



The effects of HIF-1 α overexpression on renal injury, immune disorders and mitochondrial apoptotic pathways in renal ischemia/reperfusion rats

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Background: Renal ischemia/reperfusion (RI/R) injury are a common pathogenesis of acute kidney injury, which may cause renal parenchyma damage clinically. Hypoxia-inducible factor-1 α (HIF-1 α) has protective effects on cells in regulating the metabolism, angiogenesis, erythropoiesis, and anti-apoptosis of RI/R injury. However, the specific mechanisms for HIF-1 α on RI/R injury are still unclear. This study aims to investigate the effects of HIF-1 α overexpression on renal function injury, immune disorder, and mitochondrial apoptosis in RI/R rats.

Methods: The rat model of RI/R injury was set up. The lentivirus (LV) vector of HIF-1 α overexpression was constructed, and then the LV was transfected to the model rats. The rats were randomly divided into four groups: the control group, RI/R group, RI/R + LV group, and RI/R + LV-HIF-1 α group for later experiments. The mRNA levels of HIF-1 α were detected by RT-PCR. Proteinuria, urea nitrogen, and serum creatinine levels were detected using the relative kit. Pathological damage was detected by HE staining. Apoptosis was detected by TUNEL staining. Levels of interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF- α) and interleukin-10 (IL-10) were detected by ELISA. Western blotting was used to detect the protein levels of HIF-1 α , caspase-3, caspase-9, Bax, Bcl-2, and other proteins.

Results: Compared with the control group, the mRNA and protein levels of HIF-1 α in the RI/R group were increased significantly ($P < 0.05$). Proteinuria, urea nitrogen, serum creatinine levels were increased significantly ($P < 0.05$). The levels of IL-6, IL-1 beta, TNF- α were increased significantly ($P < 0.05$). The ratios of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, and Bax/Bcl-2 were increased significantly ($P < 0.05$). There was a significant increase in apoptosis rate and renal pathological tissue damage ($P < 0.05$). Compared with RI/R+LV group, the mRNA and protein levels of HIF-1 α in the RI/R+LV-HIF-1 α group were increased significantly ($P < 0.05$). Proteinuria, urea nitrogen, serum creatinine levels were decreased significantly ($P < 0.05$). IL-6, IL-1 beta, TNF- α levels were significantly decreased ($P < 0.05$). IL-10 level was significantly increased ($P < 0.05$). The ratios of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, and Bax/Bcl-2 were significantly reduced ($P < 0.05$), showing that the pathological damage degree and the apoptosis rate was significantly lower.

Conclusions: HIF-1 α overexpression has protective effects on renal ischemia-reperfusion rats by improving pathological injury and immune function, reducing the release of inflammatory factors, and the expression of apoptotic proteins.

Keywords: Renal ischemia-reperfusion; hypoxia-inducible factor-1 α (HIF-1 α); renal injury; mitochondrial apoptosis pathway

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Introduction

Renal ischemia/reperfusion (RI/R) injury are a common pathogenesis of acute kidney injury because the kidney is a highly perfused organ and is sensitive to ischemia (1). RI/R injury happens in varying degrees during the treatment of partial renal tumor resection and renal transplantation (2). During ischemia, the activity of antioxidant enzymes decreases, leading to the formation of superoxide radicals and an increase in oxidative stress during reperfusion. Superoxide radicals destroy cell components, further damaging mitochondrial function and leading to cell death. Clinically, RI/R injury may cause renal parenchyma damage because of continuous blood flow reduction and pathological process, causing acute renal failure or chronic progressive kidney injury after kidney transplantation (3). However, because of complex pathogenesis, RI/R injury is challenging to treat. Therefore, it is necessary to dig the molecular mechanisms for causing RI/R injury or preventing it.

The precise pathophysiology of RI/R is largely unclear. Multiple pathways were involved, such as generation of reactive oxygen species (ROS) and associated inducible signaling, induction of apoptosis, and persistence of hypoxia and inflammation. The most effective method to reduce RI/R is to cool the kidney during surgery. However, there is currently no technique or drug to pretreat the kidney prior to ischemic injury. Many pharmacological reagents, such as Ca²⁺ channel blockers, mannitol, adenosine, and N-acetylcysteine, have been shown to protect against RI/R in animal models, but none passed clinical trials (4).

As heterodimer, hypoxia-inducible factor-1 α (HIF-1 α) is ubiquitously expressed in mammals and plays a role in regulating cell activity, cell growth, and apoptosis to maintain normal physiological activities of cells in a hypoxic environment (5). HIF-1 α has protective effects on cells in regulating the metabolism, angiogenesis, erythropoiesis, and anti-apoptosis of RI/R injury (6). Studies have shown that repeated hypoxic preconditioning can ease RI/R injury via the Bcl-2 anti-apoptotic pathway activated by HIF-1 α (7). However, the specific mechanisms for HIF-1 α on RI/R injury are still unclear. This study aims to investigate the effects of HIF-1 α overexpression on renal dysfunction,

immune disorder, and mitochondrial apoptosis pathway in rats with RI/R.

We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/tau-20-918>).

Methods

The animal experiments in this study were approved and supervised by the Ethics Committee of the Xiangyang No. 1 People's Hospital (SYXK (Hubei) 2016-0031). Each experiment was performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Research Council (US) Committee.

Experimental animals

Forty male Sprague-Dawley (SD) rats (aged six weeks, weight 110 \pm 5 g) were purchased from the Animal Experimental Research Center of Zhejiang University of Traditional Chinese Medicine, with the license number of SYXK (Zhejiang) 2018-0012. The animals were routinely housed in temperature control (22 \pm 1 °C) and light-controlled (200 lux, 12 hours light/dark cycle) animal facilities. The rats were accustomed for seven days before grouping and treatment.

Reagents

Hematoxylin-eosin stain (D006-1-1), urine protein quantitative kit (C035-2-1), creatinine determination kit (C011-2-1), urea nitrogen determination kit (C013-2-1) were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The TdT-mediated dUTP nick end labeling (TUNEL) apoptosis detection kit (C1091) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Antibodies against tumor necrosis factor α (TNF- α) (ab6671), IL-6 (ab100772), interleukin-1 β (IL-1 β) (ab100768), interleukin-10 (IL-10) (ab34843), Bax (ab53154), Bcl-2 (ab196495), caspase-3 (ab13847), cleaved caspase-3 (ab2302), caspase-9

(ab52298), cleaved caspase-9 (ab2324) were purchased from Abcam (UK).

Animal model establishment and grouping

Animal model was establishment as previously reported (8). The HIF-1 α lentiviral vector was constructed. The rats were divided into four groups according to the random number table method: the control group, RI/R group, RI/R + lentivirus (LV) group, and RI/R + LV-HIF-1 α group. We used HIF-1 α overexpression or vector control lentivirus (LV) to affect rats recorded as the RI/R + LV-HIF-1 α group and RI/R + LV group. All rats were anesthetized first and were dissected by midline laparotomy to expose bilateral kidneys. Both sides of the renal pedicle were clamped for 45 minutes and then restored blood flow and reperfusion. After loosening the arterial clamp, if the color of the kidney changed from dark red to bright red gradually, the modeling was determined as successful. After six hours of reperfusion, the indexes were evaluated.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA of rat myocardial tissues and H9C2 cells was extracted by Trizol agent, and then the absorbance (A260/A280) of RNA samples was measured with a Nanodrop spectrophotometer. According to the kit instructions, cDNA synthesis and PCR amplification were performed following the following steps: denatured for 5 minutes; (denatured at 94 °C for 30 seconds, annealed at 55 °C for 30 seconds, extended at 72 °C for 30 seconds) amplified for 35 cycles; extended at 72 °C for 10 minutes; stored at 4 °C. The products were separated by 2% agarose gel electrophoresis.

Renal function index

The urine and blood of rats in each group were collected. The proteinuria content in the urine and the levels of creatinine and urea nitrogen in separated serum were detected using an automatic biochemical analyzer.

Hematoxylin and eosin (HE) staining

The kidney tissues of rats in each group were first fixed with 10% formaldehyde for 48 hours and embedded in paraffin to make sections into 5 mm thick. Then the sections were stained with hematoxylin and eosin. The damage of kidney

tissue was observed under a fluorescence microscope at a magnification of 400 folds.

TUNEL staining

Kidney tissue sections of the rat were washed with xylene twice for dewaxing (5 minutes/time), dehydrated using gradient ethanol, blocked with proteinase K working solution at 37 °C for 20 minutes, and washed twice with PBS. Followed by adding 50 μ L of TUNEL reaction solution to each section and react in the dark at 37 °C for 60 minutes, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 10 minutes. The sections could be observed under a fluorescence microscope.

Enzyme-linked immunosorbent assay (ELISA)

The antigen was dissolved in a 50 mM carbonate coating buffer at 4 °C, which was added into a 96-well microtiter plate (100 μ L/well) overnight to coat the antigen. On the next day, discarding the coating solution, the coated plate was washed with 1 \times phosphate-buffered saline with Tween 20 (PBST) three times (5 minutes/time), blocked with 1% bovine serum albumin (BSA) (150 μ L/well) for 1 hour. Then they were washed with PBST three times again and added with 100 μ L of serum at different dilutions and control samples to incubate at 37 °C for 2 hours. Next, they were washed with PBST five times, added with 100 μ L of diluted Horse Radish Peroxidase (HRP)-labeled secondary antibody to incubate at 37 °C for 1 hour, washed with PBST for five times. The plate was added with the coloring agent to develop color for 20 minutes, and the absorbance was measured with a microplate reader.

Western blotting

The kidney tissues in each group were collected and lysed with lysis buffer holding protease inhibitor for total protein extraction on ice for 25 minutes. After centrifuged at 12,000 r/minutes for 10 minutes, the supernatant was collected, and the protein content was measured with the Bicinchoninic acid (BCA) kit. After denatured at 100 °C for 5 minutes, an equal amount of protein samples (20 mg) were loaded into each well and separated by the Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis, then transferred to polyvinylidene fluoride (PVDF) membrane. After blocking,

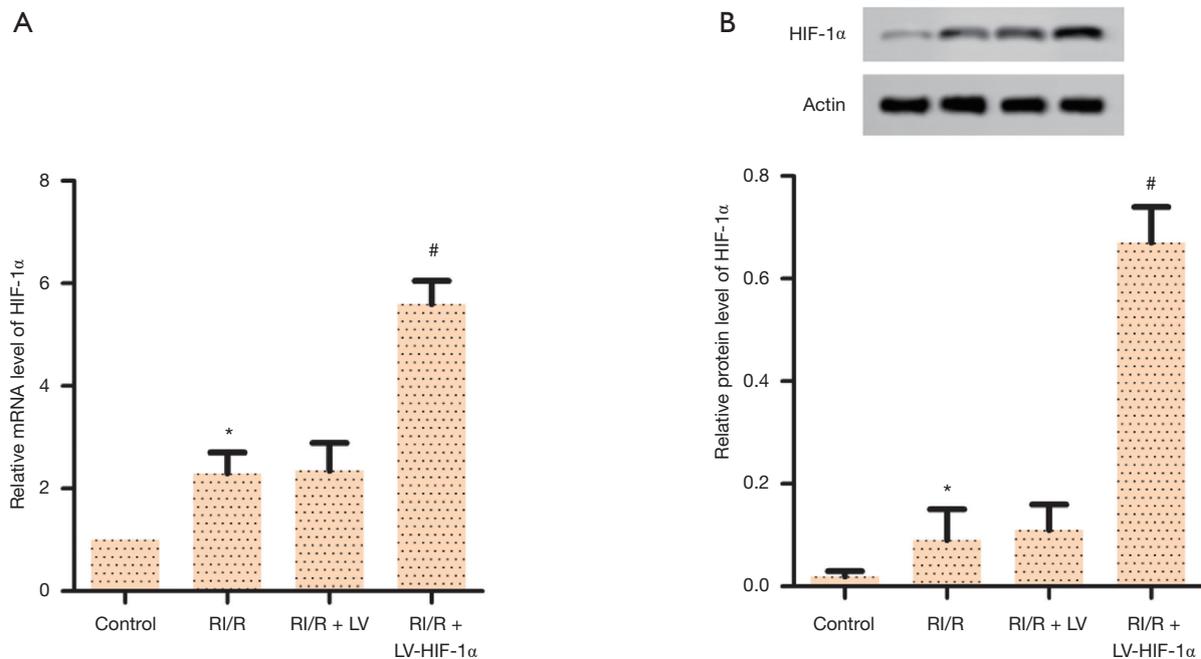


Figure 1 Validate of LV-HIF-1 α transfection. Effect of LV-HIF-1 α on HIF-1 α mRNA (A) and protein (B) expression levels in RI/R rats were tested with RT-PCR and western blotting. *, $P < 0.05$ versus control; #, $P < 0.05$ versus RI/R. HIF-1 α , hypoxia-inducible factor-1 α ; RI/R, renal ischemia/reperfusion; LV, lentivirus.

the membrane was incubated with corresponding primary antibody at 4 °C overnight, washed with TBST buffer, then added with the secondary antibody labeled with horseradish peroxidase at 4 °C for 2 hours. Enhanced chemiluminescence (ECL) reagent was used for signal detection. ImageJ software was used for the analysis of the gray value of each band.

Statistical analysis

SPSS 19.0 software was used for statistical analysis. Normal distribution measurement data was expressed as $\bar{x} \pm s$ and analyzed using a t -test. $P < 0.05$ was considered statistically significant.

Results

HIF-1 α overexpression increases mRNA and protein levels of HIF-1 α in RI/R rats

To validate the effects of HIF-1 α in RI/R, LV-HIF-1 α was established and transfected into SD rats once daily for one week before R/IR was established. The renal tissues were

collected for testing. The results of RT-qPCR and Western blotting detection showed that compared with the RI/R + LV group, the expression of HIF-1 α in the RI/R + LV-HIF-1 α group increased significantly both at the mRNA and protein levels (Figure 1, $P < 0.05$). The results supported that HIF-1 α overexpression with LV-HIF-1 α increases mRNA and protein levels of HIF-1 α in RI/R rats.

HIF-1 α overexpression reduces proteinuria, urea nitrogen and serum creatinine levels in RI/R rats

The overall effects of LV-HIF-1 α on renal injury was then evaluated. By detecting the proteinuria, urea nitrogen, and serum creatinine levels of the rats in each group with the kits, the results indicated that compared with the control group, the proteinuria (Figure 2A), urea nitrogen (Figure 2B), and serum creatinine (Figure 2C) levels of the RI/R group were significantly increased ($P < 0.05$). Compared with the RI/R + LV group, the proteinuria, urea nitrogen, and serum creatinine levels of the RI/R + LV-HIF-1 α group were significantly decreased ($P < 0.05$, Figure 2). The results indicated the protective role of LV-HIF-1 α in RI/R rats.

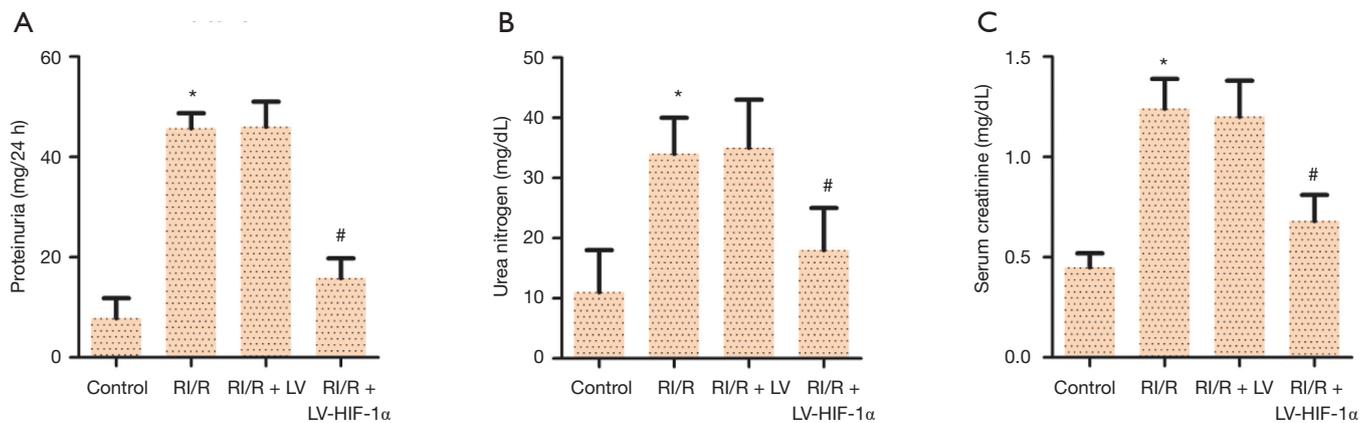


Figure 2 Effect of LV-HIF-1 α on renal function in RI/R rats. Proteinuria content in the urine (A) and the levels of creatinine (B) and urea nitrogen (C) in separated serum were detected using an automatic biochemical analyzer in RI/R rats. *, $P < 0.05$ versus control; #, $P < 0.05$ versus RI/R. HIF-1 α , hypoxia-inducible factor-1 α ; RI/R, renal ischemia/reperfusion; LV, lentivirus.

HIF-1 α overexpression improves the degree of pathological damage in RI/R rats

To further validate the protective role of LV-HIF-1 α in RI/R rats, HE and TUNEL staining was employed in renal sections. HE staining was used for detecting the pathological damage in each group. The result showed that the kidney tissue structure and glomerular cell morphology of the control group showed no abnormalities. In contrast, the renal tissue pathology of the RI/R group and the RI/R + LV group changed obviously, including the reduced number of glomeruli, edema, expansion, and vacuolation of the kidney tubule, inflammatory cell infiltration, fibrosis, cell congestion, visible protein casts in some areas, which showed apparent renal tissue pathological damage. However, the degree of pathological damage in the RI/R + LV-HIF-1 α group was significantly improved compared with the RI/R + LV group (Figure 3A). TUNEL staining was performed to detect the apoptosis of rats in each group, and the nucleus of TUNEL-positive cells was stained darkly brown. Compared with the control group, the apoptosis rate of the RI/R group was significantly increased ($P < 0.05$). While compared with the RI/R + LV group, the apoptosis rate in the RI/R + LV-HIF-1 α group was significantly decreased ($P < 0.05$, Figure 3B). The results supported the renal protective role of LV-HIF-1 α on RI/R rats.

HIF-1 α overexpression decreases the levels of IL-6, IL-1 β , and TNF- α , and increases the level of IL-10

To explore the underlined mechanism of action for LV-HIF-

1 α in alleviating RI/R, serum levels of typical inflammatory cytokines were tested. The levels of IL-6 (Figure 4A), IL-1 β (Figure 4B), TNF- α (Figure 4C), and IL-10 (Figure 4D) in each group of rats was detected by ELISA kit. The results showed that compared with the control group, the IL-6, IL-1 β , and TNF- α levels in the RI/R group increased significantly ($P < 0.05$). Compared with the RI/R + LV group, the IL-6, IL-1 β , and TNF- α levels in the RI/R + LV-HIF-1 α group were significantly decreased ($P < 0.05$), and IL-10 level was significantly increased ($P < 0.05$). These supported that LV-HIF-1 α inactivated the inflammation in RI/R rats.

HIF-1 α overexpression reduces the ratio of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, Bax/Bcl-2 in RI/R rats

Apoptosis was further investigated to show the mechanism of action for LV-HIF-1 α in RI/R rats. The protein expression levels of caspase-3, caspase-9, Bax, and Bcl-2 in each group were detected by Western blotting. The results showed that compared with the control group, the ratio of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, and Bax/Bcl-2 in the RI/R group increased significantly ($P < 0.05$). However, compared with the RI/R + LV group, the ratio of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, Bax/Bcl-2 in the RI/R + LV-HIF-1 α group was significantly decreased ($P < 0.05$, Figure 5A,B). The results showed that LV-HIF-1 α inhibited the apoptosis of renal cells in RI/R rats.

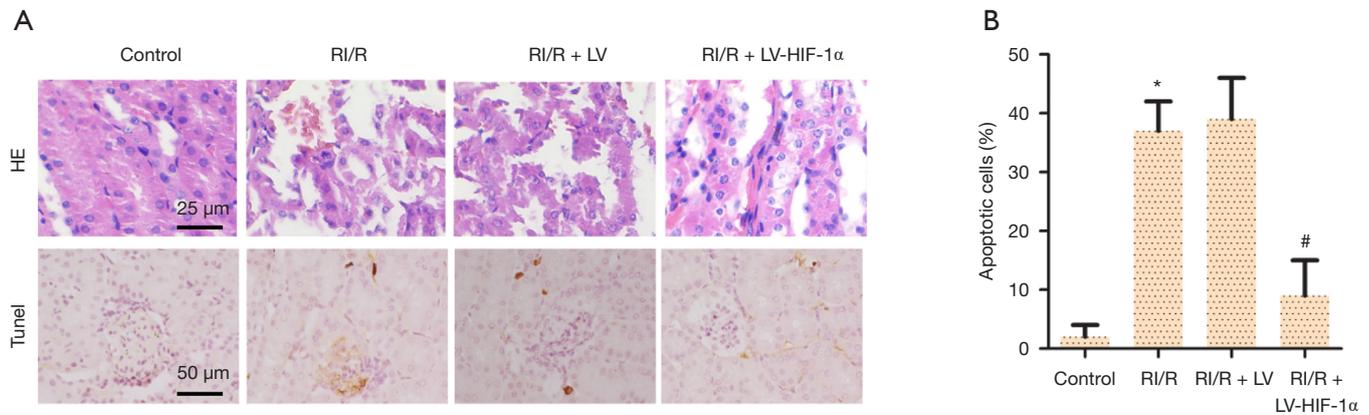


Figure 3 Effect of LV-HIF-1 α on pathological injury degree in RI/R rats. (A) Renal injury indicated with HE and TUNEL staining. (B) Apoptotic renal cells were counted. *, $P < 0.05$ versus control; #, $P < 0.05$ versus RI/R. HIF-1 α , hypoxia-inducible factor-1 α ; RI/R, renal ischemia/reperfusion; LV, lentivirus.

Discussion

As the most common cause of acute kidney injury, RI/R injury can result in a sharp decline in renal function (9). Because RI/R injury has a complex development and changing process, its pathophysiology mechanisms have not yet been sufficiently elaborated, and currently, there are no effective prevention and intervention ways for RI/R injury (10). Related studies have shown that HIF-1 α could be activated during reperfusion after renal ischemia, which is crucial for the survival of proximal tubule cells (11). HIF-1 α may help repair kidney tissues via regulating inflammation reactions after ischemia/reperfusion (12). Results in this study showed that the overexpression of HIF-1 α could improve the pathological tissue damage of RI/R rats.

RI/R can cause severe nephron damage, such as decrease of glomerular filtration rate and damage of renal tubular epithelial cells, resulting in weakened renal tubular reabsorption, which, clinically, often manifests the increase of urea nitrogen and creatinine levels in serum, and the appearance of proteinuria symptoms (13). This study found that HIF-1 α overexpression had the effects of reducing proteinuria, urea nitrogen, and serum creatinine levels in RI/R rats, suggesting that HIF-1 α overexpression has excellent protective effects on RI/R rats via alleviating the damage of glomeruli and renal tubules caused by ischemia/reperfusion.

Inflammation is a prominent feature of ischemia/reperfusion injury, which is characterized by leukocyte infiltration and renal tubular injury (14). Studies have

shown that during RI/R process, kidney tissues will produce a variety of inflammatory mediators formed after ischemia, such as TNF- α and IL-1 β , which are strictly related to ischemic injury (15). During RI/R injury, the inflammatory reactions are activated, and many proinflammatory cytokines are released. The expression of adhesion molecules on the surface of vascular endothelial cells and leukocytes are increased, resulting in the activation of leukocytes to damage the tissues by adhesion to vascular endothelial cells (16). In the inflammatory response network, IL-6, IL-1 β , and TNF- α are important proinflammatory cytokines. TNF- α increases the adhesion of vascular endothelial cells to neutrophils by up-regulating the expression of vascular endothelial adhesion factors, which leads to effusion and migration of inflammatory cells and causing the block of microcirculation of body tissues and damage. IL-6 and IL-1 β can promote granulocyte-mediated inflammation and neutrophil activation, which may aggravate the degree of kidney damage, while IL-10 plays a protective role against RI/R injury (17). This study found that HIF-1 α overexpression showed the effects of decreasing the levels of IL-6, IL-1 β , TNF- α and increasing the level of IL-10 in RI/R rats, which suggests that HIF-1 α overexpression improves RI/R injury by regulating the release of inflammatory factors.

Apoptosis is one of the programmed ways of cell death with three main pathways, including exogenous apoptosis pathways, endogenous apoptosis pathways, and endoplasmic reticulum stress pathways (18). The endogenous apoptosis pathways are also called a mitochondrial pathway, which is

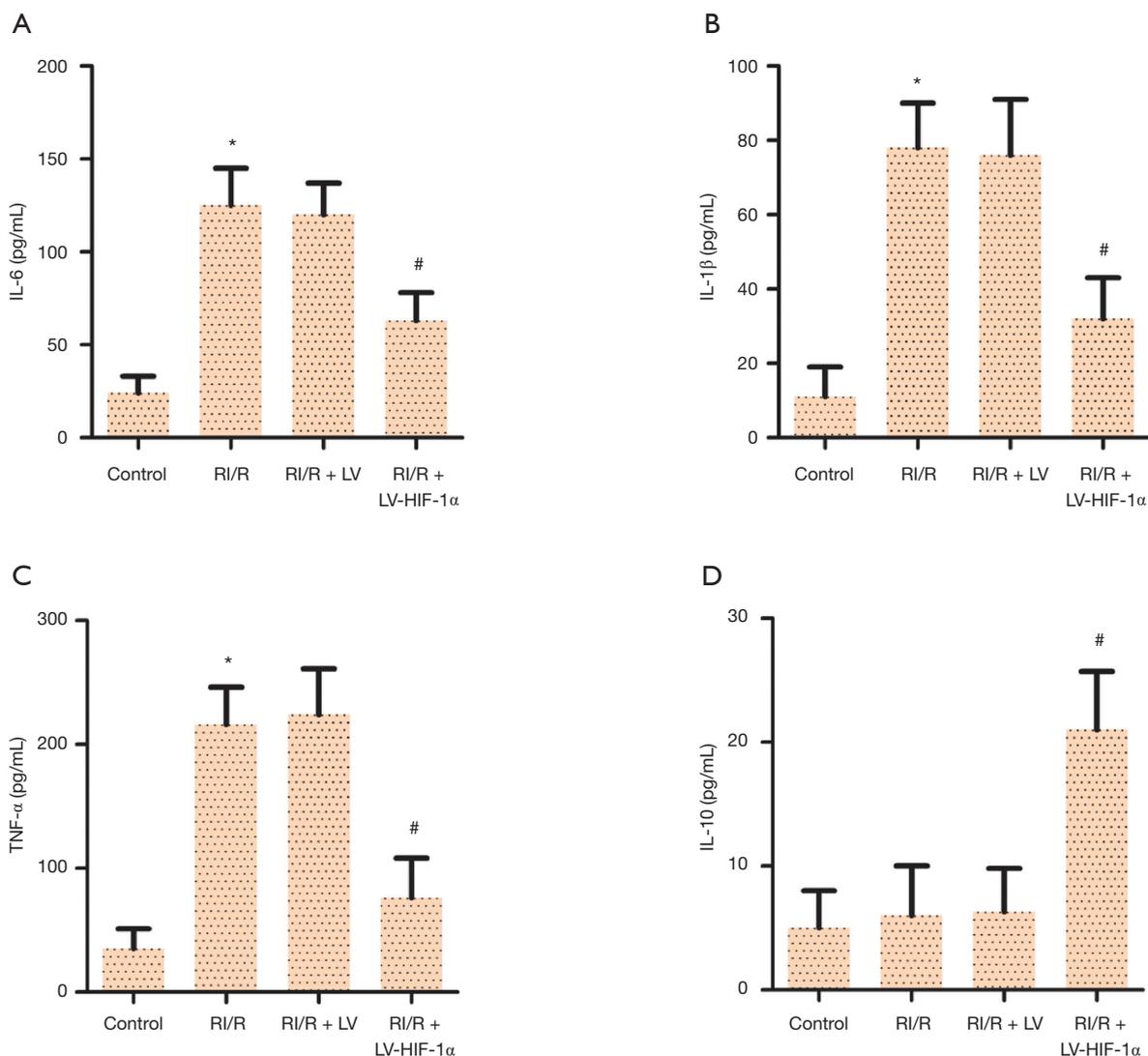


Figure 4 Effect of LV-HIF-1 α on typical inflammatory cytokines in RI/R rats. Serum levels of IL-6 (A), IL-1 β (B), TNF- α (C) and IL-10 (D) were investigated with ELISA. *, $P < 0.05$ versus control; #, $P < 0.05$ versus RI/R. HIF-1 α , hypoxia-inducible factor-1 α ; RI/R, renal ischemia/reperfusion; LV, lentivirus.

crucial to support cell physiology or pathology. Apoptosis is one way of tissue and organ damage during ischemia/reperfusion, which happens in the renal tubular epithelial cells with the most apparent apoptosis in the distal tubular epithelial cells (19). The caspase family proteins are critical elements in apoptosis, which can regulate apoptosis by interacting with many proteins and cytokines. Among the caspase family members, excessive activation of caspase-9 and caspase-3 in the apoptosis cascade pathway will promote apoptosis (20). The anti-apoptotic member Bcl-2

can prevent apoptosis, while the pro-apoptotic member Bax, in the outer mitochondrial membrane or cytoplasm, is oligomerized under stress to promote cytokines release from mitochondrial, triggering apoptosis (21). In this study, it was found that HIF-1 α overexpression reduced the ratios of cleaved caspase-3/caspase-3, cleaved caspase-9/caspase-9, and Bax/Bcl-2 in RI/R rats, suggesting that the overexpression of HIF-1 α can protect RI/R injury by regulating mitochondrial apoptosis pathway.

In summary, the overexpression of HIF-1 α has protective

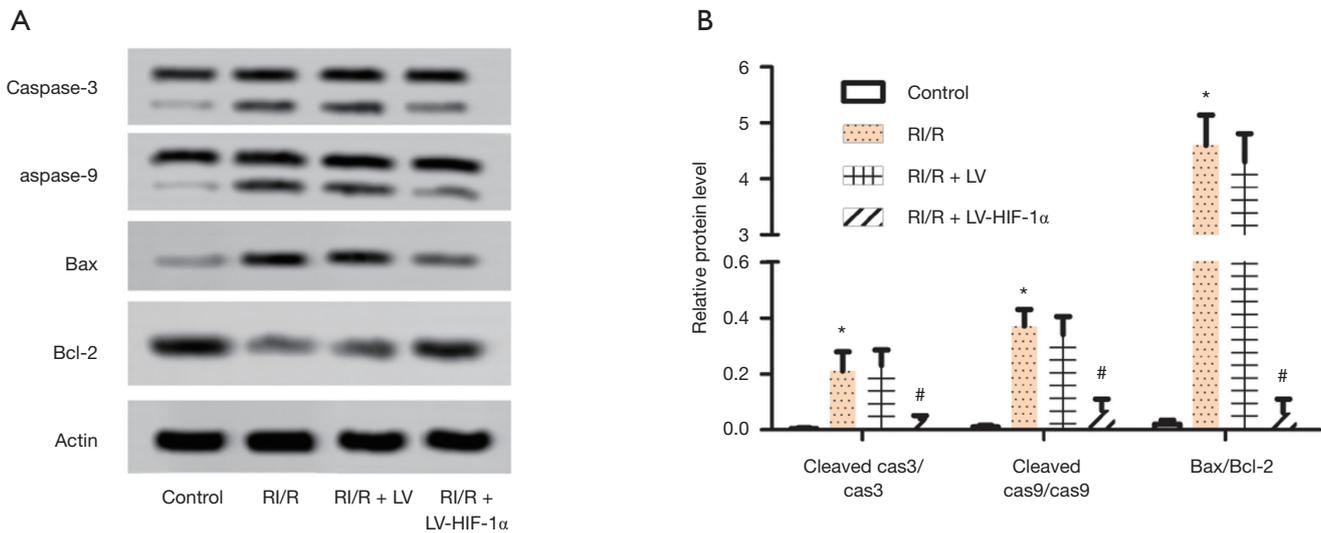


Figure 5 Effect of LV-HIF-1 α on apoptotic proteins in RI/R rats. (A) Caspase-3, Caspase-9, Bax and Bcl-2 proteins were tested with western blotting. (B) Relative protein levels of Caspase-3, Caspase-9, Bax and Bcl-2 were semi-quantified with Image J. *, $P < 0.05$ versus control; #, $P < 0.05$ versus RI/R. HIF-1 α , hypoxia-inducible factor-1 α ; RI/R, renal ischemia/reperfusion; LV, lentivirus.

effects on RI/R rats by improving renal function, inhibiting the release of inflammatory factors, and reducing the expression of apoptosis proteins during RI/R.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/tau-20-918>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tau-20-918>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tau-20-918>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The animal experiments in this study were approved by the Ethics

Committee of the Xiangyang No. 1 People's Hospital (SYXK (Hubei) 2016-0031). Each experiment was performed in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the National Research Council (US) Committee.

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