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Influences of edaravone on necroptosis-related proteins and oxidative stress in rats with lower extremity ischemia/reperfusion injury

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ABSTRACT

The study aimed to investigate the influences of edaravone on necroptosis-related proteins and oxidative stress in rats with lower extremity ischemia/reperfusion (I/R) injury. The normal group (n=10), model group (lower extremity I/R injury model, n=10), treatment group (treatment with edaravone, n=10) and intervention group [lower extremity I/R injury model intervened with necrostatin-1 (Nec-1), n=10] were set. A conventional biochemical test was adopted to detect hepatic function indexes, and an enzyme-linked immunosorbent assay was performed to measure the levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), malondialdehyde (MDA), superoxide dismutase (SOD) and myeloperoxidase (MPO). The apoptosis level in rat tissues was determined via terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. The expression levels of genes and proteins were measured via quantitative polymerase chain reaction (qPCR) and Western blotting assay. The content of serum alkaline phosphatase (ALP), glutamic-pyruvic transaminase (GPT) and creatine kinase isoenzyme (CK-MB) was remarkably higher in the model group than that in the normal group. The levels of TNF-α, IL-6 and IL-1 were increased markedly in the model group, and the content of MDA in anterior tibial muscle tissues was also raised. The SOD content was elevated in the treatment group and intervention group. The number of apoptotic cells was larger than that in other groups (p<0.05). The gene expression levels of receptor-interacting protein kinase 1 (RIPK1), RIPK3, mixed lineage kinase domain-like (MLKL) and Caspase-3 were prominently higher in the model group than those in the treatment group and intervention group (p<0.05). The expression level of SOD in the treatment group and intervention group increased remarkably compared with that in the model group (p<0.05). RIPK1 and MLKL were raised evidently in the model group (p<0.05). Edaravone may regulate necroptosis-related proteins and oxidative stress in rats with lower extremity I/R injury by inhibiting the RIPK1-MLKL signaling pathway.

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Introduction

The reperfusion in temporary ischemic tissues is crucial to maintaining survival and organ function. However, it is often related to inflammatory responses, which make the tissue injury severer than the injury caused by simple ischemia. Such a phenomenon is known as ischemia/reperfusion (I/R) injury, which frequently occurs in the lower extremity, cerebral infarction, trauma, transplantation, surgical application of tourniquets and peripheral vascular diseases (1, 2). Acute lower extremity I/R injury is a common injury after vascular reconstruction, and pathological examinations have revealed that acute I/R injury is mainly composed of tissue edema, muscle necrosis, inflammation, thrombosis, etc. The inflammation induced by I/R injury stimulates granulocytic infiltration in tissues. Recent studies have manifested that the neutrophils activated by ischemic injury can trigger the release of neutrophil extracellular traps (3, 4). The I/R injury mostly occurs in the cases of thrombotic occlusion, embolism, post-traumatic recanalization of blood vessels, and it is one of the important causes of death and disability after lower extremity revascularization. With the development of endovascular techniques, a large amount of toxins and inflammatory mediators are released during tissue ischemia, resulting in aggregation of massive leukocytes at the site of ischemia. Moreover, reperfusion can further activate such inflammatory cells as leukocytes which enter into the general blood circulation to cause damage to distant organs, including heart, lung, liver and kidney (5).

Necrosis is described as a passive, non-physiological and non-adjustable death pattern caused by accidental and acute cell injuries(6, 7). However, the latest studies have revealed the process of cell death, in which necrosis can also be triggered in a highly regulated way and operated under the influence of genetic control (8, 9). Necrosis is

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primarily dependent on receptor-interacting protein kinase 1 (RIPK1) signaling and has attracted extensive attention from the field of biomedical research in recent years (10). Necrosis is a Caspase-independent programmed cell death activated and triggered by death receptors (11). As a specific inhibitor of RIPK1, necrostatin-1 (Nec-1) has inhibitory effects on necrosis. Therefore, preventing necrosis by repressing RIPK1 can be taken as an effective method for distinguishing between necrosis and incidental necrosis (12). Research has shown that processes such as apoptosis and autophagy may serve as the backing mechanisms forthe removal of damaged cells. Nevertheless, regulating cell apoptosis and autophagy can only slightly reduce the amount of NP cell death (13). It has been discovered that the pathological process of I/R injury is very complicated, involving inflammatory cell infiltration, oxygen free radical injury, vascular endothelial dysfunction, calcium overload, endoplasmic reticulum stress and many other mechanisms, with interrelations and interactions among various factors. There has been no definite mechanism capable of properly explaining the process of I/R injury, and the clinical treatment effect is still unsatisfactory, so it is particularly important to seek the key molecules and signaling pathways that mediate, regulate or amplify the injury of cells, tissues and even organs during I/R injury as the efficacious targets of intervention and treatment.

Edaravone, a free radical scavenger, has been successfully applied in the treatment of acute cerebral ischemia (14), and it plays a vital role in cardiac I/R injury. Many studies have demonstrated that edaravone can also exert a protective effect even after medication for ischemia (15). It has been found that edaravone is able to inhibit the lipid peroxidation chain reaction in the membranes of endothelial cells, neurons and glial cells (16) and has antioxidant activity that can decrease cell apoptosis (17). In-vivo and in-vitro studies have illustrated that edaravone can effectively repress theI/R injury-induced cell apoptosis (18). It is also considered to have neuroprotective effects on transient forebrain ischemia or focal ischemia in rodent models (19). In this study, it was proposed that edaravone can affect the necrosis and oxidative stress in lower extremity I/R injury through the RIPK signaling pathway. The typical animal model of I/R injury was utilized to detect the biochemical indexes and measure the changes in the RIPK pathway-related genes and proteins in tissues via quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting assay so as to reveal the role of edaravone in the necrosis and oxidative stress in lower extremity I/R injury.

Materials and Methods

Establishment of animal model

Male Wistar rats weighing 250-320 g were selected to establish the hind limb I/R injury model by simulating complete limb ischaemia caused by the application of tourniquets during extremity surgery. A tourniquet weighing 450 g was placed underneath the femoral vessels around the thigh as high as possible to block the collateral circulation, without obstructing the femoral vein. Then the rats were subjected to 2 h of ischaemia and 24 h of reperfusion by releasing the vascular clamps and tourniquet. At the beginning of reperfusion, the rats were kept awake.All the animals in this study were fed under standard conditions and provided with water and food at any time. All the animal experiments were conducted according to the clauses of the Animal Protection Law and approved by the Laboratory Animal Committee.

Detection of hepatic function

Abnormalities of serum biochemical indexes will occur in the case of lower extremity I/R injury. To predict the occurrence of lower extremity injury in advance in clinical practices, hepatic function indexes such as glutamic-pyruvic transaminase (GPT), alkaline phosphatase (ALP) and creatine kinase isoenzyme (CK-MB) were detected to provide important references for early diagnosis. The blood samples were drawn from the femoral vein, centrifuged and separated to collect the serum, followed by examination using a biochemical analyzer.

Detection of inflammatory cytokines in each group of rats

After the rats were anesthetized and sacrificed, the anterior tibial muscle tissues were acquired and washed with normal saline. Then 0.5 g of ischemic tissues in the lower extremity were broken using a homogenizer containing prepared tissue lysis buffer, followed by centrifugation at 1,200× g and collection of supernatant. Next, the level of serum tumor necrosis factor-alpha (TNF-α) was measured via an enzyme-linked immunosorbent assay (ELISA) kit. Finally, the absorbance in each group was determined using a microplate reader according to the practical situations and instructions.

Detection of levels of oxidant and anti-oxidant indexes [superoxide dismutase (SOD), malondialdehyde (MDA) and myeloperoxidase (MPO)] in anterior tibial muscle tissues via ELISA

The tissues (150 mg) stored at -80°C in a refrigerator were taken out using anti-freezegloves and then rapidly ground in a mortar. Later, the lysis buffer was added, followed by centrifugation and separation of the supernatant, and the changes in SOD, MDA and MPO levels were detected. At last, the absorbance of indexes in each group was measured using the microplate reader, and the standard curves were plotted to analyze the changes in the content in accordance with the specific instructions.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay

The prepared paraffin-embedded sections were utilized to determine the cell apoptosis in the lower extremity by virtue of the TUNEL apoptosis assay kit (Roche). Subsequently, the mounted section samples were subjected to labeling reactions using a fluorescent chromogenic reagent. After that, the FITC-labeled TUNEL-positive cells at the wavelength of 530 nm were challenged with 488 nm fluorescence under a fluorescence microscope and counted in 10 fields of vision after imaging.

QRT-PCR

TRIzol reagent (Invitrogen) was applied to extract the total ribonucleic acid (RNA) in the anterior tibial muscle tissues of rats in each group. After meeting the purity and concentration, the total RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) strands, with attention to the use of isopropyl alcohol.

Table 1. PCR primers.

Target gene	Primer sequence
β-actin	F: 5'-CGCTAACATCAAATGGGGTG-3' R: 5'-TTGCTGACAATCTTGAGGGAG-3'
SOD	F: 5'-TTCAATAAGGAGCAGGGAC-3' R: 5'-CAGTGTAAGGCTGACGGTTT-3'
Caspase-3	F: 5'-CTACCGCACCCGGTTACTAT-3' R: 5'-TTCCGGTTAACACGAGTGAG-3'
RIPK1	F: 5'-TCCTCGTTGACCGTGAC-3' R: 5'-GCCTCCCTCTGCTTGTT-3'
RIPK3	F: 5'-CCAGCTCGTGCTCCTTGACT-3' R: 5'-TTGCGGTCCTTGTAGGTTTG-3'
Mixed lineage kinase domain-like (MLKL)	F: 5'-TCTCCCAACATCCTGCGTAT-3' R: 5'-TCCCGAGTGGTGTAACCTGTA-3'

Table 2. Serum biochemical test results.

Group	ALP (U/L)	GPT (U/L)	CK-MB (U/L)
Normal group	90.5±0.3	50.6±0.5	70.8±1.8
Model group	229.5±0.8[a]	134.8±0.2[a]	212.7±0.6[a]
Treatment group	102.5±0.4[b]	79.8±0.8[b]	99.6±0.9[b]
Intervention group	109.5±0.8[b]	89.5±0.3[b]	105.8±0.7[b]

Note: The levels of serum ALP, GPT and CK-MB are elevated markedly in the model group compared with those in the normal group, and they are lowered notably in the treatment group and intervention group, implying abnormal hepatic function indexes. [a]p<0.05 vs. normal group, [b]p<0.05 vs. model group.

Later, the primer amplification was performed using a 20 μL system (2 μL of cDNA, 10 μL of Mix, 2 μL of primer and 6 μL of ddH_2O) for 40 cycles, and then PCR amplification was conducted. The primer sequences of target genes and internal reference were designed according to those on GenBank (Table 1). The expression levels of target genes were detected via qRT-PCR, and the mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method.

Western blotting assay

The anterior tibial muscle tissues of the rats were cut into pieces, weighed and added with RIPA lysis buffer at a ratio of 100 mg: 1 mL for tissue homogenization. Then the concentration of total proteins extracted from the anterior tibial muscle tissues of rats in each group was measured via a BCA protein assay kit (Pierce). After that, samples and gel were prepared and loaded for electrophoresis, and the proteins were transferred onto a membrane and sealed. The primary antibody and secondary antibody were added for incubation, and a freshly prepared ECL mixture was added for image development in a dark room, followed by treatment of bands with software. The protein bands were scanned and quantified using an Odyssey scanner, and the level of proteins to be detected was corrected via GAPDH. Image Lab software was employed to quantify the bands of Western blotting. The expression levels of corresponding proteins in each group were calculated.

Statistical analysis

All the raw data recorded during experiments were processed using SPSS 20.0 software andsubjected to multiple comparisons. The experimental results obtained were presented as mean ± standard deviation (χ±SD), and p<0.05 suggested that the difference was statistically significant. The histograms were plotted by means of GraphPad Prism 5.0.

Results

Results of serum biochemical test

The detection results of serum GPT, ALP and CK-MB are shown in Table 2. The levels of serum ALP, GPT and CK-MB were elevated markedly in the model group compared with those in the normal group (p<0.05), and they were lowered notably in the treatment group and intervention group (p<0.05), implying that the hepatic function indexes are increased significantly during the occurrence and development of I/R injury and apoptosis in anterior tibial muscle tissues, which can provide an important reference for early diagnosis.

Detection results of cytokines in each group

The levels of inflammatory factors TNF-α, IL-6 and IL-1 were obviously higher in model group than those in the other groups, and they declined evidently in treatment group and intervention group (p<0.05), manifesting that a large quantity of inflammatory factors are produced in lower extremity I/R injury and further indicating the deve-

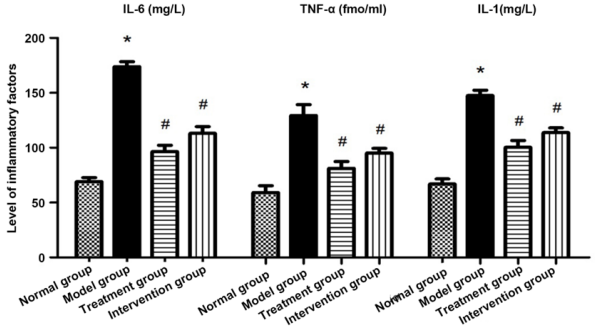

Figure 1. The levels of inflammatory factors IL-6, TNF-α and IL-1 are obviously higher in model group. *p<0.05 vs. model group, #p<0.05 vs. normal group, the same as below.

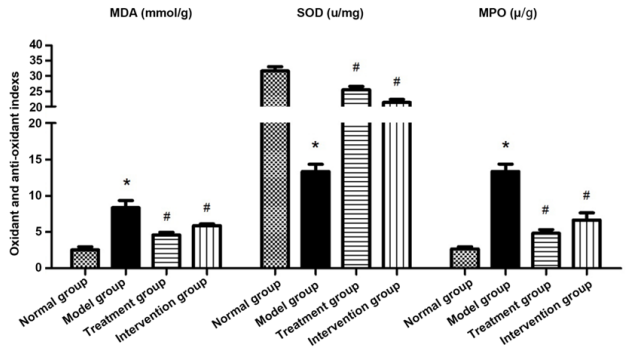

Figure 2. Oxidant and anti-oxidant indexes: the content of MDA and MPO is raised in model group (p<0.05), but the opposite trend of SOD is detected (p<0.05).

lopment of lower extremity I/R injury (Figure 1).

Results of SOD, MDA and MPO

According to Figure 2, the content of MDA and MPO was raised in model group (p<0.05) and reduced in the treatment group and intervention group (p<0.05), but the opposite trend of SOD was detected (p<0.05).

Results of TUNEL apoptosis assay

The results of the TUNEL assay (Figure 3) showed that there was massive cell apoptosis in the model group, while it decreased markedly in the treatment group after edaravone treatment. Besides, decreased apoptotic cells were observed in the intervention group.

Results of qRT-PCR

As shown in Figure 4, the content of SOD declined prominently in the model group (p<0.05) but rose in the treatment group (p<0.05). Moreover, the model group had distinctly increased levels of Caspase-3, RIPK1, RIPK3 and MLKL (p<0.05), while the treatment group and intervention group manifestedlower levels of those indexes (p<0.05).

Related proteins detected in anterior tibial muscle tissues

The levels of RIPK1 and MLKL proteins were elevated remarkably in model group (p<0.05) and lowered notably in the treatment group and intervention group (p<0.05) (Figure 5).

Discussion

The therapeutic principle of limb ischemia is to minimize the I/R injury. The mechanism of reperfusion injury is complex, including strong inflammatory responses to reperfusion, in which the innate immune system plays the central role. The I/R injury is partially mediated by the pro-inflammatory cytokines, activation of endothelial cells, reactive oxygen species (ROS) and neutrophil infiltration and activation (20). Neutrophils play a key role in resisting the inflammatory responses in I/R injury. The accumulation of neutrophils at the site of inflammation is triggered by cytokines, which is composed of genomic DNA in neutrophils and granular proteins embedded in the cytoplasm released into the extracellular matrix (21). Necroptosis is mostly likely to occur in acute ischemia, inflammation and trauma as well as some viral infections

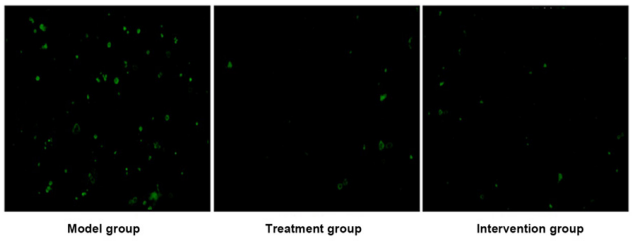

Figure 2. TUNEL staining. There is massive cell apoptosis in model group (×100), while it is decreased markedly after edaravone treatment (×100). Besides, decreased apoptotic cells are observed in intervention group (×100).

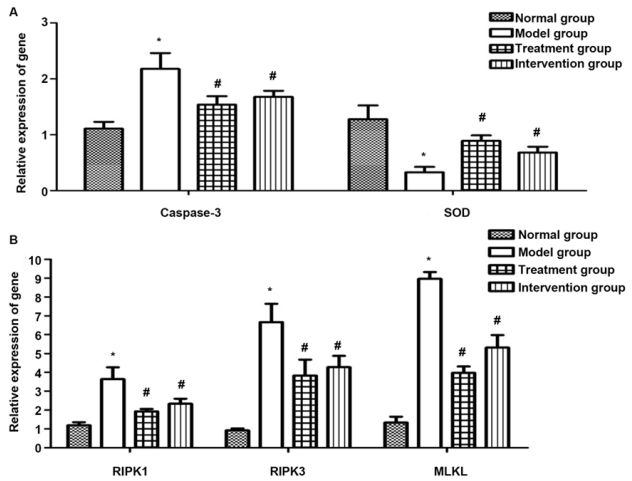

Figure 4. Results of qRT-PCR. The content of SOD declines prominently in model group (p<0.05) but rises in treatment group (p<0.05). The trends of Caspase-3, RIPK1, RIPK3 and MLKL are opposite. *p<0.05 vs. model group, #p<0.05 vs. normal group.

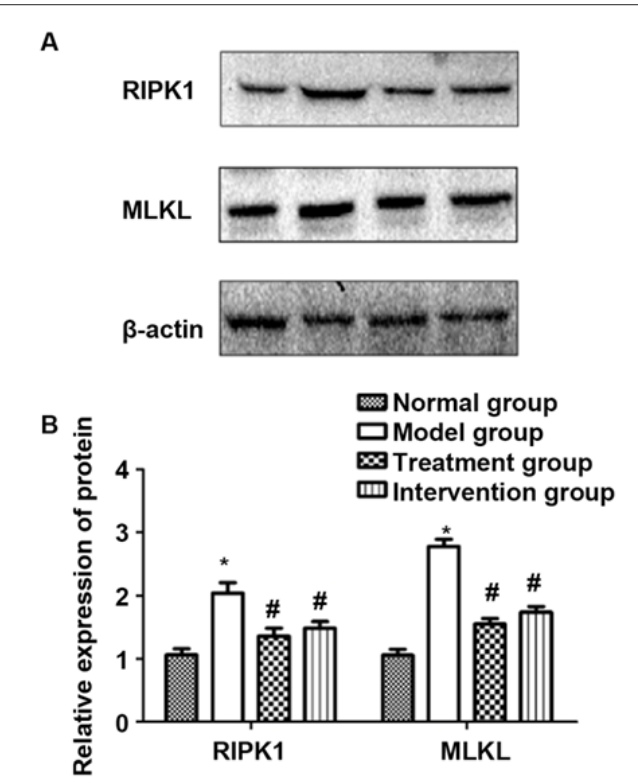

Figure 5. Results of proteins. The levels of RIPK1 and MLKL proteins are elevated remarkably in the model group (p<0.05) and lowered notably in the treatment group and intervention group (p<0.05). *p<0.05 vs. model group, #p<0.05 vs. normal group.

and tumors. A study hasdiscovered through histopathological observations that there is a large amount of neutrophil infiltration in the anterior tibial muscle tissues of I/R injury model rats (22). Akar et al observed neutrophil infiltration accompanied by muscle cell necrosis, karyopyknosis and other changes in the soleus tissues of the rat model of I/R injury (23, 24). Inflammations can cause the loss of membrane integrality, and intra-cellular myocardial enzymes such as LDH and CK-MB are released in the extracellular fluid (25, 26). In the present study, the results of serum biochemistry showed that the levels of ALP, GPT and CK-MB were elevated markedly in the model group compared with those in the normal group ($p<0.05$), implying that the hepatic function and myocardial function indexes are increased significantly during the occurrence and development of myocardial ischemia, thus providing important references for early diagnosis.

According to some research findings, the myocardial I/R injury can increase the inflammation level in the serum (27), and I/R injury is involved in the inflammatory responses including gene activation in cells. In this study, the levels of IL-6, IL-1 and TNF-α were raised in the model group, suggesting that the increased levels of IL-6 and TNF-α can further promote the development of I/R injury and aggravate the inflammatory responses. However, the levels of those three factors were decreased after the treatment with edaravone, indicating that the disease condition is improved after such a treatment, which is consistent with the results of previous studies. The role of oxidative stress in lower extremity I/R injury has been widely paid attention to, and the existence of SOD can prevent the I/R abnormality. MDA can resist the effect of SOD and has cytotoxicity (28), and edaravone can decrease the concentration of oxygen free radicals and inhibit delayed neuronal death. It was found in this study that the content of MDA in the model group was notably higher than that in other groups, but that of SOD was evidently lower, suggesting that the symptoms are improved after the treatment with edaravone. In addition, it was observed that MPO activity and tissue injury were decreased in the treatment group, which is in consistence with the experimental results of neutrophil depletion in myocardial and intestinal I/R injury models (29). Corresponding reactions of apoptosis can be triggered by invasion to the cell body, which can be regarded as crucial guidelines for multiple clinical diseases such as tumors and myocardial apoptosis. Apoptosis is regulated by apoptosis-related genes. In the present study, the results of the TUNEL assay manifested that the apoptosis level was increased remarkably in the model group compared with that in other groups, and the expression level of apoptosis-related gene Caspase-3 was elevated obviously in the model group, indicating prominent apoptosis in the muscle cells with I/R injury. Similar results are also obtained in previous studies (30, 31).

The RIPK1-RIPK3-MLKL signaling pathway plays a vital role in necrosis in a variety of cases (32). The pigmentation in the nucleus, decentralization in chromatin and reticular formation are unique characteristics of studies of cell death, and they seemingly have defined an independent research category of cell death. Data have revealed that the reticular formation of suicide includes the downstream RIPK1-RIPK3-MLKL signaling pathway generated by ROS in cells. Since the effects of different doses of Nec-1 are contradictory, the theory that neutrophil death may represent neutrophil necrosis still remains controversial (33). However, consistent evidence is provided by investigations in RIPK3 -/- mice and MLKL inhibition, illustrating that the RIPK1-RIPK3-MLKL signaling pathway participates in the neutrophil death and reticular formation at least, but it is unlikely to participate in the so-called important reticular formation, namely the net release without neutrophil death (34). Nec-1, a type of necroptosis-specific inhibitor discovered in recent studies, can specifically suppress the activity of RIPK1, thereby destroying the construction of RIPK1-RIPK3 complex. As a result, it can repress the process of I/R injury(10). According to the gene detection results in this study, the gene levels of RIPK1, RIPK3 and MLKL were increased prominently in model group, and the same changes in corresponding proteins were also detected. The above results demonstrate that edaravone can prevent further impacts on the rats with lower extremity I/R injury by inhibiting the overproduction of the RIPK1-RIPK3-MLKL signaling pathway and inflammatory factors, and such an effect was proven in the present study.

In conclusion, edaravone can relieve the infiltration of inflammatory cells and affect the apoptosis of muscle cells, and it exerts such effects mainly by mediating the RIPK1-RIPK3-MLKL signaling pathway. This study provided a theoretical basis for the prevention and treatment of lower extremity I/R injury as well as new ideas for subsequent in-depth studies.

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Not applicable.

Availability of data and material
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
GZwrote the manuscript. LZ and LY were responsible for establishment of animal model. LW contributed hepatic function detection and performed ELISA. ZH helped with statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the ethics committee of the General Hospital of Ningxia Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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