μm). Data are presented as mean ± SEM. *P<.05 vs control group; # P<.05 vs H/R group; △P<.05 vs H/R + HPO group or H/R + OP group. HPO, hypoxic postconditioning; MDA, malondialdehyde; OP, ozone postconditioning; SEM, standard error of the mean; SOD, superoxide dismutase.
induced by H/R and inhibited pyroptosis induced by H/R in NRK-52E cells.

CONCLUSION

In conclusion, the present study provides, for the first time, evidence that the combination of IPO and OP synergistically protected the kidney from I/R by attenuating pyroptosis in kidney cells.

References