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Preconditioning with Hyperbaric Oxygen Induces Tolerance Against Renal Ischemia-Reperfusion Injury *Via* Increased Expression of Heme Oxygenase-1

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Objective. **Renal ischemia/reperfusion (I/R) injury occurs in both native and transplanted kidneys. Hyperbaric oxygen (HBO) has been shown to prevent I/R injury in different tissues. The aim of this study was to evaluate the effect of HBO on renal I/R injury in rats.**

Materials and methods. **Male Sprague-Dawley rats were randomly assigned to three groups. The sham group ($n = 8$) received right nephrectomy. The I/R ($n = 8$) and HBO + I/R groups ($n = 8$) received 45 min left renal ischemia followed by 24 h of reperfusion after right nephrectomy. The HBO + I/R group ($n = 8$) received 100% oxygen at 2.5 atmosphere absolute (ATA), for 1 h at every 12 h interval for 2 d. Reperfusion was performed 24 h later after the last HBO exposure.**

Results. **In HBO + I/R group, blood urea nitrogen (BUN) and creatinine levels decreased significantly compared with the sham and I/R groups ($P < 0.01$). Activities of superoxide dismutase (SOD) were increased in renal tissue in the HBO + I/R groups. The content of malondialdehyde (MDA) were decreased in the HBO + I/R groups. Kidney samples from HBO + I/R group rats revealed markedly reduced histological damage under histopathological examination. The animals treated with HBO showed significantly elevated heme oxygenase-1 (HO-1) protein and mRNA levels expression compared with I/R group ($P < 0.05$).**

Conclusions. **Hyperbaric oxygen preconditioning (HBO-PC) can protect renal I/R injury against oxidative stress, and the up-regulation of HO-1 expression plays an essential role in HBO induced preconditioning effect.**

Key Words: **ischemia/reperfusion (I/R) injury; hyperbaric oxygen preconditioning (HBO); heme oxygenase-1 (HO-1); reactive oxygen species (ROS); malondialdehyde (MDA); superoxide dismutase (SOD).**

INTRODUCTION

Ischemia reperfusion injury is commonly seen in clinical practice, usually in renal surgeries, kidney transplantation, acute renal arterial occlusion and hypovolemic shock, which can lead to acute renal failure or delayed functional recovery of the transplanted kidney [1–3]. Therefore, to effectively prevent all the acute or chronic kidney diseases resulting from kidney I/R injury remains to be solved urgently. The pathogenesis of ischemia-reperfusion injury involves calcium overload, generation of oxygen free radicals, activation of the apoptosis gene, and the disorder of mitochondria function. Oxygen free radicals are considered to be one of the important factors involved in the pathophysiology of ischemia-reperfusion. Thus, reducing the production of oxygen free radicals in the kidney after reperfusion may alleviate the I/R injury.

Hyperbaric oxygen preconditioning induces tolerance against brain ischemia reperfusion injury by up-regulation of antioxidant enzymes [4], and induces ischemia tolerance in other organs including the spinal cord [5], myocardium [6], and liver [7]. The HO-1 system, the rate-limiting step in the conversion of heme,

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is one of the most critical cytoprotective mechanisms activated during cellular stress [8]. HO-1 protein overexpression protects the kidney from free radical-mediated injury [9]. To induce the overexpression of HO-1 chemically or physically is an effective method for preventing or treating I/R injury. Huang *et al.* [10]. found that HBO pretreatment significantly induced the expression of HO-1 in lung injury. However, the effect of HBO pretreatment on HO-1 expression in the kidneys of rats experiencing renal I/R injury remains unstudied. The aim of the current study was to determine whether HBO preconditioning could induce tolerance against on renal I/R injury, and, if so, to identify the mechanism for the tolerance induction.

MATERIALS AND METHODS

Animals

A total of 24 male Sprague-Dawley rats weighing 225–250 g were used in the present study, which was approved by the Institutional Animal Care and Use Committee. All experiments were performed in accordance with the National Institute of Health guidelines (NIH pub. no. 86-23, revised 1985). Before the experiments, the animals were fed a standard rat chow, drank water *ad libitum*, and were housed in metabolic cages under controlled temperature in 12-h light/dark cycles for at least 1 wk. The rats were randomly assigned to one of three groups. The sham group ($n = 8$) received right nephrectomy. Animals in the I/R ($n = 8$) group received 45 min left renal ischemia followed by 24 h of reperfusion after right nephrectomy. In the HBO + I/R ($n = 8$) group, 1 h at every 12 h interval for pretreatment with hyperbaric oxygen was performed for 2 d. I/R models were performed 24 h later after the last HBO.

Surgical Procedure

All procedures were performed aseptically. The animals were anesthetized with a combination of ketamine hydrochloride (85 mg/kg) and xylazine hydrochloride (15 mg/kg), and placed on a heating pad. Laparotomy was performed through a midline incision. The right renal artery and right ureter were ligated and right nephrectomy was performed. The left renal pedicle was exposed and occluded with a non-traumatic vascular clamp for 45 min (except for the sham group) and left kidneys were reperfused for 24 h in the I/R and HBO + I/R groups. Twenty-four hours after the initiation of renal ischemia, rats were sacrificed under anesthesia, blood was drawn, and the left kidneys were harvested and frozen in liquid nitrogen.

HBO Treatment Procedure

A special animal hyperbaric chamber was used for HBO-PC. The HBO-PC procedure was conducted by consecutive four times of 1 h HBO exposure (2.5 ATA, 100% O_2) at an interval of 12 h. The sham group was not exposed to HBO in order to determine the basal levels of antioxidative enzymes. Before pressurization, 100% medical oxygen was flushed through the chamber for 10 min to displace ambient air. Oxygen pressure was then increased slowly and reached 2.5 ATA in 5 min. The chamber was ventilated during HBO therapy to avoid carbon dioxide accumulation. After 60 min at 2.5 ATA, the chamber was decompressed to normal atmospheric pressure in 5 min. Accumulation of CO_2 was prevented by using a small container with calcium carbonate crystals. Chamber temperature was maintained in the range 22–25°C. No seizures were observed in any animals during all HBO-PC procedures. I/R group rats were placed in the same rodent chamber for 1 h at 12 h interval for 2 d in room air without increased pressure. All HBO-PC administrations were started at the same time in the morning (08:00 AM) to prevent biological rhythm changes.

Analysis of Renal Function

Twenty-four hours after renal ischemia, blood was used to assess renal function by measuring serum blood urea nitrogen (BUN) and creatinine (Cr). The samples were analyzed on a COBAS Mira chemical analyzer (Roche, Basel, Switzerland) with commercial kits from Sigma (St. Louis, MO).

Histopathologic Evaluation

Four-micron-thick sections were cut and stained with hematoxylin and eosin (HE). Samples were blindly analyzed by a pathologist who determined the extent of kidney injury based on a technique outlined by Erdogan *et al.* [11]. Briefly, 24 areas corresponding to the kidney proximal tubules were graded for the degree of renal damage based on each of the following parameters: tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage, and tubular dilatation. Specifically, one whole deep coronal section was examined under the microscope and graded according to the extent of damage, based on the percentage of involvement of the kidney. Higher scores represent more severe damage, with maximum score being 4. [0, normal kidney; 1, minimal damage (0%–5% injury); 2, mild damage (5%–25% injury); 3, moderate damage (25%–75% injury); and 4, severe damage (75%–100% injury)]. The mean score for each parameter was determined and subjected to statistical analysis.

Immunohistochemistry

Kidney tissue sections (4-μm thick) were subjected to immunohistochemical analyses. Sections were dewaxed in xylene, rehydrated through graded ethanol solutions, rinsed in PBS for 5 min, and immersed in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. The slides were then rinsed in PBS for 5 min, blocked with 5% BSA at room temperature for 15 min, then incubated with primary monoclonal antibody against HO-1 (1:100 dilution; Boster Biotechnology Ltd. Co., Wuhan, China) at 4°C overnight. Rabbit IgG Isotype was used as the negative control. The slides were then incubated with biotinylated mouse anti-rabbit IgG secondary antibody (Maixin Biotechnology Ltd. Co., Fuzhou, China). Finally, incubated with H_2O_2-DAB for 1 min. Sections were counterstained with hematoxylin. The estimates were performed by a blinded observer on coded sections (3 to 4 sections per kidney and 10 to 12 fields per section). The observer performed light microscopy and scored semiquantitatively the quantity of HO-1 staining in the whole section (0 = none, 1 = weak, 2 = moderate, 3 = strong).

Measurement of Antioxidant Enzyme Activity and Malondialdehyde Content

Kidney MDA and SOD content were performed according to the technical manual of the detection kits (Nanjing Jiancheng Biochemistry Co., Nanjing, China). Kidney tissue was homogenized in phosphate buffer (pH 7.4). After centrifugation at 12,000 *g* for 20 min, the MDA and SOD content in the supernatant were measured using the corresponding kits. MDA content was measured with thiobarbituric acid (TBA) reaction. The method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 532 nm; estimated MDA level was expressed as nmol/mg-protein. SOD activity was measured using nitroblue tetrazolium (NBT) reduction assay following the reduction of nitrite by a xanthine-xanthine oxidase system, which is a superoxide anion generator. One unit of SOD is defined as the amount that shows 50% inhibition.

TABLE 1

Primers and Probes of Rat HO-1 and ACTIN

Gene	Primers and probes	Sequence (5′ to 3′)
HO-1	Forward primer	GCCTGCTAGCCTGGTTCAAGATACTAC
	Reverse primer	AGGAAACTGAGTGTGAGGACCCATC
	Probe	FAM-AGAGACGCCCCGAGGAAAATCCCAGA-TAMRA
ACTIN	Forward primer	GCCACTGCCGCATCCTCT
	Reverse primer	CTGGAAGAGAGCCTCGGGG
	Probe	FAM- AGCTGCCTGACGGTCAGGTCATCACTATC-TAMRA

Real-Time RT-PCR

The mRNA levels were determined by real-time RT-PCR methods. Primers and probes are presented in Table 1. Total RNA was extracted from tissues using a total RNA purification kit (Shenergy Biocolor BioScience and Technology Co., Shanghai, China) according to the manufacturer's instructions. The purity and concentration of total RNA was measured by spectrophotometer. With the first strand cDNA synthetic kit (Fermantas, Vilnius, Lithuania) according to the manufacturer's instructions, 2 μg total RNA was reverse transcribed to cDNA. All PCRs were performed in the LightCycler (Roche, Switzerland) with a final volume of 25 μL. Optimum conditions were obtained with 2.5 μL buffer, 1.5 μL $MgCl_2$, 0.5 μL dNTPs, 0.25 μL Taq DNA polymerase, 0.1 μL of specific sense primer(s), 0.1 μL specific antisense primer(s), 0.1 μL specific probe(s), and 2 μL template cDNA. Finally, 17.95 μL ddH_2O was added to the reaction mixture. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and 60°C for 12 s, collecting the fluorescence signal at 40°C. The standard substances for target gene were performed using PCR with templates at the same time. From each amplification plot, a threshold cycle (Ct) value (defined as the number of PCR cycles where the fluorescence signal exceeded the detection threshold value) was calculated while the number of molecules of the genes was calculated. All samples were amplified in duplicate and the average values were then exported to a Microsoft Excel for further analysis.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 4.0 software package (GraphPad Software, Inc., San Diego, CA). All data were expressed as mean ± SD. Statistical analyses were made using one-way ANOVA, followed by a Student-Newman-Keuls (SNK) test for multiple comparisons. A P value <0.05 was considered as statistically significance.

RESULTS

Renal Functions

Renal functions of animals were assessed with serum BUN and creatinine levels in plasma (Table 2). In the I/R group, BUN (24.27 ± 7.45 mmol/L) and creatinine levels (238.70 ± 47.29 μmol/L) significantly increased compared with the sham group (5.87 ± 3.17 mmol/L, 63.39 ± 22.79μmol/L), indicating renal failure development ($P < 0.05$). In the HBO + I/R group, plasma urea nitrogen (14.89 ± 7.02 mmol/L) and creatinine levels (117.90 ± 35.61 μmol/L) exhibited significant decreases compared with the I/R group ($P < 0.01$). The difference of Plasma urea nitrogen and creatinine levels between HBO + I/R and sham group was not significant ($P > 0.05$).

Histopathologic Findings

Light microscopy revealed normal kidney morphology in the non-injured sham group (Fig. 1A). However, tubular cell necrosis, cytoplasmic vacuoles, hemorrhage, and tubular dilatation were observed in histological specimens from the I/R group (Fig. 1B). These four types of histological alterations were markedly reduced in specimens from the HBO +I/R group (Fig. 1C) compared with I/R group (Fig. 1G; 1.67 ± 0.72 *versus* 3.05 ± 0.62, $P < 0.01$).

Immunohistochemistry of HO-1

Positive staining of HO-1was observed in both the cortex and the outer medulla in the HBO + I/R group (Fig. 2E, F). HO-1 immunostaining was only marginally in both the cortex and the outer medulla in the sham group (Fig. 2A, B) as well as in the I/R groups (Fig. 2C, D), HO-1 immunostaining in the HBO +I/R group was significantly increased than that in the I/R group (2.29 ± 0.72 *versus.* 1.32 ± 0.38, $P < 0.01$), HO-1 immunostaining in the I/R group (Fig. 2C, D) was increased than that in the sham groups, however,

TABLE 2

Beneficial Effect of HBO Preconditioning on Renal Function (mean ± SD; $n = 8$)

	Sham	I/R	HBO + I/R
BUN (mmol/L)	5.87 ± 3.17	24.27 ± 7.45†	14.89 ± 7.02*
Cr (μmol/L)	63.39 ± 22.79	238.70 ± 47.29†	117.90 ± 35.61*

Both serum Cr and BUN increased in I/R group compared with those of sham group, but there was no difference between HBO + I/R and sham groups. HBO treatment significantly decreased Cr and BUN.

*$P < 0.01$, *versus* I/R group.

†$P < 0.01$, *versus* sham group.

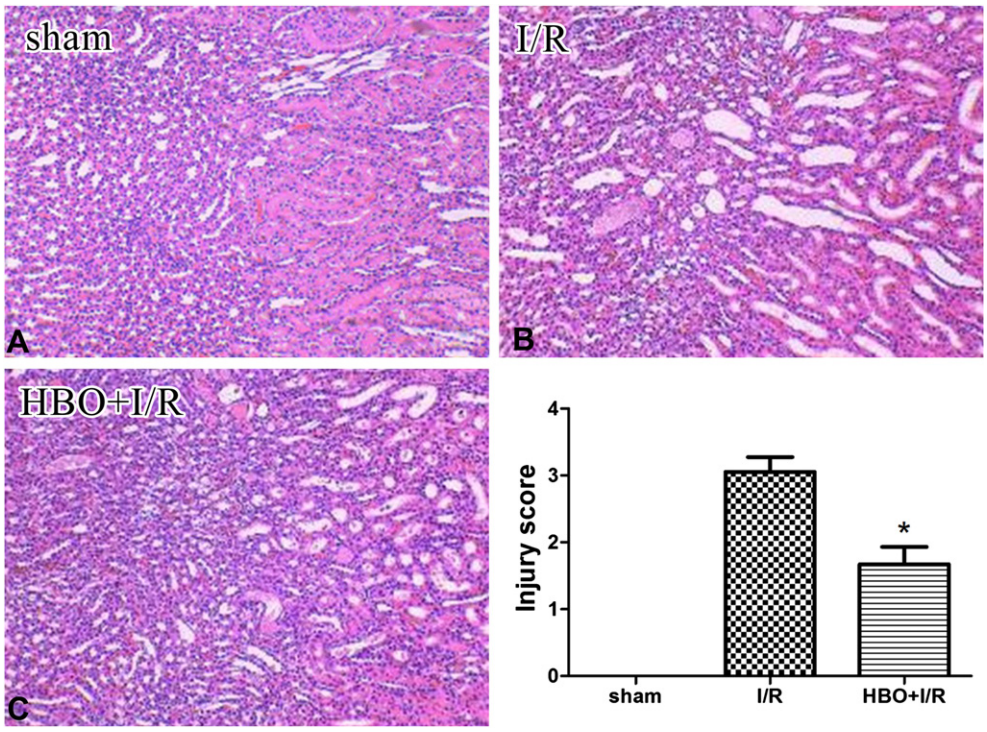

FIG. 1. Histologic changes in the kidney at the end of 24 h of reperfusion .Kidney tissues from rats of the various treatment groups were fixed, embedded, sectioned, and stained with hematoxylin and eosin. Shown are representative histological specimens from the sham group (A); renal I/R injury group (B); and HBO + I/R groups (C). Original magnification ×100. Histologic changes observed 24 h after renal I/R injury included tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage, and tubular dilatation. Injury scores are expressed as mean ± SD. The middle bar represents the injury score obtained from the I/R group, while the right bar represents the injury score from the HBO + I/R group, *$P < 0.01$, *versus* I/R group. The sham group (no bar depicted) had a mean score of 0.

the difference was not significant (1.32 ± 0.38 *versus.* 0.91 ± 0.64, $P > 0.05$).

Renal Oxidative Stress

As shown in Fig. 3, HBO preconditioning could significantly decrease MDA levels .The MDA content of the HBO + I/R group was significantly lower than that of the I/R group (15.38 ± 8.63 *versus* 28.30 ± 11.17, $P < 0.05$). No difference was observed between the HBO + I/R group and sham group (15.38 ± 8.63 *versus.* 12.04 ± 4.53, $P > 0.05$). HBO preconditioning significantly elevated SOD activities. The activity of SOD of the HBO + I/R group were higher than that of I/R group (190.20 ± 63.75 *versus.* 122.80 ± 49.42, $P < 0.05$). No difference was observed between the I/R group and sham group (122.80 ± 49.42 *versus.* 105.7 ± 41.09, $P > 0.05$).

Renal Tissue HO-1 Gene Expression

The mRNA levels in the sham, I/R and HBO + I/R groups were determined with real-time RT-PCR and β-actin was used as a reference gene (Fig. 4). The HO-1 mRNA levels of the HBO + I/R group were higher than that of I/R group (99.88 ± 53.30 *versus* 47.13 ± 30.91, $P < 0.05$). The levels of HO-1 mRNA in the I/R and sham group was not significantly different (47.13 ± 30.91 *versus* 23.83 ± 21.47, $P > 0.05$).

DISCUSSION

In this study, we investigated the effect of HBO on renal I/R injury and demonstrated that HBO efficiently suppressed I/R-induced renal dysfunction and tissue injury. We used a two-day short term HBO-PC in this study which is clinically feasible. Both renal function tests and histopathologic scores were relatively better in hyperbaric oxygen group. We found that this short term HBO-PC protocol decreased the content of MDA, and increased the activity of antioxidant enzymes. Moreover, renal tissues HO-1 protein and mRNA levels were increased in rats treated with HBO.

In our experiment, repeated HBO-PC induced an increase in the activity of SOD in the kidney and there was significant difference between the HBO + I/R group and I/R group at 24 h after I/R injury. In mammals, three isoforms of SODs are known: cytosolic copper-zinc SOD, mitochondrial manganese SOD (Mn-SOD), and extracellular SOD, which are products of distinct genes but catalyze the same reaction. Among them Mn-SOD localizes in mitochondria and is considered to be a first line of

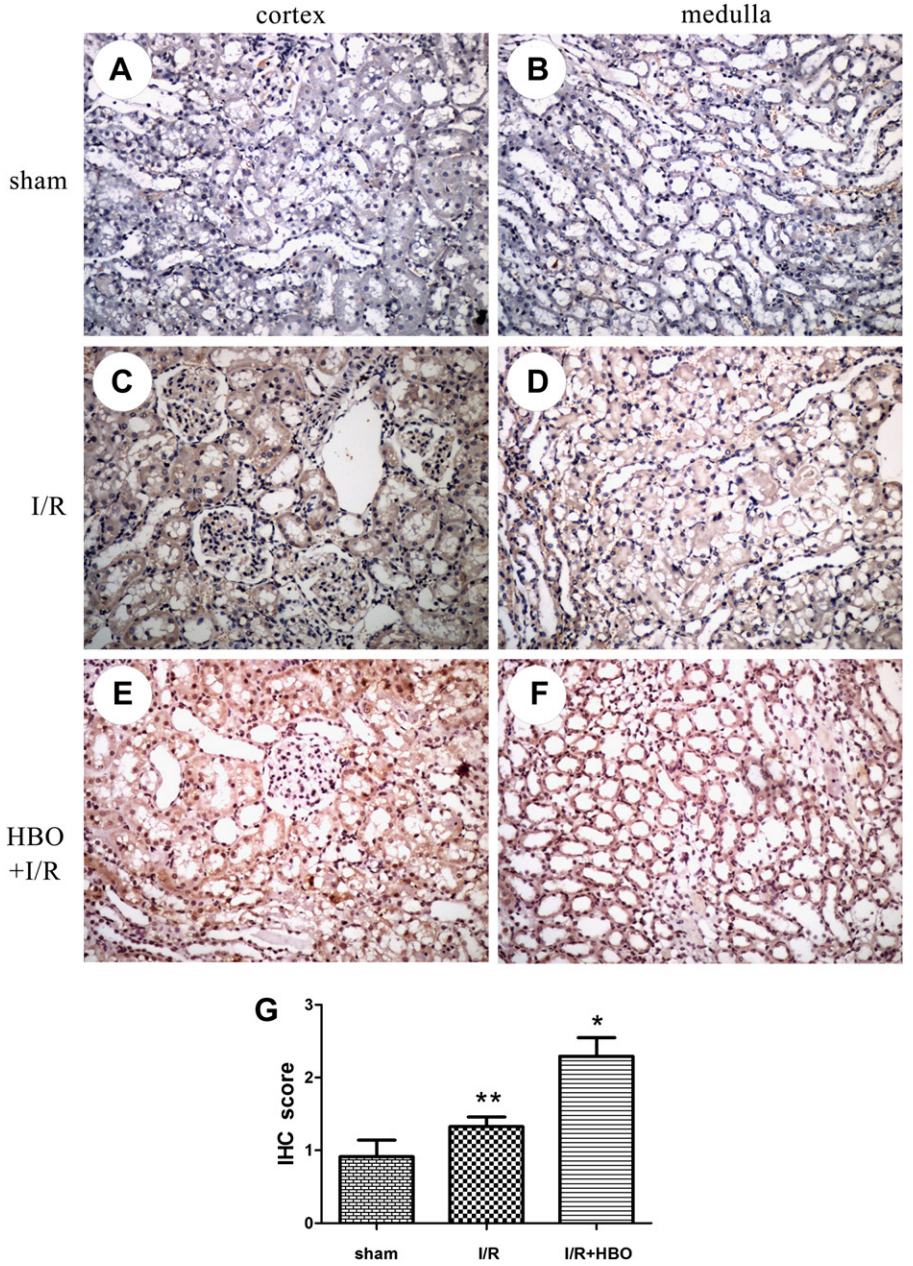

FIG. 2. Immunohistochemistry of HO-1 in the kidney at the end of 24 h of reperfusion. Each photograph represents at least three experiments. Cortex (A) and outer medulla (B) of the kidney of the sham group; cortex (C) and outer medulla (D) of the kidney of the I/R injury group; cortex (E) and outer medulla (F) of the kidney HBO + I/R groups. Immunohistochemical staining was carried out as described in materials and methods with original magnification ×100. (G) Data of the renal tissue HO-1 protein levels in the sham, I/R group and I/R + HBO group were expressed as means ± SD after replicate independent experiments ($n = 8$ per group), $^{*}P < 0.05$, *versus* I/R group; $^{**}P > 0.05$, *versus* sham group.

defense against oxidative stress [12]. It has been proved that the 5′-flank region of the Mn-SOD gene contains a putative binding site for NF-κB, which is activated by ROS and this suggests that Mn-SOD expression can be regulated by ROS through NF-κB [13]. Even though we measured total SOD activity, it is likely Mn-SOD is involved in HBO-PC.

Although the precise mechanisms of renal injury caused by ischemia-reperfusion have not been defined. ROS is thought to play an important role in the pathogenesis of I/R injury [14]. The radicals cause lipid peroxidation of cellular membranes resulting in increased microvascular permeability, interstitial edema, inflammatory cell infiltration, neutrophil activation, and eventually cell death. MDA is an important product of lipid peroxidation. In accordance with the increase in toxic oxygen metabolites. Renal MDA levels were also significantly increased, indicating the presence of enhanced lipid peroxidation due to I/R injury. The results obtained in the present study showed a significant

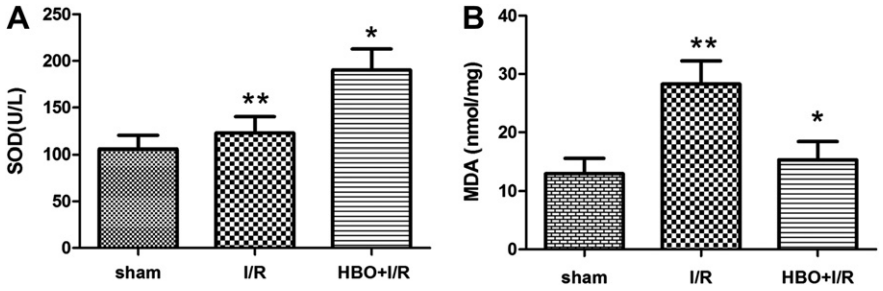

FIG. 3. (A) SOD activities in the kidney at the end of 24 h of reperfusion. The activities of SOD in HBO + I/R group were significantly higher than those in the I/R group and sham group. The activity of SOD in the I/R and sham group was not significantly different, $^{*}P < 0.05$, *versus* I/R group; $^{**}P > 0.05$, *versus* sham group. (B) MDA levels in the kidney at the end of 24 h of reperfusion. The levels of MDA in HBO + I/R group and sham group were significantly lower than in the I/R group. The difference between HBO + I/R and sham group was not significant, $^{*}P < 0.05$, *versus* I/R group; $^{**}P < 0.01$, *versus* sham group.

reduction of MDA content between the HBO + I/R group and the I/R group. These data indicated that HBO against renal I/R could have a relationship with decreased lipid peroxidation caused by oxidative stress. One of the possible explanations is that elevated activities of CAT and SOD scavenged excessive ROS and attenuated the lipid peroxidation.

The IARF injury is thought to be due to ROS generated by reperfusion, which has been suggested to be a result of the rapid release of heme from microsomal cytochrome P450 [15]. Yogaratnam *et al.* [16] found that both HO-1 mRNA and its enzyme activity were significantly increased in the reversible IARF model . Upregulation of HO-1 confered increased resistance against oxidative threat [17–20]. Inhibition of HO activity by Sn-MP resulted both in a marked increase in intracellular heme content and the aggravation of renal function in this model. HO-1 induction thus played an important role in the protection against renal dysfunction due to oxidative damages caused by heme. Using a cultured lens epithelium model, Padgaonkar *et al.* [21] showed that HBO induced HO-1 expression. Rothfuss *et al.* [22] further indicated that HBO increased the level of HO-1 in human lymphocytes consistent with previous studies. We found that both HO-1 mRNA and protein levels in the kidney of the HBO+ I/R group were significantly enhanced after exposure to HBO. The mechanism by which HO-1 exerts its beneficial effects *via* several mechanisms. Increased HO-1 activity results in degradation of the heme moiety, which is a pro-oxidant and potentially toxic to several cellular targets, including lipid bilayers, mitochondria, cytoskeleton, and components of the nucleus [23, 24]. In addition to detoxifying heme, elevated HO-1 activity results in the increased generation of bilirubin, an antioxidant capable of scavenging peroxy radicals and inhibiting lipid peroxidation [25]. Finally, HO-1 generates CO gas as a byproduct of the breakdown of heme. Several studies have demonstrated the protective role of CO itself in limiting renal damage in ischemia-induced acute kidney injury [26, 27].

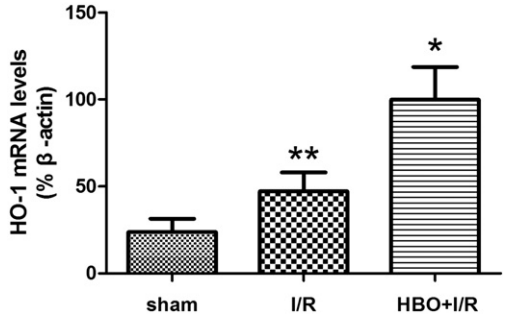

FIG. 4. The mRNA levels of HO-1 in the kidney at the end of 24 h of reperfusion. Real-time RT-PCR showed that the HO-1 mRNA level of the I/R + HBO group was higher than that of I/R group, the mRNA level of HO-1 in the I/R and sham group was not significantly different, $^{*}P < 0.05$, *versus* I/R group; $^{**}P > 0.05$, *versus* sham group.

The results also demonstrated that insufficient HO-1 expressed in the I/R group was not able to prevent the injury, presenting with the compromised renal functions and kidney oxidative injuries. While the HBO preconditioning induced significant HO-1 expression after the kidney ischemia and reperfusion with less kidney injuries and better kidney functions, which provided experimental evidence for renal ischemia and reperfusion injury prevention and treatment.

It is usually accepted that a critical ischemic window exists during which the tissue is vulnerable to reperfusion injury. Reperfusion leads to complete recovery before this critical window. However, if it is exceeded reperfusion leads to no recovery [28]. Some investigators have speculated that the altered microcirculatory environment after HBO therapy changed the response to ischemia-reperfusion by inactivating radical generation in parenchymal cells and endothelium [29, 30]. HBO-induced oxidative effects depend not only on therapeutic time windows but also on the exposure protocol. The salvage rate has been 100% if HBO treatment was initiated within 24 h of ischemia, compared with 0% when treatment was delayed 72 h after ischemia-induced skeletal muscle injury [31]. Another study

investigating the chronic effects of HBO using a standard therapy protocol of 2.4 ATA for 90 min as daily sessions for 21 d also defined functional reduction [32]. In most previous experiments, the HBO-PC protocol comprises three or five consecutive cycles of 1-h HBO exposure 2.5 ATA at a 1-d interval. Similarly, HBO preconditioning was performed with 100% oxygen at 2.5 ATA for 1 h at 12-h interval for four times in 2 d in our experiments. The last HBO preconditioning was performed at 24 h before renal ischemic. However, a 2-d short term HBO-PC was used in this study, which is more clinically feasible than a previous 5-d HBO-PC protocol.

Ischemic acute renal failure is very common in clinical practice. In nephrolithotomy, nephron-sparing surgery for renal tumor and living donor nephrectomy, ischemia reperfusion injury could lead to ARF. Some patients could develop into end-stage renal disease (ESRD) gradually after the surgery. HBO preconditioning has been proved to be able to induce ischemia tolerance, which ushers in a new way of prevent renal ischemia injury. Due to the mild toxicity, side effects and easy acceptability, HBO has a more promising future compared with other techniques such as ischemia and drugs etc.

In conclusion, HBO preconditioning has a protective effect on I/R by reducing the free oxygen radical peroxidation of lipid membranes, and increasing the activity of antioxidative enzymes. The up-regulation of HO-1 expression plays an essential role in HBO induced preconditioning effect.

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