Effect of ozone oxidative preconditioning on inflammation and oxidative stress injury in rat model of renal transplantation[1]

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Abstract

Purpose: To investigate the effect of ozone oxidative preconditioning (OzoneOP) on inflammation and oxidative stress injury in rat model of renal transplantation.
Methods: Thirty six male Sprague Dawley (SD) rats were randomly divided into three groups. Sham group: rats were treated with opening and closing abdomen. Kidney transplantation group (KT group): SD rat received the donor's left kidney derived from another SD rat. Ozone oxidative preconditioning and kidney transplantation (OOP+KT group): donor SD rats received OzoneOP treatments by transrectal insufflations before kidney transplantation. After transplantation, parameters of renal function of recipients were determined. Morphology and pathological changes of renal allograft were examined. Expression of NF-κBp65, HMGB-1 were also determined by Western-blot.
Results: Compared to KT group, the morphology and pathological damages of renal allograft were less serious in OOP+KT group. Meanwhile, levels of SOD and GSH-Px of renal allograft in OOP+KT group were higher than those in KT group respectively. Western-blot showed that the expressions of NF-κBp65 and HMGB-1 in OOP+KT group were obviously less than those in KT group.
Conclusion: Ozone oxidative preconditioning could attenuate the inflammatory reaction and oxidative stress injury in renal allograft, which might be related with the enhancement of anti-oxidative system and suppression of inflammatory reaction.
Key words: Kidney Transplantation. Ozone. Oxidative Stress. Rats.

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■ Introduction

The most effective treatment measure for end-stage renal disease patients who need dialysis is still the renal transplantation. There are growing kinds of effective immunosuppressive agents which show powerful abilities on suppressing and controlling the acute rejection during the perioperative period of kidney transplantation. However, there were still many adverse factors which were difficult to avoid during the operation, and one of main disadvantage factors is ischemia reperfusion injury. Ischemia reperfusion injury involves complex pathological process, which contains many factors, such as oxidative stress injury, intracellular inflammatory response, cell apoptosis and necrosis, calcium overload and so on. Therefore, treatment measures which could attenuate the inflammation reaction and oxidative stress injury may be beneficial for the renal function recovery of renal allograft.

Ozone as one kind of powerful oxidizing gas, which is widely used to the disinfection of water and food. Some report has showed that ozone had numerous abilities, ozone therapy could be used to treat infected wound and empyrosis[1]. Furthermore, researches have showed that ozone therapy could enhance the function of anti-oxidative system, which could mitigate the damage coused by oxidative stress[2,3]. However, to our knowledge, the effect of ozone oxidative preconditioning on inflammation and oxidative stress injury in the homologous kidney transplantation has not been studied up till now.

Therefore, the current study tried to research the effect of ozone oxidative preconditioning on inflammation and oxidative stress injury in homologous kidney transplant in rat model.

■ Methods

The experimental protocol was approved by the Animal Ethics Review Committee of Wuhan University, and the procedures were carried out accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Thirty six males, adult SD rats (body weight 250-300g) were acquired from the Experimental Animal Center of Medical College of Wuhan University (Wuhan, China). Animals were housed at the Central Animal Facility of Affiliated Renmin Hospital of Wuhan University based on standard guidelines. Rats were kept in an air-filtered, homoiothermal (20–22°C), and light-controlled (light for 8a.m.-8p.m.) room, and allowed free access to a standard diet.

Experimental protocol

Kidney donor rats were randomly divided into three groups: OOP+KT group (n=6): before the kidney transplant procedure, donor rats received 15 OzoneOP treatments by transrectal insufflations (1mg/kg), once a day, at an ozone concentration of 50 μg/ml; KT group: SD rat received the donor's left kidney derived from another SD rat; Sham group: rats were treated with opening and closing abdomen. All donors were intraperitoneal with atropine (0.01 mg/kg), buprenorphine (0.04 mg/kg), diazepam (10 mg/kg). Ten minutes later, they were anesthetized with pentobarbital (45 mg/kg). Then, the donor's blood vessels and ureter were fully separated. Kidneys were flushed through the aorta with 3 ml of 4°C cold Ringer lactate solution with heparin (50U/ml) until homogeneously pale. Left kidneys were removed with vascular and ureter with an ureterocystic flap and placed in cold Ringer lactate solution at 4°C for 180

min. In recipient rats, after left nephrectomy, renal arterial and venous anastomoses were performed as end-to-end anastomoses to the renal arterial and venous of recipient, respectively. Then, anastomoses of donor ureterocystic flap to recipient's bladder was constructed. Finally, recipient rats received right nephrectomy. During the surgery, body temperature was monitored and constantly kept between 35 °C and 37 °C. Animals were placed on a warm blanket with free access to water and standard laboratory chow *ad libitum* after transplantation. Twenty-four hours after transplantation, blood was drawn for analysis, animals were sacrificed and kidneys were harvested for different determinations.

Serum assays

To assess Cr and BUN, blood samples were collected, centrifuged and kept at –20˚C until analyses, adopting standard techniques using an Olympus AU 2700 Analyzer (Olympus Optical Co. Ltd., Tokyo, Japan).

Histological examination

The kidney was fixed in 10% neutral-buffered formalin, paraffin embedded and sectioned at 4-µm thick according to standard procedure. Sections were deparaffinized and hydrated gradually and examined by H&E staining. Morphological assessment was performed by an experienced renal pathologist who was unaware of the treatment. A grading scale of 0-4, as outlined by Jablonski[4], was used for the histopathological assessment of isogeneic renal transplantation induced damage of the renal proximal tubules.

Periodic Acid Schiff (PAS) Staining

Serial sections (thickness, 4 µm) were washed with distilled water, incubated in 0.5–1 % (v/v) aqueous periodate for 5–10 min, and washed three times with distilled water. Sections were differentiated, followed by incubation in Schiff 's reagent for 10–30 min. After staining, sections were subjected to washing three times with sulfite, then with distilled water. Sections were counterstained with hematoxylin to identify nuclei.

Immunohistochemistry

The expression of NF-κBp65, HMGB1 were conducted by 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 (Boster Biological Technology, Ltd., Wuhan, China) in Tris-buffered saline (TBS) for 30 min at 37˚C. Subsequently, they were incubated overnight at 4˚C with the rabbit polyclonal anti-rat antibody (NF-κBp65; dilution at 1:300; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-rat antibody (HMGB1; dilution at 1:1000; Proteintech Group, Wuhan, China). After washing three times with phosphate-buffered saline (PBS), these sections were incubated with the HRP-conjugated anti-rabbit secondary antibody (dilution at 1:5000, Boster, Wuhan, China) for 30 min at room temperature, followed by color reagent 3,3'-diaminobenzidine (DAB). For the negative control group, the procedures were performed with the exception of the addition of the primary antibody.

Enzyme-Linked Immunosorbent Assay

Detecting the levels of serum IL-6, IL-18 and COX-2 strictly according to the instructions of ELISA kits produced by Elabscience Biotechnology Co.,Ltd.

Measurement of MDA, SOD and GSH-Px in Kidney

Renal tissue malondialdehyde (MDA) concentration was measured by the thiobarbituric acid (TBA) method. Amounts of lipid peroxides (LPO) were measured as the production of MDA. Absorbance was measured at 532 nm using a spectrometer (assay kit; Nanjing Jiancheng Bioengineering Institute). Superoxide dismutase (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. Glutathione peroxidase (GSH-Px) activity was determined by the colorimetric method using a GSH-Px kit (Nanjing Jiancheng Bioengineering Institute). Absorbance was determined at 412 nm using a spectrometer. All protein concentrations of renal tissue homogenate samples were determined with Coomassie blue method (assay kit; Nanjing Jiancheng Bioengineering Institute).

Western Blot analysis

Proteins were extracted and purificated from renal tissue as previously described. In brief, protein samples were prepared for gel electrophoresis to be separated on 12.5% sodium dodecyl sulfate-polyacrylamide gels (40μg/lane) and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in TBST buffer and then incubated with primary antibodies overnight at homoiothermy of 4˚C. After rinsing with TBST buffer extensively, the blots were incubated with secondary antibodies, and developed with the use of an enhanced chemiluminescence system (ECL kit; Pierce Biotechnology Inc., Rockford, IL) and captured on light-sensitive imaging film (Kodak) to analyze. The following kits were used as primary antibodies: the rabbit polyclonal anti-NF-κBp65 (1:300; Santa Cruz, CA), HMGB1 (1:1000; Proteintech Group, Wuhan, China). HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (Boster, Wuhan, China) was used as secondary antibody.

Statistical analysis

All data was statistically analyzed using the statistical package for the social sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL, USA). The means of the different groups were compared using the Student's *t*-test and differences were considered statistically significant when $p<0.05$.

■ Results

Effect of ozone oxidative preconditioning on renal function after renal transplantation

The renal function parameters of rats were detected at 24h after renal transplantation. Rats subjected to isogeneic renal transplantation showed significant increase in blood urea nitrogen and serum creatinine compared with sham operated rats. Though the renal function changes induced by renal transplantation were slightly improved by ozone oxidative preconditioning, there were no significant differences between group KT and OOP+KT on the levels of BUN and serum Cr (Figure 1).

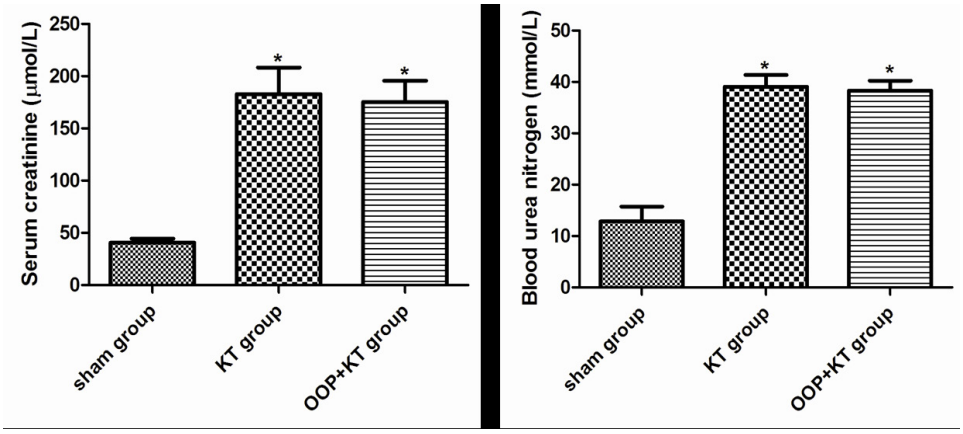

Figure 1 - Effects of ozone oxidative preconditioning on the renal function after kidney transplantation in rat model. Both serum Cr and BUN concentrations in groups KT and OOP+KT were significantly higher than those in group sham. Though the levels of serum Cr and BUN in OOP+KT group were slightly lower than those in group KT, there were no significant differences in the levels of serum Cr and BUN between groups KT and OOP+KT. *p<0.05 *vs*. sham group.

Effect of ozone oxidative preconditioning on morphological lesions after renal transplantation

Histopathological examination revealed that morphological lesions existed in the allograft kidney tissue after isogeneic renal transplantation. However, OzoneOP could alleviate the extent of renal morphology damages. The isogeneic renal transplantation resulted in significant renal injury as evidenced by loss of brush border, tubular cell swelling and necrosis, tubular dilation. However, these renal damages could be attenuated by OzoneOP (Figures 2 and 3). Furthermore, there were less renal tubular necrotizing changes in the OOP+KT group than those in KT group. The Jablonski grade analysis of severe acute tubular necrosis also showed that Jablonski grade in OOP+KT group was obviously lower than that in KT group (Figure 4).

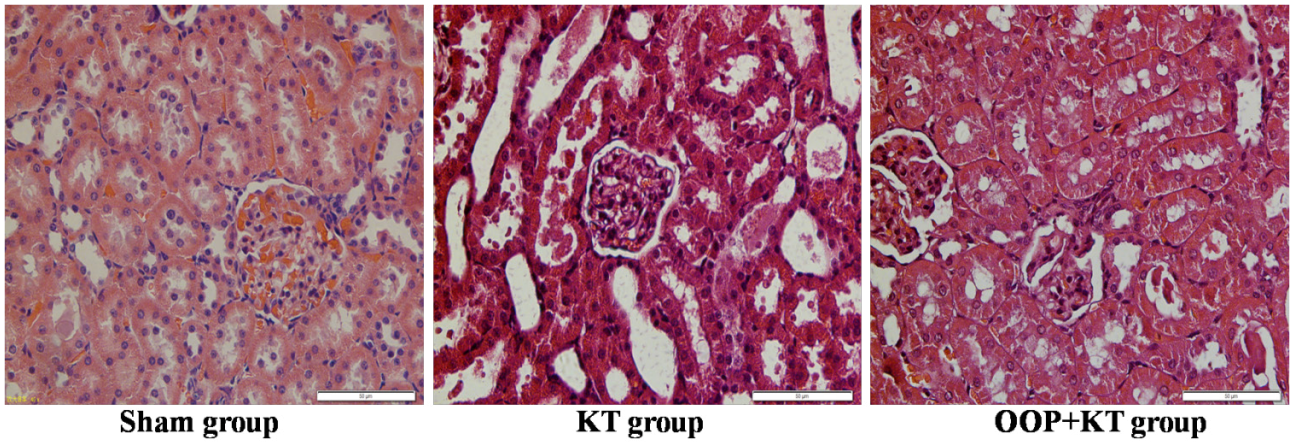

Figure 2 - Ozone oxidative preconditioning alleviated the morphological damages after renal transplantation according to the representive micrographs of hematoxylin-eosin staining (×400). There were serious morphological lesions in KT group, including renal tubular swelling, necrosis and the distruction of renal tubular normal construction. Furthermore, compared to group KT, morphological damages in group OOP+KT were obviously attenuated.

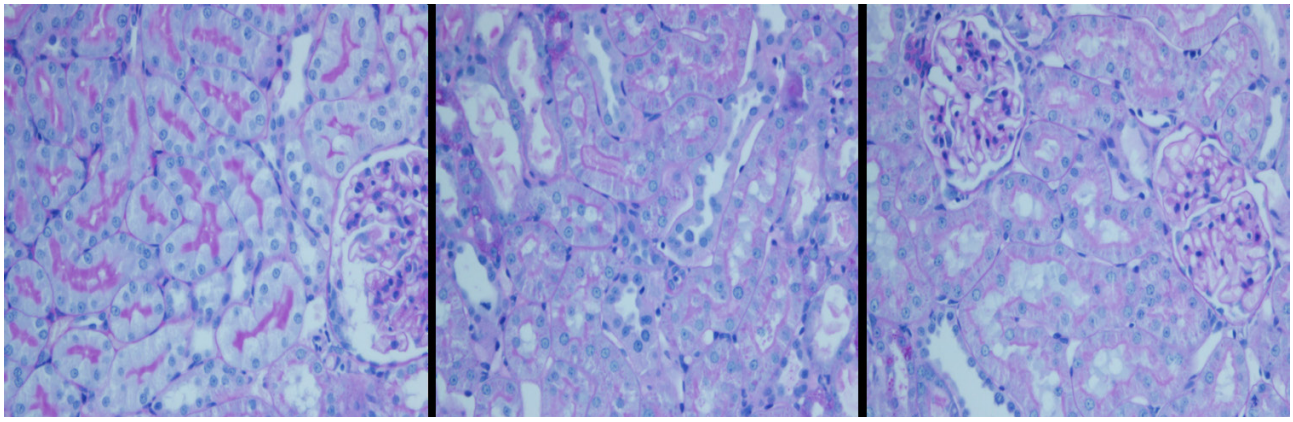

Figure 3 - Ozone oxidative preconditioning attenuated the injury of brush border of proximal renal tubular after kidney transplantation. Representive micrographs of PAS staining showed that severe renal damages including tubular necrosis and distruction of proximal renal tubular brush border were obviously slighter in OOP+KT group than those in KT group (×400).

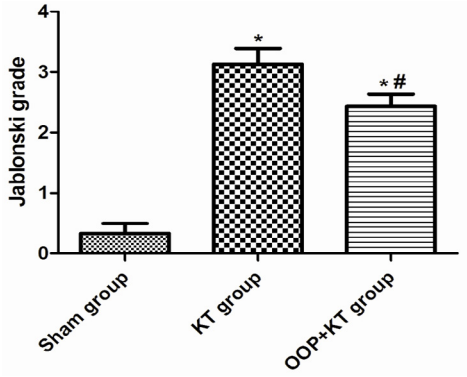

Figure 4 - Ozone oxidative preconditioning reduced the Jablonski grade after kidney transplantation. The Jablonski grades in groups KT and OOP+KT were obviously higher than those in sham group. Furthermore, Jablonski grade in group OOP+KT were significantly lower than that in KT group. *p<0.05 *vs*. sham group, # p<0.05*vs*. KT group.

Effect of ozone oxidative preconditioning on the levels of serum inflammatory markers after renal transplantation

ELISA results showed that serum levels of IL-6, IL-18 and COX-2 in both groups KT and OOP+KT were much higher than those in sham group. However, levels of IL-6, IL-18 and COX-2 could be significantly down-regulated by ozone oxidative preconditioning according to the results in groups KT and OOP+KT (Figure 5).

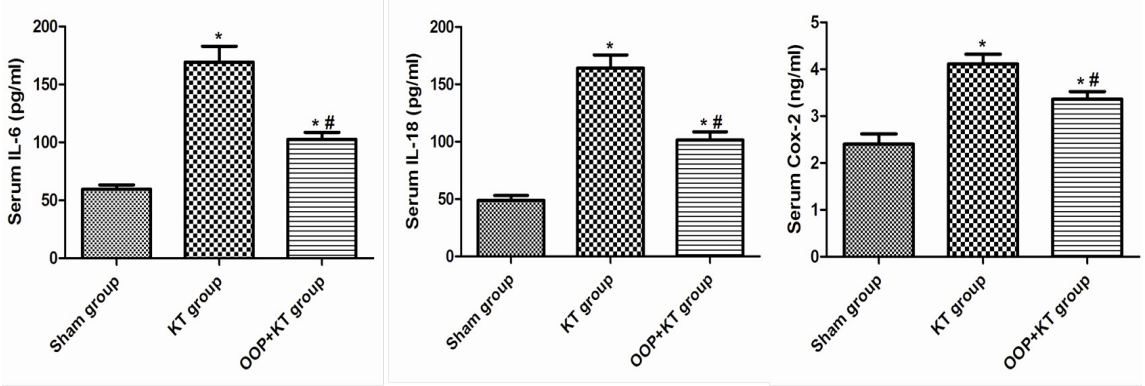

Figure 5 - Ozone oxidative preconditioning down-regulated the level of serum inflammatory factors after kidney transplantation according to the results of ELISA. The levels of serum IL-6, IL-18 and Cox-2 were all significantly higher than those in sham group. Furthermore, compared to group KT, the levels of serum IL-6, IL-18 and Cox-2 in group OOP+KT were all largely decreased. *p<0.05 *vs*. sham group, # p<0.05 *vs*. KT group.

Effect of ozone oxidative preconditioning on oxidative stress injury after kidney transplantation

As shown in Table 1, the level of MDA content which was an index of lipid peroxidation was obviously higher in groups KT and OOP+KT than that in sham group. Furthermore, the level of MDA in group OOP+KT was significantly lower than that in group KT. Meanwhile, the levels of SOD and GSH-Px in the kidney tissue were largely lower in KT group than those in sham group. Furthermore, compared to group KT, the reduction of the levels of SOD, GSH-Px after kidney transplantation could be significantly attenuated in OOP+KT group.

Table 1 - Activities of SOD, MDA, GSH-Px in renal tissues.

Groups	SOD(units/mgprot)	MDA(nmol/mgprot)	GSH-Px (U/mgprot)
Sham	86.24±7.12	3.52±1.13	7.61±0.92
KT	38.96±8.55*	6.48±1.69*	3.24±0.85*
OOP+KT	56.89±8.76*,#	4.92±1.78*,#	4.64±0.59*,#

The results are means ± SD for 6 rats in each group. *p<0.05 *vs*. Sham group , # p<0.05 *vs*. KT group. Superoxide Dismutase = SOD; Malonaldehyde = MDA; Glutathione peroxidase = GSH-Px.

Effect of ozone oxidative preconditioning on the expression levels of inflammatory markers after renal transplantation

The results of the immunohistochemistry analysis showed that expression levels of NF-κBp65 and HMGB1 in OOP+KT group were significantly lower than those in the KT group (Figures 6 and 7). Furthermore, western blot analysis also

Revealed that the expression levels of NF-κBp65 and HMGB1 in OOP+KT group were obviously lower than those in the KT group (Figure 8) (p<0.05).

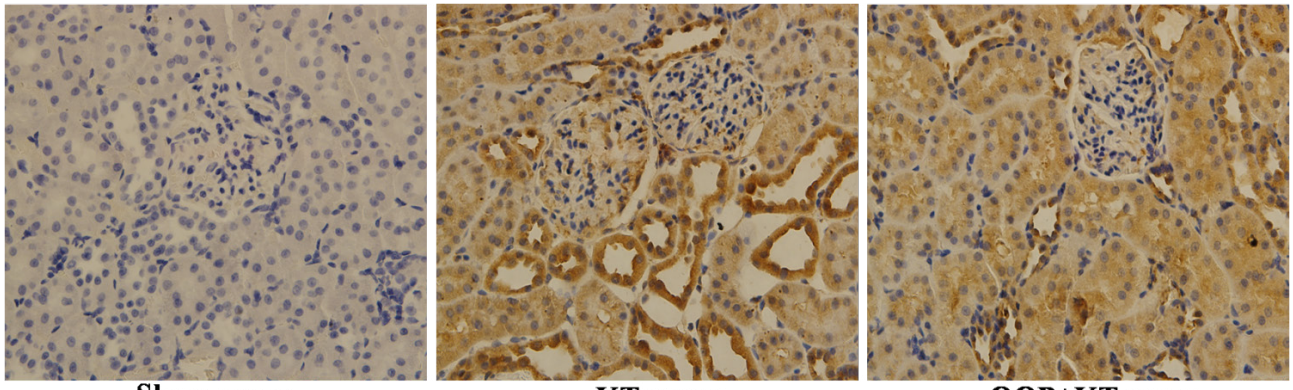

Figure 6 - Ozone oxidative preconditioning restrained the expression level of NF- κBp65 in renal tissue after kidney transplantation according to the representive micrographs of immunohistochemistry (×400). The expression level of NF-κBp65 in renal tissue were significantly higher in groups KT and OOP+KT than that in sham group. Furthermore, the expression level of NF-κBp65 in group OOP+KT was obviously lower than that in group KT.

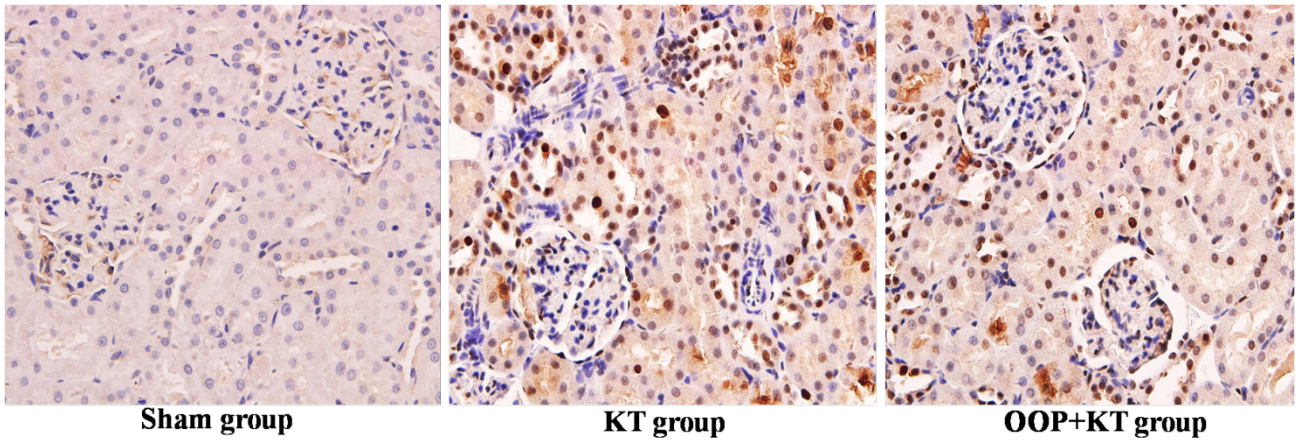

Figure 7 - Ozone oxidative preconditioning suppressed the expression of HMGB1 in renal tissue after kidney transplantation according to the representive micrographs of immunohistochemistry (×400). The expression level of HMGB1 in renal tissue were obviously higher in groups KT and OOP+KT than that in sham group. Furthermore, the expression level of HMGB1 in group OOP+KT was significantly lower than that in group KT.

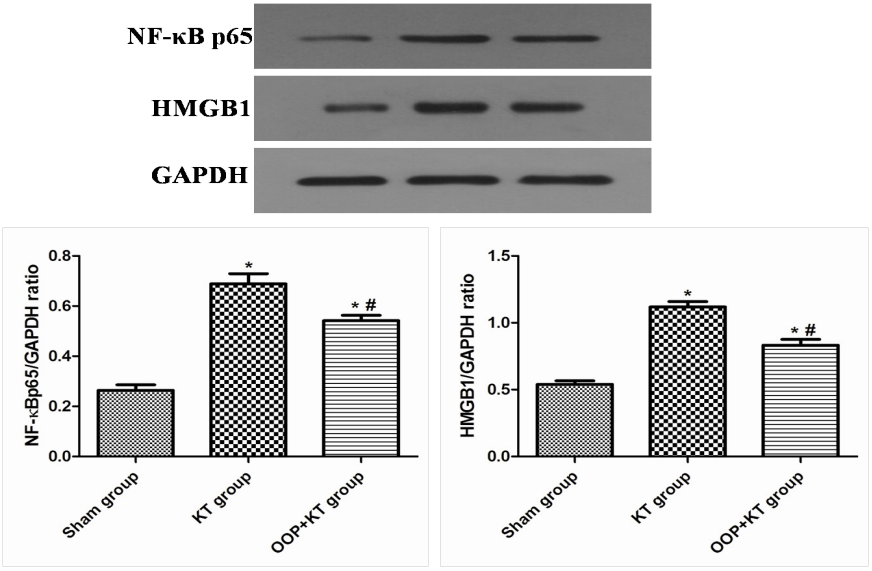

Figure 8 - Ozone oxidative preconditioning inhibited the expression levels of NF-κBp65 and HMGB1 in renal tissue after kidney transplantation according to the results of western blot. The expression levels of NF-κBp65 and HMGB1in renal tissues were obviously higher in groups KT and OOP+KT than those in sham group. Furthermore, both the expression levels of NF-κBp65 and HMGB1 in group OOP+KT were significantly lower than those in group KT. *p<0.05 *vs*. Sham group, #p<0.05 *vs*. KT group.

■ Discussion

Kidney transplantation provides an effective and important option for the patients who have end-stage renal disease. Furthermore, it has been shown that renal transplantation provide a more considerable improvement in health-related quality of life and a more favorable cost-effectiveness ratio when compared with dialysis[5,6]. Currently, many kinds of immunosuppressive agents which can effectively help to improve the success rate of renal transplantation and graft survival rate. However, there are still some

unfavourable factors during the perioperative period of kidney transplantation, and one important adverse factor of them is ischemia reperfusion injury, which is hard to avoid.

Ischemia reperfusion injury is involved with a cascade of cellular events which contain cell apoptosis, necrosis, release of reactive oxygen species (ROS), infiltration of inflammatory cells and release of active mediators, and those factors may result in serious injury of tissue. Though the exact mechanisms of ischemia reperfusion injury have not been fully revealed. It has been shown that increasing ROS and pro-inflammatory mediators seems to play an important role during the reperfusion phase[7]. Therefore, reducing the generation or release of ROS and suppressing the release of inflammatory mediators may be benefit for the renal function recovery and long-term survival of renal allograft.

Rodríguez *et al*.[8] have shown that ozone as an powerful oxidant gas could promote organ stress by inducing enhancement of endogenous protective parameters such as SOD, CAT, GSH-Px and GSH, in order to alleviate organ injury. In our study, oxidative stress preconditioning could enhance the expression of SOD and GSH-Px in renal tissue after kidney transplantation compared with KT group. These results maybe indicated that ozone oxidative preconditioning could enhance the activity of endogenous anti-oxidant system. Therefore, ozone oxidative preconditioning might help to induce the tolerance to ROS generated by some injury factors or toxic agents, this effect might be analogous to other protective measures such as ischemia preconditioning[9], chemical preconditioning[10], thermal preconditioning[11]. It was deserved to show that all these treatments were involved with a repeated and nonlethal stress, which could provide protection against a prolonged and severe stress[8].

High-mobility group box-1 protein (HMGB1) is a bi-functional protein, which functions as chromatin-associated proteins to regulate transcription in the nucleus. Meanwhile, it can also be released extracellularly to mediate the response to inflammatory stimuli such as infection and injury[12]. It has been identified that HMGB1 could be passively released from necrotic cells and actively secreted by certain cells such as killer cells, monocytes and macrophages[13,14], which plays a pro-inflammatory role as damage-associated molecular patterns (DAMPs)[15]. It has been reported that Ozone oxidative preconditioning could inhibited the increase in TNF-α production in the model of partial hepatectomy in rats[16]. Furthermore, moderate ozone oxidative stress may also play a role in suppressing NF-κB and inflammatory response[17]. In this study, the expression level of HMGB1 in group OOP+KT was obviously lower than that in KT group. Therefore, the inhibition of HMGB1 production by ozone oxidative preconditioning in the kidney tissue may be one of the factors related to the alleviation of inflammatory response.

Nuclear factor κB (NF-κB) is a transcription factor that contains five members: P65, P50, P52, RelB, and Rel[18,19], which plays a key role in the expression of pro-inflammatory cytokine genes[20]. In resting status, NF-κB is an inactive form and sequestered in the cytoplasm by binding the inhibitory protein IκB. Once NF-κB is activated, it will be phosphorylated and ubiquitinated, meanwhile, the inhibitory proteins will be degraded, and the released NF-κB dimers are further activated through modifications and translocated to the nucleus where they bind to specific DNA sequences and thus enhance transcriptional activity of target genes[19,21,22]. Furthermore, León Fernández *et al*.[23] have showed that ozone oxidative preconditioning could largely reduced the

intensity of the p65 expression in the rat model of liver ischemia/reperfusion. Xing *et al*.[24] also has showed that ozone oxidative preconditioning had potent anti-inflammatory properties by the modulation of the TLR4-NF-κB pathway in renal ischemia/reperfusion injury. In this study, the expression level of NF-κBp65 in group OOP+KT is significantly lower than that in group KT, which is coincident with the results of previous researches.

Pro-inflammatory cytokines also play a vital role in the development of kidney injury during the kidney ischemia/reperfusion process. It has been reported that inflammation reaction after kidney ischemia-reperfusion is characterized by upregulation of the production of inflammation cytokines and infiltration of macrophage[25]. IL-6 is an important pleiotropic cytokine, which has been identified that the level of IL-6 in urine correlates with the severity of ischemia-reperfusion injury in human renal allografts[26]. In the present study, the serum level of IL-6 in group OOP+KT was apparently lower than that in group KT. IL-18 is an pleiotropic inflammatory cytokine,whose expression is up-regulated in numerous conditions, such as infection and inflammation[27]. Furthermore, it has been reported that IL-18 was a sensitive and specific biomarker to diagnosis acute kidney injury and predict the mortality of patients[28]. In our study, ozone oxidative preconditioning could largely reduce the level of serum IL-18 in group OOP+KT compared with that in group KT. Another well-know factor is COX-2, which is an inducible enzyme. Research showed that COX-2 expressed at sites of infection, inflammation and tumor which generated prostanoids that drive disease pathogenesis[29,30]. It has been showed that COX-2 can be rapidly and robustly expressed in response to diverse range of pro-inflammation cytokines and mediators[31]. In this study, serum level of COX-2 in OOP+KT group was obviously lower than that in group KT.

■ Conclusions

Ozone oxidative preconditioning could attenuated the kidney inflammatory injury and the oxidative stress injury of renal tubular epithelium cells in the rat model of kidney transplantation, which may be related with the inhibitions of NF-κBp65 and HMGB1 upregulation, and reductions of some pro-inflammatory cytokines. Furthermore, the enhancement of anti-oxidative stress system induced by ozone oxidative preconditioning may be another favour factor which helped to alleviate the kidney ischemia reperfusion injury. However, further research are needed to show the effect of ozone oxidative preconditioning on the model of renal transplantation between different strains rodents or other mammal models, especially the acute immunological rejection model.

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