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Original Research

Effects of rhEPO on Nrf2 and HO-1 expression in rats with acute kidney injury and its protective effects on kidney

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Abstract: If the kidney suddenly loses its ability to remove waste, acute kidney injury (AKI) will occur that dangerous levels of waste may accumulate, and the chemical composition of the blood may become unbalanced. AKI usually develops rapidly within a few days and is very common in hospitalized patients, especially those in urgent need of intensive care. AKI can be fatal and requires serious treatment. However, it can be reversible. The purpose of this research was to investigate the effects of recombinant human erythropoietin (rhEPO) on the expression of nuclear factor E2-related factor 2 (Nuclear factor E2, Nrf2) and Heme oxygenase (HO-1) in rats AKI and its protective effects on the kidney. For this purpose, 40 SD rats were averagely and randomly divided into 4 groups: control group, sham operation group, model group, and rhEPO group. The rhEPO group was injected with 5% glucose mixed with rhEPO to form 3000 IU/ (kg/d) rhEPO. Except for the rhEPO group, three groups were injected with 5% glucose at the same dose level as the rhEPO group respectively. Before the third administration, the renal pedicle was clamped for 60 min and then perfused for 24 hours. Changes of Serum creatinine (Scr) and Urea nitrogen (BUN) of rats in each group were detected before and after modeling. Twenty-four hours after modeling, renal tissues of rats in each group were taken, and expressions of Nrf2 and HO-1 in renal tissues were detected by qRT-PCR and Western blot methods. There were no significant differences in Scr and BUN contents in the four groups before modeling ($p > 0.05$). There were no significant differences in Scr and BUN contents in the control group and sham operation group after modeling compared with those before modeling ($p > 0.05$). Expressions of Nrf2 and HO-1 in the rhEPO group were higher than those in the model group, the sham operation group and the control group ($p < 0.05$), while expressions of Nrf2 and HO-1 in model group were higher than those in sham operation group and control group ($p < 0.05$). There were no significant differences in expressions of Nrf2 and HO-1 between the sham operation group and the control group ($p > 0.05$). rhEPO can induce expressions of Nrf2 and HO-1 in AKI rats. RhEPO has a protective effect on the kidney, which may be related to expressions of Nrf2 and HO-1.

Key words: rhEPO; Acute kidney injury; Serum creatinine; Urea nitrogen; Nrf2; HO-1.

Introduction

Kidney injure means a drastic reduction in kidney function. Kidney injure is in two types, acute and chronic. Various causes can cause acute renal injure, which can be divided into three categories: pre-renal causes (insufficient blood supply to the kidneys, such as severe renal artery stenosis), renal causes (such as glomerulonephritis, and acute tubular necrosis), and post-renal causes (Urinary tract obstruction, for example, due to kidney stones or benign prostatic hyperplasia). The main symptoms of this problem are a sharp decrease in urine volume and an increase in blood urea and creatinine levels. Kidney disease, when it reaches the final stages of severe failure, regardless of whether the cause is acute or chronic), they have similar treatments. The principles of dietary treatment include taking certain supplements, kidney transplants, and hemodialysis or peritoneal dialysis (1-5).

At present, studies have reported an increase in the morbidity of acute kidney injury (AKI) among hospitalized patients. The morbidity of patients in the intensive care unit is about 30-40%, among which AKI occurs in most patients within 2 days after admission to the intensive care unit (ICU). The mortality of AKI is relatively high, and the morbidity is on the rise as the mortality rate (1-4). Besides, AKI is also one of the key causes of chronic kidney disease and chronic renal failure. About 35% of AKI patients may develop chronic kidney disease after the acute onset is treated and improved, and nearly 5% of them develop advanced renal disease (5). Therefore, studies on drugs that have protective functions on the kidney have important clinical value.

Recombinant human erythropoietin (rhEPO), which is commonly used for improving anemia status, not only has the function of promoting hematopoiesis but also has the function of slowing down cell apoptosis, reducing inflammatory reaction, promoting tissue oxygenation and promoting the growth of renal tubular epithelial cells (6-10). However, there is little research on protective functions of rhEPO on AKI, so we have studied protective functions of rhEPO in kidneys of AKI rats and further understood how rhEPO performs protective functions in kidneys of AKI through influences

of expressions of nuclear factor E2 (Nrf2) and Heme oxygenase (HO-1) in kidneys of AKI rats.

Nrf2 is a newly discovered gene transcription factor that causes highly sensitive oxidative stress. When Nrf2 transcription factors are deleted, cells' oxidative stress sensitivity is significantly improved (11). Antioxidase genes such as HO-1 interact with Nrf2 in oxidative stress to stabilize it and prolong the accumulation of Nrf2 in the nucleus, thus regulating its expression levels and activating the antioxidant system to play an antioxidant role (12). There are usually some symptoms of ischemia and anoxia in acute kidney injury (13). At present, there are few related literature reports on whether rhEPO regulates renal ischemia and anoxia by affecting Nrf2 and HO-1 expressions in patients with acute kidney injury. This study intended to treat ischemia-reperfusion AKI rat models by rhEPO intervention and to explore the effects of rhEPO on Nrf2 and HO-1 expression levels in AKI rats and its protective effects on the kidney.

Materials and Methods

Experimental animals and materials

Forty male SD rats weighing 180~240 g were provided by Shanghai Slack Experimental Animal Co., Ltd. with production license SCXK (Shanghai) 2012-0002. They were kept at a constant temperature of 23 ℃, with normal circadian rhythm for 12 hours, and were free to eat and drink. The operation of the modeling process on rats conforms to standards of the Laboratory Animal Ethics Committee of Weifang People's Hospital. RhEPO was purchased from Guangzhou Yuexunkang Pharmaceutical Co., Ltd. Desktop high-speed refrigerated centrifuges were purchased from Hunan Xiangxin Instruments Co., Ltd. and full-automatic biochemical analyzers were purchased from Shandong Boko Biological Industry Co., Ltd. Primers were designed and synthesized by Shanghai Bio-engineering Co., Ltd., Trizol reagent was purchased from Beijing Soledad Technology Co., Ltd., UV spectrophotometer was purchased from Shanghai Olay scientific instruments Co., Ltd., RIPA lysate, reverse transcription kit and PCR kit were all purchased from Beijing Ovia Biotechnology Co., Ltd., PVDF membrane was purchased from Thermo Fisher Scientific (China) Co., Ltd., Nrf2 and HO-1 rabbit anti-mouse polyclonal antibodies were purchased from American Baiqi Biological Company, GAPDH antibody was purchased from Ai Bixin (Shanghai) Biotechnology Co., Ltd., secondary anti-goat anti-mouse was purchased from China Biological Equipment Network, and ECL developer was purchased from Shanghai Shifeng Biotechnology Co., Ltd.

Experimental methods

Grouping and administration of experimental animals

Forty selected SD rats were numbered and randomly divided into the control group, sham operation group, model group, and rhEPO group, with 10 rats in each group. RhEPO group was injected with 5% glucose mixed with rhEPO to form 3000 iu/(kg d) rhEPO. Except for the rhEPO group, three groups were injected with 5% glucose at the same dose level as the rhEPO group respectively. The administration site was in the tail vein of rats. The administration time was once per day. Eight hours after the third administration, the AKI model was established by the ischemia-reperfusion injury method.

Modeling

Ischemia-reperfusion rat models were established 8 hours before the third administration. Preoperative fasting was 8 hours and water deprivation was 4 hours. Preoperative two-dimensional ultrasound was used to locate bilateral kidney positions, with skin preservation for disinfection and intraperitoneal injection of 10% chloral hydrate (2 mL/kg, specification 1 mL: 100 mg). A longitudinal incision was made from 1 cm above the pubic symphysis to below the xiphoid to protect the abdominal intestine tube, ureter and expose bilateral kidneys and renal pedicles, and bilateral renal pedicles were clipped with non-invasive arterial clips, and the ischemic was judged according to the color of the kidney. The color gradually changes from bright red to dark red. The abdominal cavity is clipped by a vascular clamp, kidneys are exposed by removing vascular clamp after 1 hour, the arterial clamp is loosened, perfusion is resumed, and kidneys restored to bright red and reperfusion is successful; normal activities and eating ability of rats after operation indicate that modeling was successfully built. The sham operation group has the same operation steps as a model group except that renal ischemia is not performed. After the operation, rats were fed freely and placed in an observation environment of about 26 ℃to observe their vital signs (14).

Detection indicators

Detection of Scr and BUN contents in four groups before and after modeling

Altogether 3ml abdominal venous blood was drawn before and after rat models were established, and blood samples were centrifuged to obtain serum. Contents of Scr and BUN were detected by an automatic biochemical analyzer.

Detection of Nrf2mRNA and HO-1 mRNA after modeling in four groups of rats by QRT-PCR

After 24 hours of modeling, 2ml venous blood of rats from the four groups was extracted, Nrf2mRNA and HO-1 mRNA in the blood was purified by addition Trizol reagent, and the concentration and quality of total RNA were identified by ultraviolet spectrophotometer. Altogether 2μl total RNA was taken and cDNA was synthesized strictly according to the instructions of reverse transcription kit. Altogether 2 μl synthesized

Table 1. Sequences of specific primers for Nrf2, HO-1 and β-actin.

Factor	Forward primer	Reverse primer
Nrf2	5′-CA-CATTCCCAAACAAGATGC-3′	5′-TCTT-TTTCCAGCGAGGAGAT-3′
HO-1	5'-CGTGCAGAGAATTCTGAGTTC-3'	5'-AGACGCTTTACGTAGTGCTG-3'
β-actin	5'-CCTGTATGCCTCTGGTCGTA-3'	5'-CCATCTCTTGCTCGAAGTCT-3'

cDNA was taken for qPCR. Nrf2 reaction conditions were as follows: 90 ℃ for 10 min, then 90 ℃ for 60 s, 58 ℃ for 60 s, 72 ℃ extension for 25 s, with 32 cycles. HO-1 reaction conditions were as follows: 95 ℃ for 9 min, then 95 ℃ for 10 s, 61 ℃ for 25 s, 72 ℃ for 20 s, with 35 cycles. Expression levels of Nrf2mRNA and HO-1 mRNA were detected with β-actin as an internal reference substance. The primer sequences were shown in Table 1. Relative expression levels were expressed by $2^{-\Delta CT}$, and the experiment was repeated three times.

Detection of expressions of Nrf2 protein and HO-1 protein in renal tissue 24 hours after modeling by western blot method

Rats were anesthetized by intraperitoneal injection 24 hours after modeling, kidney tissues were taken out, RIPA lysate was added to tissues for lysis, the supernatant of the lysed tissues was centrifuged, and the supernatant was subjected to a water bath at 100 ℃ for 5 minutes to denature protein, then total protein was collected, then protein was separated by 10% SDS-PAGE, transferred to PVDF membrane, and then sealed with 5% skim milk at room temperature for 1 hour. Then Nrf2 (1:1000) and HO-1 (1:1000) primary antibodies were added, GAPDH primary antibody (1:1500) was added respectively at a temperature of 4 ℃ overnight, then secondary anti-goat anti-mouse (1:1500) was added for incubation at 37 ℃ for 1.5 hours, finally, ECL developer was used for color development, and expression levels of the protein were semi-quantitatively determined by the ratio of the grayscale of the target protein to the grayscale of the internal reference protein (GAPDH).

Observation indicators

The Observation Indicators are 1) Comparison of Scr Levels of Four Groups before and after Modeling, 2) Comparison of BUN Levels of Four Groups before and after Modeling, 3) Detection of Expressions of Nrf2 and HO-1 in Four Groups of Renal Tissues 24 Hours after Modeling by QRT-PCR and 4) Detection of Nrf2 and HO-1 expressions in four groups of renal tissues 24 hours after modeling by Western blot method.

Statistical methods

In this experiment, SPSS 19.0 statistical software (Beijing NDTimes Technology Co., Ltd.) was used to analyze the experimental data statistically. Measurement data were reported as mean±standard deviation. The comparison between the two groups was conducted by t-test, the comparison between multiple groups was conducted by one-way analysis of variance, and the comparison before and after modeling was conducted by paired t-test. In this experiment, Graphpad Prism8 was used to draw pictures, with $p<0.05$ being differences with statistical significance.

Results

Comparison of Scr levels of four groups before and after modeling

There were no significant differences in Scr content among the four groups before modeling ($p> 0.05$). There were no significant differences in Scr content between the control group and the sham operation group after modeling ($p> 0.05$). Scr content in the model group and rhEPO group was higher than that before modeling ($p< 0.05$). The content of serum Scr in the model group was significantly higher than the sham operation group ($p< 0.05$). The content of serum Scr in the rhEPO group was significantly lower than the model group ($p< 0.05$), and the content of serum Scr in rhEPO group was significantly higher than the sham operation group and control group ($p< 0.05$, Table 2 and Figure 1).

Based on Figure 1, There were no significant differences in Scr content among the four groups before modeling ($p> 0.05$). There were no significant differences in Scr content between the groups of the control and sham operation after modeling ($p> 0.05$). The Scr content in the model group and rhEPO group was higher than that before modeling ($p< 0.05$). Compared with the sham operation group, the serum Scr content in the model group was significantly increased ($p< 0.05$). Compared with the model group, the serum Scr content in the rhEPO group was significantly decreased, and the serum Scr content in the rhEPO group was significantly higher than the sham operation group and control group ($p<0.05$).

Comparison of BUN levels of four groups before and after modeling

There were no significant differences in BUN content between the four groups before modeling ($p> 0.05$).

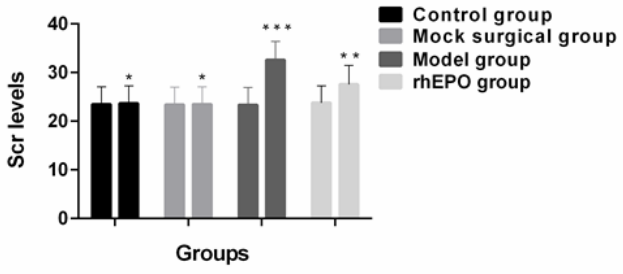

Figure 1. Comparative Analysis of Scr Expression Levels of Four Groups before and after Modeling; Note: $p> 0.05$ between * and *, $p< 0.05$ between * and **, ***, $p< 0.05$ between ** and ***.

Table 2. Comparison of Scr Levels of Four Groups before and after Modeling (n=10, μmol/L).

Group	Before molding	After molding	t	*p*
Control group	23.42±3.63	23.64±3.59*	0.136	0.893
Sham operation group	23.39±3.59	23.46±3.56*	0.044	0.966
Model group	23.32±3.57	32.54±3.84***	5.561	<0.001
RhEPO group	23.75±3.46	27.56±3.85**	2.328	0.032
F	0.029	13.220		
p	0.993	<0.001		

Note: $p> 0.05$ between * and *, $p< 0.05$ between * and **, ***, $p< 0.05$ between ** and ***.

There were no significant differences in BUN content between the groups of control and sham operation after modeling (p> 0.05). BUN content in the model group and rhEPO group was higher than that before modeling (p< 0.05). Levels of serum BUN in the model group were significantly higher than those in the sham operation group and the control group (p< 0.05). The content of serum BUN in the rhEPO group was significantly lower than the model group (p< 0.05), and the content of serum BUN in the rhEPO group was significantly higher than the sham operation group and control group (p< 0.05, Table 3 and Figure 2).

According to Figure 2, there were no significant differences in terms of BUN content between the four groups before modeling (p> 0.05). There were no significant differences in BUN content between the control group and the sham operation group after modeling (p> 0.05). The BUN content in the model group and the rhEPO group was higher than that before modeling (p< 0.05). Compared with the sham operation group, the serum BUN content in the model group was significantly increased (p< 0.05). Compared with the model group, the serum BUN content in the rhEPO group was significantly decreased, and the serum BUN content in the rhEPO group was significantly higher than the sham operation group and control group (p<0.05).

Expressions of Nrf2mRNA and HO-1mRNA in four groups of kidney tissues 24 hours after modeling

Expressions of Nrf2mRNA and HO-1mRNA in the rhEPO group were higher than those in the model group, sham operation group, and control group (p<0.05), while expressions of Nrf2mRNA and HO-1mRNA in model group were higher than those in sham operation group and control group (p< 0.05). Expressions of Nrf2mRNA and HO-1mRNA in the sham operation group were not significantly different from those in the control group (p>0.05, Table 4).

Expressions of Nrf2 protein and HO-1 protein in four groups of kidney tissues 24 hours after modeling

After 24 hours of modeling, expressions of Nrf2 protein and HO-1 protein in the rhEPO group were higher than those in the model group, sham operation group and control group (p<0.05). Expressions of Nrf2 protein and HO-1 protein in the model group were higher than those in the sham operation group and the control group (p<0.05). Expressions of Nrf2 protein and HO-1 protein in the sham operation group were not significantly different from those in the control group (p>0.05, Table 5).

Discussion

Studies have found that the main cause of inducing AKI is cell apoptosis or necrosis caused by ischemia and hypoxia (13). When kidney injury is in a severe state, extensive damage, inflammatory cell infiltration, fibroblast proliferation, damaged extracellular matrix repair disorder, and continuous inflammation and fibrosis occur in epithelial cell peritubular capillaries, endothelial

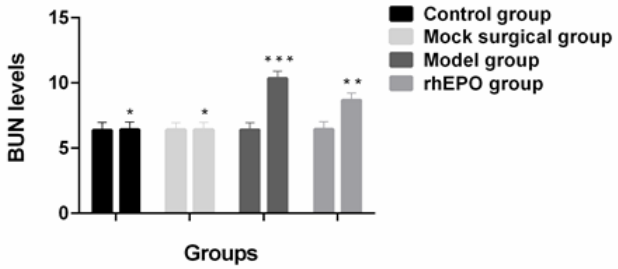

Figure 2. Comparative Analysis of BUN Expression Levels of Four Groups before and after Modeling; Note: p> 0.05 between * and *, p< 0.05 between * and **, ***, p< 0.05 between ** and ***.

Table 3. Comparison of BUN Levels of Four Groups before and after Modeling (n=10, mmol/L).

Group	Before molding	After molding	t	*p*
Control group	6.36±0.59	6.41±0.57*	0.193	0.849
Sham operation group	6.37±0.58	6.38±0.59*	0.038	0.970
Model group	6.39±0.56	10.33±0.55***	15.870	<0.001
RhEPO group	6.43±0.58	8.67±0.54**	8.939	<0.001
F	0.029	116.000		
p	0.993	<0.001		

Note: p> 0.05 between * and *, p< 0.05 between * and **, ***, p< 0.05 between ** and ***.

Table 4. Expressions of NRF2mRNA and HO-1mRNA in four groups of kidney tissues 24 hours after modeling.

Group	Control group	Sham operation group	Model group	RhEPO group	F	*p*
Nrf2mRNA	0.92±0.05*	0.93±0.04*	1.24±0.04***	1.54±0.04**	477.200	< 0.001
HO-1mRNA	1.09±0.06*	1.08±0.05*	1.36±0.05***	1.57±0.06**	171.500	< 0.001

Note: p> 0.05 between * and *, p< 0.05 between * and **, ***, p< 0.05 between ** and ***.

Table 5. Expressions of Nrf2 Protein and HO-1 protein in four groups of kidney tissues 24 hours after modeling.

Group	Control group	Sham operation group	Model group	RhEPO group	F	*p*
Nrf2 protein	0.55±0.03*	0.56±0.04*	0.82±0.05***	0.97±0.07**	170.900	< 0.001
HO-1 protein	1.03±0.04*	1.04±0.03*	1.23±0.04***	1.38±0.05**	170.100	< 0.001

Note: p> 0.05 between * and *, p< 0.05 between * and **, ***, p< 0.05 between ** and ***.

cells, and pericytes, posing certain difficulties and challenges to diagnosis and treatment (14-18). Due to renal tubular atrophy and fibrosis, a vicious circle is formed, which leads to AKI gradually developing into chronic kidney disease. Patients may die if serious, greatly threatening the living standard and working quality of patients and their families. At present, AKI is still based on symptomatic support therapy and renal replacement therapy (19). Up to now, although many drugs and therapeutic methods have shown good renal protection in animal experimental models, almost none of them have been successfully applied to the clinic (20). Therefore, there is an urgent need to study drugs that can protect the kidneys of AKI patients to reduce mortality and improve prognosis.

After grouping rats and modeling rhEPO drug intervention therapy, the results showed that there were no significant differences in Scr and BUN contents in the four groups before modeling ($p> 0.05$). After modeling, there were no significant differences in Scr and BUN contents in the control group and sham operation group compared with those before modeling ($p> 0.05$). Scr and BUN contents in the model group and rhEPO group were higher than those before modeling ($p< 0.05$). Levels of serum Scr and BUN in the model group were significantly higher than those in the sham operation group ($p< 0.05$). Contents of serum Scr and BUN in the rhEPO group were significantly lower than those in the model group ($p< 0.05$), while contents of serum Scr and BUN in the rhEPO group were significantly higher than those in sham operation group and control group ($p< 0.05$). It is suggested that clipping bilateral renal pedicle leads to the successful establishment of acute renal injury models of ischemia-reperfusion and rhEPO has protective effects on renal ischemia-reperfusion injury in AKI rats. Some studies have found that EPO expression increases when ischemia and hypoxia occur, which is mainly affected by hypoxia-inducible factors (21). Many studies have found that rhEPO induces the expression of inhibitor of apoptosis protein to reduce protein apoptosis, exert the effect of promoting cell survival and resisting cell apoptosis, exert antioxidant effect by reducing the activity of renal oxidase, and reduce inflammatory reaction (22-25). All the above reports indicate the potential medicinal value of rhEPO in clinical renal tubular injury diseases, which has a renal protective function on renal injury rats, and has a certain similarity with our research. Further study on the relationship between rhEPO and expressions of Nrf2 and HO-1 in AKI rats showed that expressions of Nrf2 and HO-1 in rhEPO group were higher than those in the model group, sham operation group and control group ($p< 0.05$) 24 hours after modeling detected by qRT-PCR and Western blot method. These results suggest that rhEPO increases expressions of Nrf2 and HO-1 during the treatment of AKI rats, thus regulating renal ischemia and hypoxia. Previous studies have shown that (26, 27), Nrf2/HO-1 expression increases and its corresponding signal pathway is activated, which plays an inhibitory role in renal tubular apoptosis due to ischemia-reperfusion injury, plays a defense function against oxidative stress, and plays a role in renal protection. Previous studies have shown that Epo can increase HO-1 mRNA expression in neuroblastoma SH-SY5Y cells by activating phosphatidylinositol -3 kinase (PI3K), mitogen-activated protein kinase (MAPK) and Nrf2 pathway to induce nerve HO-1 expression (28). There have also been studies that rhEPO can up-regulate Nrf2mRNA, thus reducing cerebral hemorrhage injury (29). In this regard, gene and genome editing technology can be very effective (30). It is proved from the side that rhEPO can regulate expressions of HO-1 and Nrf2 in ischemia-hypoxia rats, and rhEPO can play a protective role in renal function in AKI rat models (31-48).

To sum up, rhEPO therapy has a significant effect on AKI rats, which can effectively improve the renal function evaluation of AKI rats and expression levels of Nrf2 and HO-1 in rats, promote the recovery of renal function in rats, and is worthy of clinical promotion. However, due to the lack of relevant research in this experiment and the fact that the specific mechanism of rhEPO treatment improving expression levels of Nrf2 and HO-1 in AKI rats has not been explored, it is hoped that the majority of scholars can further carry out more in-depth experiments in the future.

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