Effect of ozone oxidative preconditioning on oxidative stress injury in a rat model of kidney transplantation

TAO QIU1*, ZHI-SHUN WANG2*, XIU-HENG LIU2, HUI CHEN2, JIANG-QIAO ZHOU1, ZHI-YUAN CHEN2, MIN WANG2, GUAN-JUN JIANG2, LEI WANG2, GANG YU3, LONG ZHANG3, YE SHEN2, LU ZHANG2, LI HE2, HUA-XIN WANG3 and WEN-JING ZHANG4

Departments of 1Organ Transplantation, 2Urology and 3Anesthesiology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; 4Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430022, P.R. China

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Abstract. The aim of the present study was to investigate the protective effect of ozone oxidative preconditioning (OOP) on renal oxidative stress injury in a rat model of kidney transplantation. Thirty-six male Sprague Dawley (SD) rats were randomly divided into three groups: A sham (S) group, a kidney transplantation (KT) group and an OOP and kidney transplantation (OOP+KT) group. In the S group, the rats’ abdomens were opened and closed without transplantation. In the KT group, the rats received a left kidney from donor SD rats. In the OOP+KT group, donor SD rats received 15 OOP treatments by transrectal insufflations (1 mg/kg), once a day, at an ozone concentration of 50 µg/ml, before the kidney transplantation. Twenty-four hours after transplantation, the parameters of renal function of the recipients were measured. The morphology and pathological effects of renal allograft were examined using hematoxylin and eosin staining, periodic acid-Schiff staining, a terminal deoxynucleotidyl transferase dUTP nick end labeling assay and immunohistochemistry. Markers of oxidative stress were also detected using the thiobarbituric acid method, and expression levels of Nrf-2 and HO-1 were determined by western blot analysis. Blood urea nitrogen and creatinine levels were significantly decreased in the OOP+KT group compared with the KT group, and the morphology and pathological changes of renal allograft were also less severe. Meanwhile, the renal allograft cell apoptosis index was significantly higher in the KT group compared to the OOP+KT group (P<0.05). Levels of superoxide dismutase, glutathione and catalase in the renal allografts were significantly higher in the OOP+KT group compared to those in the KT group (P<0.05), while malondialdehyde levels were significantly lower in the OOP+KT group compared to those in the KT group (P<0.05). Western blot analysis indicated that the expression levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) and heme oxygenase 1 (HO-1) were significantly higher in the OOP+KT group compared to the KT group (P<0.05). In conclusion, the mechanism by which OOP alleviates oxidative stress injury in renal transplantation may be related to the activation of the signaling pathways of Nrf-2/HO-1 and inhibition of renal tubular epithelial cell apoptosis.

Introduction

Kidney transplantation is the most effective treatment for end-stage renal disease in patients who require dialysis, helping to improve their quality of life and prolong their survival (1). There are several immunosuppressive agents currently used for renal transplantation, which are effective at preventing acute rejection throughout the transplantation process. However, the process still has some adverse effects, including ischemia/reperfusion injury (IRI), which occurs after blood flow recovery and restoration to tissues and organs. It has previously been identified that IRI is associated with an increased incidence of acute rejection and decreased long-term allograft survival, and is considered to be one of the leading causes of early allograft dysfunction (2,3). Therefore, it is necessary to adopt effective measures to reduce or alleviate IRI during kidney transplantation.

Ozone is a powerful oxidizing gas, which is commonly used as a disinfectant in the water and food industries (4). Although ozone is a potentially toxic agent, it can modulate many biochemical pathways at low and controlled non-toxic doses (5). Medical ozone therapy involves delivering a mixture of gaseous ozone and oxygen to the body (6). It has previously been identified that ozone therapy can be useful in treating inflammation-mediated diseases, such as infected wounds, burns, and advanced ischemic diseases (7). Ozone therapy also results in resistance to oxidative stress by inducing...
antioxidant systems (8,9). Furthermore, it has been reported that ozone oxidative preconditioning (OOP), a type of ozone therapy (10) has a protective effect against IRI in the liver and kidney (11,12). To our knowledge, the current study is the first to investigate the effect of OOP on IRI in a homologous kidney transplantation in a rat model.

Materials and methods

Animal preparation. The experimental protocol used in the present study was approved by the Animal Ethics Review Committee of Wuhan University (Wuhan, China), and the procedures were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. A total of 36 clean male, 7-week-old, Sprague Dawley (SD) rats (250-300 g) were purchased from Huazhong University of Science and Technology, all of them were kept in an air-filtered, homeothermal (20-22˚C), and light-controlled (light between 7:00 a.m. and 7:00 p.m.) room, and allowed free access to a standard diet.

Experimental protocol. Kidney donor rats were randomly divided into three groups, 12 rats in each, with 6 donors and 6 recipients. In the OOP and kidney transplantation (OOP+KT) group, donor rats (n=6) received 15 OOP treatments by transrectal insufflations (1 mg/kg), once a day, at an ozone concentration of 50 µg/ml, before their left kidneys were transplanted into recipient rats. In the kidney transplantation (KT) group, recipient rats each received a left kidney from a donor rat that had not undergone OOP treatment. The experiments that follow were only on the donated kidneys in groups OOP+KT (n=6) and KT (n=6). In the sham (S) group (n=12), the rats' abdomens were opened and closed without transplantation; six kidneys from six rats were used in the subsequent experiments.

Surgical procedure. In all groups, donors were injected intraperitoneally with atropine (0.01 mg/kg), buprenorphine (0.04 mg/kg) and diazepam (10 mg/kg), all drugs were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). After 10 min, they were anesthetized with pentobarbital (45 mg/kg), buprenorphine (0.04 mg/kg) and diazepam (10 mg/kg), blood of recipient rats was drawn for analysis 24 h following kidney transplantation. The renal allografts were harvested for subsequent experiments. While under anesthesia, the animals were sacrificed by cervical dislocation. All rats underwent a laparotomy and nephrectomy to harvest the kidneys. Kidneys were then fixed in 10% phosphate-buffered formalin or immediately frozen, and stored at -80˚C for subsequent experiments.

Serum assays. To assess creatinine (Cr) and blood urea nitrogen (BUN) serum levels, blood samples were collected, centrifuged and kept at -20˚C until analysis. The samples were examined using an Olympus AU 2700 Analyzer (Olympus Corporation, Tokyo, Japan), following standard techniques.

Histological examination. The kidney was fixed in 10% neutral-buffered formalin, embedded in paraffin wax and cut into 4-µm thick sections according to standard procedure. Sections were deparaffinized and hydrated gradually and examined using hematoxylin and eosin staining and immunohistochemistry. Morphological assessment was performed by an experienced renal pathologist who was unaware of the treatment that had been carried out in each case, images were captured using a BX53F light microscope (Olympus Corporation), at a magnification of x400. A grading scale of 0-4, as outlined by Jablonski et al (13), was used for the histopathological assessment of isogenic renal transplantation-induced damage of the proximal tubules.

Periodic acid-Schiff staining. Paraffin sections were routinely de waxed to water. Serial sections (4-µm) were washed with distilled water, incubated in 0.5-1% (v/v) aqueous periodate for 5-10 min, and washed a further three times with distilled water. The sections were then incubated in Schiff's reagent (Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China) for 10-30 min. After staining, the sections were washed three times with sulfite, then Rinsed with running water for 10 min, and then washed with distilled water once. The sections were then counterstained with hematoxylin to identify nuclei.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. To observe cell apoptosis induced by ischemia, an in situ apoptosis detection kit (Promega Corporation, Madison, WI, USA) was used. The TUNEL assay was performed based on the manufacturer's instructions. Briefly, after being fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS; pH 7.4) solution at 4˚C overnight, the whole specimens were extensively washed with 1X PBS solution three times, then immersed into 70% ethanol for at least 24 h at 20˚C. After being washed a further three times with PBS solution, the samples were immersed into a permeabilization buffer for 15 min on ice then washed again with PBS solution. Subsequently they were incubated in 50 ml reaction buffer (5 ml terminal deoxynucleotidyl transferase enzyme and 45 ml Labeling Safe Buffer) (Promega Corporation) for 90 min at 37˚C. The labeling procedure was stopped by washing with PBS solution. The image was analyzed using a Zeiss LSM

Preservation of kidneys. After all the recipient rats were anesthetized with atropine (0.01 mg/kg), buprenorphine (0.04 mg/kg) and diazepam (10 mg/kg), blood of recipient rats was drawn for analysis 24 h following kidney transplantation. The renal allografts were harvested for subsequent experiments. While under anesthesia, the animals were sacrificed by cervical dislocation. All rats underwent a laparotomy and nephrectomy to harvest the kidneys. Kidneys were then fixed in 10% phosphate-buffered formalin or immediately frozen, and stored at -80˚C for subsequent experiments.
510 Confocal laser scanning microscope (Carl Zeiss AG, Oberkoden, Germany) with a 488 nm excitation line and a 530 nm emission filter. Five high-power fields of vision in the distribution areas of the apoptotic cells in each slide were randomly selected, and the average number of apoptotic cells per 100 cells was calculated. The apoptotic index (AI) was expressed as a percentage.

Measurement of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) levels in the kidney. Renal tissue MDA concentration was measured using the thiobarbituric acid method. Levels of lipid peroxides were measured as an indicator of MDA production rates (assay kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Absorbance was measured at 532 nm using a spectrometer. SOD activity in renal tissue was measured using a commercialized chemical assay kit (Nanjing Jiancheng Bioengineering Institute) by the xanthine oxidase method. Absorbance was determined at 550 nm using a spectrometer. GSH content was detected using a colorimetric GSH detection kit (Nanjing Jiancheng Bioengineering Institute). Briefly, the renal homogenate was mixed with reagent A, reagent B and reagent C. Absorbance of the yellow solution was measured at 412 nm. CAT activity in renal tissue was measured using a commercialized chemical assay kit (Nanjing Jiancheng Bioengineering Institute) by the visible light method. Absorbance was determined at 405 nm using a spectrometer. All protein concentrations of renal tissue homogenate samples were determined using the Coomassie Blue method (assay kit; Nanjing Jiancheng Bioengineering Institute).

Immunohistochemistry. The expression levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) and heme oxygenase 1 (HO-1) were evaluated using immunohistochemical staining. Briefly, 5-µm sections were deparaffinized, and endogenous peroxidase activity was blocked with 3% hydrogen peroxide at 37°C for 10 min. Then, the sections were treated with 10% normal goat serum (Wuhan Boster Biological Technology, Ltd., Wuhan, China) in Tris-buffered saline for 30 min at 37°C. Subsequently, they were incubated overnight at 4°C with a rabbit polyclonal anti-rabbit antibody to Nrf-2 (catalogue no. sc-722, 1:300; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), a rabbit polyclonal antibody to HO-1 (catalogue no. 10701-1-AP, 1:1,000; Wuhan Sanying Biotechnology, Wuhan, China) or anti-mouse secondary antibodies (BA-1054, 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China). The expression levels following the western blot analysis of each protein was assessed for the three groups using ImageJ software. To quantify the results, the average gray value of each protein was calculated.

Statistical analysis. All the data were statistically analyzed using SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA) and presented as the mean ± standard deviation. Statistical differences were analyzed using the Student’s t-test and P<0.05 was considered to indicate a statistically significant difference.

Results

**Effect of OOP on renal function after kidney transplantation.** The renal functional parameters of rats were detected 24 h after renal transplantation. Rats subjected to isogeneic renal transplantation showed a significant increase in BUN and Cr levels compared with sham-operated rats (P<0.05). The negative effects on renal function induced by renal transplantation were significantly reduced in the OOP+KT group compared with the KT group, in relation to levels of BUN or serum Cr (P<0.05; Fig. 1).

**Effect of OOP on morphological features of renal cells.** Histopathological examination revealed that morphological lesions existed in the allograft kidney tissue after isogeneic renal transplantation. Renal injury was evidenced by loss of tubular integrity, dilated tubules, congestion, and tubular cell swelling and tubular dilation. However, this renal damage was attenuated by OOP (Figs. 2 and 3). Furthermore, the Jablonski grade analysis of severe acute tubular necrosis results showed that the OOP+KT group had significantly lower levels of damage compared with the KT group (Table I). In addition, OOP significantly reduced the AI in the OOP+KT group compared with the KT group (P<0.05; Table I and Fig. 4).

**Levels of SOD, MDA, GSH and CAT in renal tissue.** As shown in Table II, the level of MDA content, which was indicated...
The level of lipid peroxidation, was significantly higher in the KT and OOP+KT groups than in the S group (P<0.05). However, the level of MDA in the OOP+KT group was significantly lower than in the KT group (P<0.05). Meanwhile, the level of MDA in the OOP+KT group was significantly lower than in the KT group (P<0.05).
levels of SOD, GSH and CAT in the kidney tissue were significantly decreased after kidney transplantation in the KT group and the OOP+KT group compared with the S group (P<0.05). However, the levels of SOD, GSH and CAT after kidney transplantation were significantly higher in the OOP+KT group compared with the KT group (P<0.05).

**Expression levels of Nrf-2 and HO-1.** The results of the immunohistochemistry analysis showed that the expression levels of Nrf-2 and HO-1 in the OOP+KT group were higher than those in the KT group (Figs. 5 and 6). Furthermore, the western blot analysis confirmed that the expression levels of Nrf-2 and HO-1 in the OOP+KT group were significantly higher than those in the KT group (P<0.05; Fig. 7).

**Discussion**

Compared with dialysis treatment, kidney transplantation is often seen as preferable for patients with end-stage kidney disease, and previous research confirms that renal transplant patients have a longer survival time, with a better quality of life, than dialysis patients (15,16). However, despite a low
occurrence of acute allograft rejection due to the application of novel anti-immunity drugs, some detrimental factors still exist during the perioperative period of renal transplantation, such as IRI and chronic graft rejection (17).

IRI is a pathological process that involves oxidative stress, intracellular calcium overloading, inflammation reaction and cell apoptosis. Oxidative stress is one of the most important components of IRI, and is primarily caused by excessive reactive oxygen species (ROS) being generated in ischemic tissue after reperfusion (18). Naturally generated antioxidant enzymes can counteract the cellular effects of oxygen free radicals under normal conditions (18,19), but excessive ROS generation during the period of ischemia reperfusion cannot be effectively controlled by this system (18). It has been reported that inflammation cytokines and chemokines are released during oxygen radical generation and lipid peroxidation. Together with adhesion molecules, these attract inflammatory cells, such as monocytes and neutrophils, which in turn help to release more ROS and aggravate renal damage (2). Therefore, effective measures or treatments to attenuate oxidative stress or help balance the oxidative and anti-oxidative systems may be useful in alleviating IRI during renal transplantation.

In the present study, the effects of OOP on kidney transplantation were investigated in a rat model. It was found that pretreatment with a controlled concentration of ozone before kidney transplantation could decrease oxidative stress injury, demonstrated by a significantly reduced renal tissue concentration of MDA in the OOP+KT group compared with the KT group. Furthermore, compared with the KT group, indicators of anti-oxidative stress significantly increased in the allograft kidney tissue, such as SOD, GSH and CAT. These results were consistent with a prior study, which identified that OOP improves organ stress by enhancing endogenous protective mechanisms (21).

Nrf2 belongs to the cap ‘n’ collar family, a small group of transcription factors that contain a unique basic leucine zipper motif (22,23). Nrf2 principally regulates transcriptional activation through an antioxidant responsive element (ARE) (24), which is considered to be a cis-acting regulatory element in promoter regions of many cytoprotective genes. Under basal conditions, Nrf2 is constantly targeted for Keap-1-mediated ubiquitination and subsequent proteasomal degradation to maintain low Nrf2 protein levels (25). However, upon activation, Nrf2 can activate downstream antioxidant proteins, phase II metabolizing/detoxifying enzymes and phase III ATP-dependent drug efflux pump-encoded genes (26-28). It has been reported that Nrf2 activates numerous types of enzymes with antioxidation and detoxifying activities that serve a key function in the protection of cells against various environmental stresses, such as electrophiles, ROS and reactive nitrogen species (29). In the present study, the expression level of Nrf2 in the OOP+KT group was significantly higher than that in the KT group. A proposed explanation for this is the low levels of OOP in the KT group, which could have helped to trigger the activation of Nrf2/Keap-1. Furthermore, a prior study indicates that overexpression of Nrf2 increases ARE transcriptional activity and enhances the expression of several ARE-dependent antioxidant and cytoprotective enzymes, including HO-1, glutamate cysteine ligase, glutathione peroxidase and NAD(P)H quinone oxidoreductase 1 (30). In the present study, the expression level of HO-1 in the OOP+KT group was significantly higher than that in the KT group, which could help to explain why the OOP+KT group displayed less severe oxidative stress injury than the KT group. In
addition, it has previously been proposed that Nrf2-dependent HO-1 expression could inhibit the activation of nuclear factor κB, stimulated by tumor necrosis factor alpha and monocyte chemoattractant protein-1 secretion in endothelial cells (31).

In addition, the present results suggest that OOP could reduce the rate of apoptosis in renal tubular epithelial cells caused by oxidative stress injury during the process of renal transplantation. It has been demonstrated that excessive ROS overwhelming the scavenging capacity of the endogenous antioxidant system can lead to cellular damage (32), such as blocking cellular mitochondrial respiration (33), or facilitating the formation of mitochondrial transition pores, which help to increase the release of apoptosis-related proteins after ischemia reperfusion (34,35). A previous study outlines several measures to prevent oxidative stress-induced apoptosis, such as increasing the activity of the endogenous antioxidant system, applying exogenous antioxidant enzymes or free radical scavengers, or decreasing the lipid peroxidation byproducts (36). In this study, it was indicated that OOP reduces the apoptosis or necrosis of renal tubular epithelial cells, which may be related to the strengthening of endogenous antioxidant systems, such as the Nrf2/HO-1 pathway.

In conclusion, this study demonstrated that OOP could mitigate oxidative stress injury and apoptosis of renal tubular epithelial cells during kidney transplantation in a rat model, which may be related to activation of the Nrf2/HO-1 signaling pathway and reduction of renal tubular epithelial cell apoptosis. However, the long-term effect of OOP on renal transplantation has not been studied here, such as the differences in survival rate between groups. Furthermore, the effects of OOP on the immune response and inflammation between different strains of rats, especially in the acute immunological rejection model, need to be investigated further.

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References


