

Oxymatrine ameliorates renal ischemia-reperfusion injury from oxidative stress through Nrf2/HO-1 pathway¹

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ABSTRACT

PURPOSE: To investigate if oxymatrine pretreatment could ameliorate renal I/R injury induced in rats and explore the possible role of oxymatrine in Nrf2/HO-1 pathway.

METHODS: Unilaterally nephrectomized rats were insulted by I/R in their left kidney. Twenty four rats were randomly divided into three groups: sham group, I/R + saline-treated group, I/R + OMT-treated group. Oxymatrine or vehicle solution was administered intraperitoneally injected 60 min before renal ischemia, respectively. Renal function, histology, makers of oxidative stress, cell apoptosis and Nrf2/HO-1 expressions were assessed.

RESULTS: Oxymatrine pretreatment exhibited an improved renal functional recovery, alleviated histological injury and oxidative stress, inhibiting tubular apoptosis, and accompanied by upregulated the expression of Nrf2/HO-1 proteins.

CONCLUSION: Oxymatrine may attenuate renal ischemia/reperfusion injury, and this renoprotective effect may be through activating the Nrf2/HO-1 pathway.

Key words: Ischemia. Reperfusion Injury. Oxidative Stress. Kidney. Rats.

Introduction

Ischemia-reperfusion (I/R) injury of the kidney, a serious clinical condition, is a commonly encountered problem in renal transplantation, hemorrhagic shock, vascular surgery, partial nephrectomy, and accidental or iatrogenic trauma, which causes a serious injury to tissues and organs¹. The mechanisms underlying renal I/R injury are complex and not completely understood. One important pathway that contributes to the pathogenesis of renal I/R injury is oxidative stress². Interruption of blood flow to the kidney and the subsequent reperfusion lead to an acute oxidative stress response that may cause the generation of reactive oxygen species (ROS). Consequently, the overproduction of ROS causes lipid peroxidation, DNA mutation, and induced apoptotic and necrotic cascades, ultimately resulting in cell death in various ways^{3,4}.

The nuclear factor erythroid-2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway was found to be related to antioxidative stress and scavenging of ROS under conditions of oxidative stress. Nrf2 (a transcription factor) responsible for the expression of phase II enzymes the primary function of which is to reduce redox stress⁵. Especially, Nrf2 binds to anti-oxidant response element (ARE) localized in the promoter regions of a battery anti-oxidant and detoxifying genes including HO-1⁶. Previous studies have demonstrated the therapeutic potential of targeting the Nrf2/Ho-1 pathway in renal ischemia-reperfusion⁷.

Oxymatrine (OMT), extracted from a traditional Chinese herb, *Sophora flavescens* Ait, has a variety of pharmacological properties such as anti-inflammatory, anti-oxidative, antiviral effects and immunological regulation, and has been used for the treatment of chronic hepatitis⁸, bronchial asthma⁹, myocardial ischemic injuries¹⁰, and lung¹¹, liver¹², intestinal¹³ and brain¹⁴⁻¹⁶ ischemia/reperfusion injury in animal models. OMT also have a protective role in adriamycin-induced chronic renal fibrosis¹⁷. More recently, OMT has been reported to have neuroprotection in cerebral ischemia-reperfusion injury in rats, which is related to Nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response¹⁸. However, the relationship between OMT-induced renoprotection and signal transduction pathway has not been elucidated. Thus, the purpose of the current study was to investigate whether OMT can protect the kidney against ischemic injury through Nrf2/HO-1 signal pathway.

Methods

The experimental protocol used in this study 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.

Male Sprague-Dawley rats (220-250g) were purchased from the center of Experimental Animals in Medical College, Wuhan University. The animals were kept under standard conditions and allowed free access to feed and tap water.

Experimental design

The I/R injury rat model was induced as previously described¹⁹. Briefly, after one week of acclimation, rats were fasted for 12 h before surgery with free access to tap water. 24 rats were randomly divided into three groups: sham group (n=8), I/R + saline-treated group (I/R group, n=8), I/R + OMT-treated group (n=8). Under the pentobarbital sodium (50mg/kg, intraperitoneally) anesthesia, rat underwent a median laparotomy to expose kidneys, followed by a right nephrectomy. The left renal hilus was occluded using a non-traumatic microvascular clamp for 45 min to effect complete cessation of renal blood flow, followed by 24 h reperfusion. The sham operated rat underwent the same procedure without vessel occlusion. At the end of the reperfusion period, Blood samples were collected by cardiac puncture for detection of blood urea nitrogen (BUN), serum creatinine (SCr) and lactate dehydrogenase (LDH) levels. Left nephrectomy was performed and renal tissue samples were fixed in 4% paraformaldehyde or snap-froze them in liquid nitrogen, and stored at -80°C for the subsequent measurement.

Drug administration

OMT was purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd (CAS 16837-52-8, purity ≥ 98%, molecular formula: C₁₅H₂₄N₂O₂). OMT was dissolved in 0.9% sodium chloride and stored at 4°C. We intraperitoneally injected OMT (150mg/kg) 60 min before renal ischemia. We chose the dose on the basis of previous studies and our preliminary experiment¹¹. For control purposes, I/R groups were injected with an equal volume of 0.9% sodium chloride.

Assessment of renal function

The SCr, BUN and LDH levels were measured with an automated analyzer (Siemens ADVIA 2400).

Renal tissues SOD and MDA assay

The activity of superoxide dismutase (SOD) was detected using commercialized assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The malondialdehyde (MDA) concentrations were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instrument, and the optical densities were read at 535nm. The results are expressed as nanomoles per milligram protein.

Histologic examination

We embedded kidneys in paraffin, 4- μ m thickness sections were stained with hematoxylin-eosin and examined under the microscope (Olympus, Tokyo, Japan) in a blinded manner by pathologist who was unaware of the treatment. The degree of tubular damage was evaluated using a semiquantitative scale according to the criteria described previously²⁰: 0 = normal kidney; 1 = minimal damage (< 5% area, outer medulla or the cortex); 2 = mild damage (5%-25% area, outer medulla or cortex); 3 = moderate damage (25%-75% area outer medulla or cortex); and 4 = severe damage (>75% area, outer medulla or cortex).

TUNEL staining

Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. TUNEL staining were performed using an situ terminal deoxynucleotidyl transferase mediated-dUTP nick end labeling (TUNEL) assay with Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturers' instructions. The nucleus was stained using 3,3-diaminobenzidine (DAB) as a substrate for the peroxidase. For each paraffin section, ten areas were randomly selected and the numbers of TUNEL-positive cells were counted at $\times 400$ magnification in a blinder manner.

Renal Nrf2 and HO-1 immunohistochemical assays

Immunohistochemical staining for Nrf2 and HO-1 detection was performed on formalin-fixed paraffin sections

using the streptavidin-biotin-peroxidase method. Under a light microscope (Olympus, Tokyo, Japan), the yellowish-brown color were accounted as positive staining.

Renal tissues Nrf2 and HO-1 Western Blot analysis

Samples (50 μ g/lane) were separated on a 10% SDS-PAGE gels and then electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat milk solution for 2 h at room temperature with gently shaking, and then incubated with primary antibodies overnight at 4°C. Subsequently, the membranes were washed and incubated for 2 h at room temperature with a secondary antibody. The protein bands were detected by chemiluminescence technology.

Statistical analyses

Data were expressed as mean \pm standard deviation values. Statistical analysis of the results was carried out by one-way analysis of variance (GraphPad Prism™ 5.0, San Diego, USA). $p < 0.05$ were considered significant.

Results

Effects of OMT on renal function induced by reperfusion after renal ischemia

BUN and SCr levels, two important indexes of kidney function, were significantly increased in the saline-treated I/R group after 24 h of reperfusion, while in the OMT-treated group, these levels of BUN and SCr were markedly decreased ($p < 0.05$, Figure 1A and B). We also measured lactate dehydrogenase (LDH) levels in these experimental groups. LDH levels in the saline-treated I/R group were obviously increased after 24 h of reperfusion. However, the LDH levels in the OMT-treated group were significantly decreased by 24 h of reperfusion ($p < 0.05$, Figure 1C).

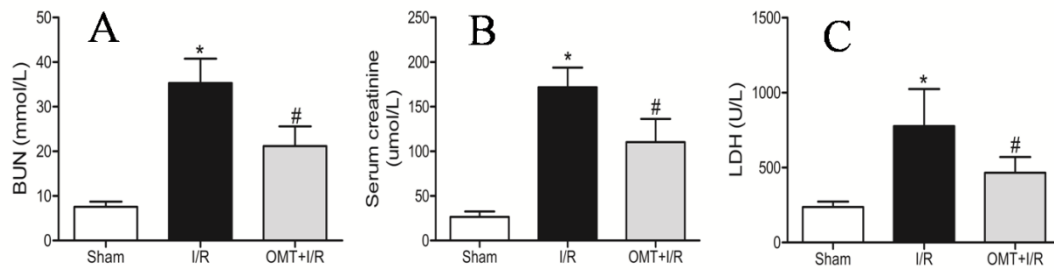


FIGURE 1 - OMT attenuates renal injury after renal I/R. Blood were collected 24h after reperfusion. BUN (A), SCr (B) and LDH (C) were significantly higher in the saline-treated I/R group than sham-operated group. OMT treatment inhibited renal dysfunction after renal I/R injury. * $p < 0.05$ compared with the sham-operated group and # $p < 0.05$ versus the I/R group.

Effects of OMT on SOD and MDA levels in renal tissues

Usually antioxidative ability of tissue is estimated by SOD activity and MDA content, which were the sensitive indicators of oxidative stress²¹. SOD is the most important endogenous antioxidant enzymes that scavenges oxygen free radicals and protects mitochondria against damage caused by cytotoxic reactions. MDA is one of the products of lipid peroxidation

which is induced by the attack of reactive oxygen species on polyunsaturated fatty acid. SOD activity and MDA concentration in kidney tissue was measured as shown in Figure 2, following 45 min of ischemia, reperfusion obviously decreased SOD activity and increased MDA concentration in the saline-treated I/R group compared with those in sham group ($p < 0.05$). In the OMT-treated group, the SOD levels increased and the MDA levels declined compared to the saline-treated I/R group ($p < 0.05$).

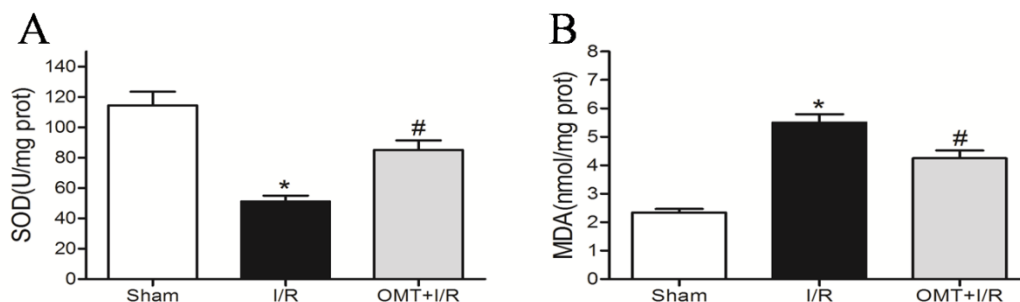


FIGURE 2 - OMT attenuates I/R-induced oxidative stress. We measured SOD levels (A) and MDA (B) activity in kidneys at 24h after reperfusion. * $p < 0.05$ compared with the sham-operated group and # $p < 0.05$ versus the I/R group.

Effect of OMT on I/R-induced histopathological damage

OMT could alleviate renal histology injury at 24 h after renal reperfusion. In Figure 3, the renal tubules in the saline-

treated I/R group showed pathological changes, including loss of brush border, congestion, tubular cell swelling, tubular dilation, and inflammatory cell infiltration. However, a significant amelioration of histological damage was seen in the OMT-treated I/R groups.

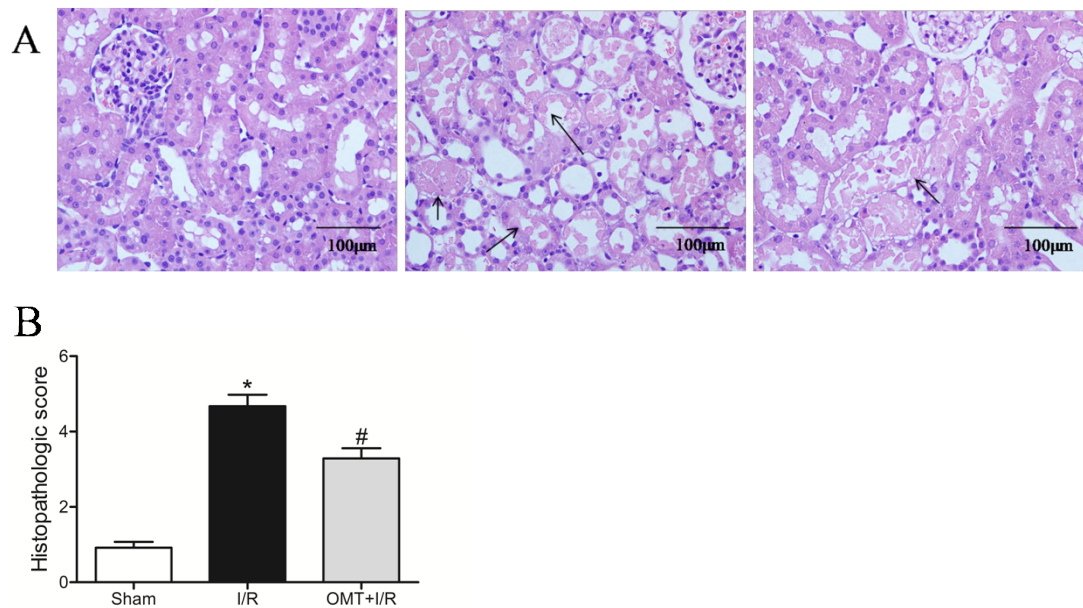


FIGURE 3 - OMT attenuates I/R-induced histopathologic renal damage. The sham group did not observed morphological changes; In the I/R group, kidney section shows tubular cell swelling, vacuolization, cast formation and tubular necrosis; and less damage was observed in the OMT+I/R group compared to the I/R group. (A) Representative hematoxylin-eosin stained kidney sections (magnification $\times 400$). (B) Histopathologic score measure at 24h after reperfusion. Scale bar = 100 μm . * $p < 0.05$ compared with the sham-operated group and # $p < 0.05$ versus the I/R group.

Effect of OMT on apoptosis of tubular epithelial cells

We investigated the ability of OMT pretreatment to mediate protection against ischemia induced apoptotic cell death. After 24 h reperfusion, TUNEL assay was performed

on renal tissue sections. As shown in Figure 4, a significant number of apoptosis cells in saline-treated I/R group were observed compared with the sham group. Few apoptosis cells were observed in the sham group. Pretreatment with OMT significantly decreased apoptosis cells as compared to the saline-treated I/R group ($p < 0.05$).

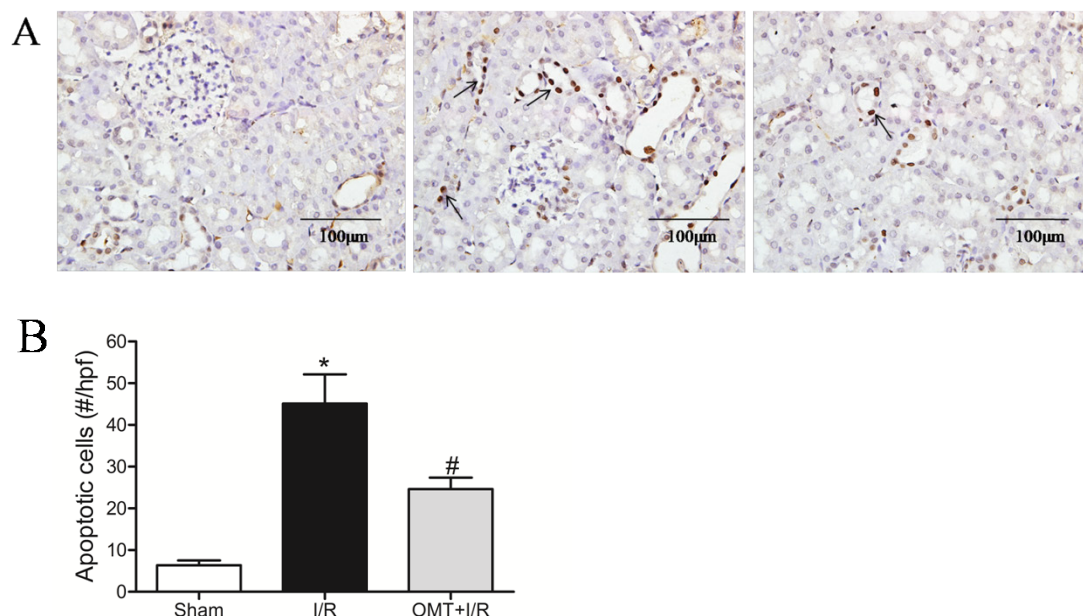


FIGURE 4 - OMT attenuates I/R-induced tubular cell apoptosis at 24h after reperfusion by TUNEL staining. At 24h after reperfusion, the numbers of apoptotic positive cells dramatically increased when compared with the sham group. In contrast to the I/R group, an increase significantly reduced by treatment with OMT. (A) Representative photomicrographs of TUNEL assay (magnification $\times 400$). (B) Quantitative analyses of apoptotic positive cells per field. Scale bar = 100 μm . * $p < 0.05$ compared with the sham-operated group and # $p < 0.05$ versus the I/R group.

Effect of OMT on Nrf2 and HO-1 expression after renal ischemia-reperfusion

To identify whether Nrf2/HO-1 signaling pathway is involved in the renoprotective effect of OMT, we analyzed ischemic renal tissue by immunohistochemical technique and Western blot in each group. Immunohistochemical analysis of renal tissues showed that the expression of Nrf2 and HO-1 was upregulated by OMT at 24 h after reperfusion (Figure 5A and B). In the sham group, few cells were stained by Nrf2 and HO-1.

In the saline-treated I/R group, the numbers of cells stained by Nrf2 and HO-1 increased. The number of cells labeled with Nrf2 and HO-1 in the OMT-treated I/R group was obviously increased compared with the saline-treated group. Consistently, Western blot analysis also showed that Nrf2 and HO-1 protein expressions were increased in saline-treated group while they were significantly further increased in OMT-treated group (Figure 5C and D) ($p<0.05$), which indicated that the Nrf2/HO-1 pathway may have an important role in the OMT-mediated renoprotection against I/R injuries in rats.

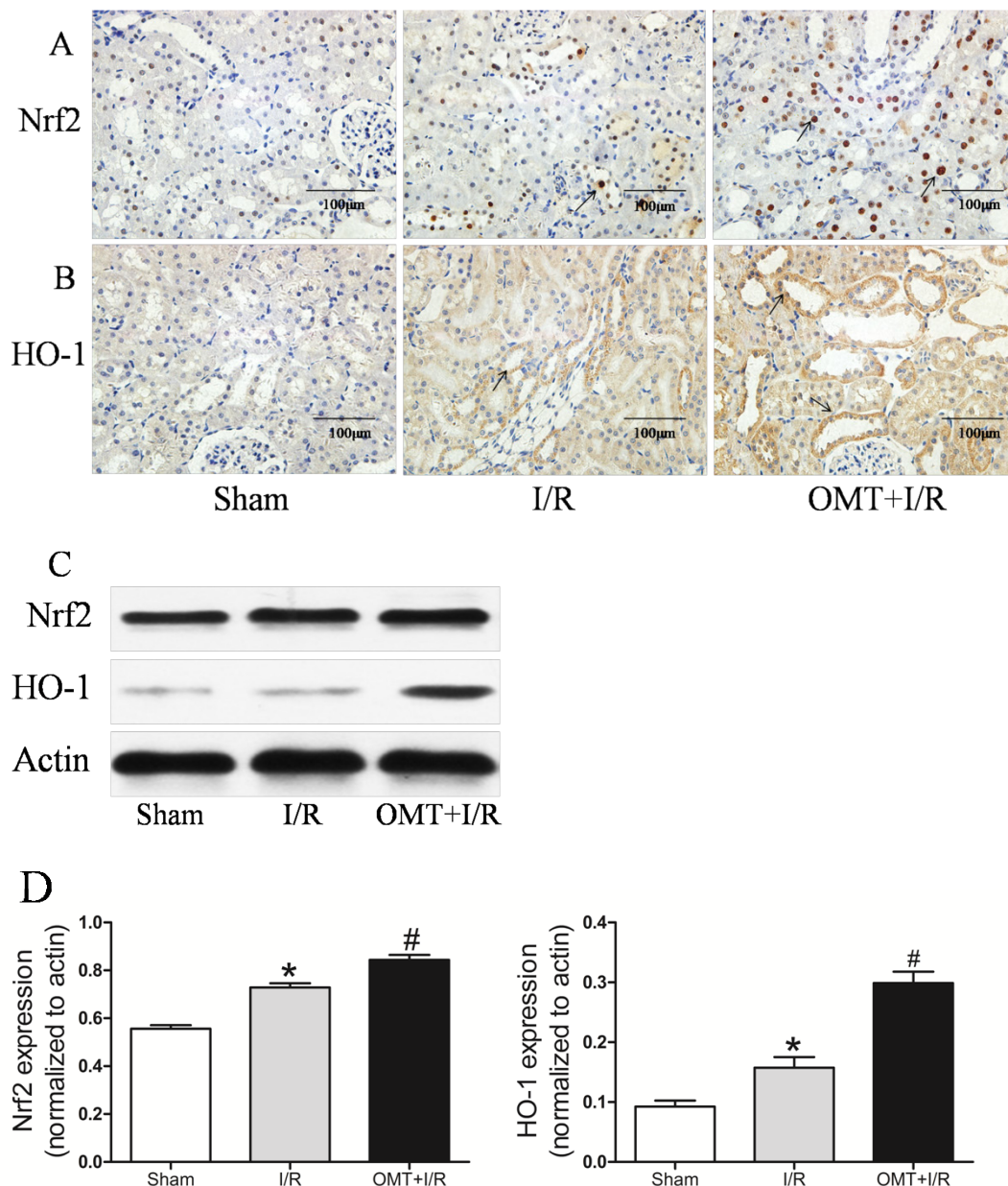


FIGURE 5 - Effect of OMT on I/R-induced Nrf2 and HO-1 expression. We detected Nrf2 and HO-1 expression in kidneys at 24h after reperfusion by immunohistochemical analysis and western blots. The results showed that the expression of Nrf2 and HO-1 significantly increased in the OMT-treated groups, compared to that in the I/R group. (A and B) Representative photomicrographs of immunohistochemical stained kidney sections for Nrf2 and HO-1 (magnification $\times 400$). (C) Representative western blots of Nrf2 and HO-1. (D) Quantitative analyses of the band density of Nrf2 and HO-1 (relative to Actin). Scale bar = 100 μm. * $p<0.05$ compared with the sham-operated group and # $p<0.05$ versus the I/R group.

Discussion

In this study a well-characterized rat model of renal I/R injury was used to demonstrate that the renal ischemic insults were associated with an increased production of free radicals, specifically superoxide, higher lipid peroxidation, and lower enzymatic antioxidant defenses. Targeting these damage factors or pathways may be a potential therapeutic platform for renal ischemia. Thus a number of studies have been targeted at investigating antioxidative effects on protection against ischemic damage.

OMT has been proved to have a variety of biological effects in treatment of viral hepatitis⁸, bronchial asthma⁹ and ischemic injuries^{10-16,22}, and plays a role in scavenging activity against reactive oxygen species¹⁸. In view of these considerations, we tested the therapeutic potentials of OMT on renal I/R injury rat model. After 24h reperfusion, the saline-treated I/R group rats had an obvious change in renal function, morphology and apoptosis. But administration of OMT before operation could protect renal function, decrease the histopathologic damage and alleviate apoptosis as compared with the saline-treated I/R group, indicating the lesser histological damage was observed as compared with the saline-treated group. These results suggest that OMT exerts renoprotective effects against renal ischemia-reperfusion injury.

Reperfusion produces excess reactive oxygen species (ROS) such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\cdot), while downregulating the expression of some endogenous antioxidant enzymes^{23,24} such as SOD, resulting in oxidative stress in the kidney. Among endogenous antioxidant defense mechanisms, SOD catalyzes the reduction of superoxide anions to H_2O_2 , and H_2O_2 is converted to H_2O and O_2 by catalase (CAT). The improved activities of these endogenous antioxidant enzymes provide a degree of protection against oxidative stress. Usually antioxidative ability of tissue is estimated by SOD activity and MDA content, which is the sensitive indicator of oxidative stress²¹. Therefore, we detected oxidative stress-related biochemical parameters in the kidney. Consistent with previous studies¹⁰, our results suggest that OMT could significantly reverse the I/R-induced decrease in the activities of SOD and the I/R-induced increase in the levels of MDA. This indicated that OMT could attenuate oxidative stress via enhance the endogenous antioxidant capacity and reducing lipid peroxidation.

The signaling pathways related to renal ischemia-reperfusion injury remains not fully understood. Recently, the Nrf2/HO-1 pathway that plays an important role in oxidative stress attracted more attention. Under normal conditions, Nrf2 signaling is

sequestered in the cytoplasm via binding to its repressor molecule, Kelch-like ECH-associated protein 1 (Keap1)²⁵. However, in pathological conditions just like oxidative stress could induce the dissociation of the Nrf2-Keap 1 complex and translocates into the nuclear, subsequently binds to the antioxidant response element (ARE) and induces phase II defense enzymes including HO-1, to offset cellular oxidative stress²⁶. The previous study reported that OMT significantly reduced Nrf2 and HO-1 expression in brain I/R injury¹⁸. However, the role of OMT on Nrf2 and HO-1 expression in ischemic kidney has not been determined yet. Therefore, in this study, the activity of Nrf2 and HO-1 in kidneys was detected by immunohistochemistry and Western blot. Here, our findings showed pretreatment of OMT significantly increased the expression of Nrf2, accompanied by up-regulation of HO-1 in the I/R-induced kidneys, suggesting that OMT preconditioning may gain renoprotection by regulating Nrf2 and its target genes, HO-1. Together, we conclude that OMT could upregulate the expression of HO-1 via the activation of the Nrf2 pathway.

Conclusions

Oxymatrine exhibits renoprotective effects against renal ischemia-reperfusion injury via the inhibition of oxidative stress. The potential mechanism of this renoprotection is due to the inactivation of the apoptotic pathway, and activation of the Nrf2/HO-1 pathway. OMT might be a promising preventive agent for acute ischemic renal failure.

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