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Effect of rosiglitazone on myocardial injury in septic rats through NF-κB pathway

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Abstract. – INTRODUCTION: To explore the effect of rosiglitazone on myocardial injury in septic rats through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway.

MATERIALS AND METHODS: A total of 60 healthy adult female Sprague-Dawley (SD) rats were randomly divided into 4 groups, namely, group A (sepsis model group, n=15), group B (sham operation group, n=15), group C (sepsis model + 3 mg/kg rosiglitazone, n=15), and group D (sham operation group + 3 mg/kg rosiglitazone, n=15), respectively. After the sepsis model was successfully established, the rats were administered with 3 mg/kg rosiglitazone by gavage, with a gavage volume of 1 mL, once a day for a total of 3 days. Blood was taken from the abdominal aorta, while lactate dehydrogenase (LDH) and creatine phosphokinase kits were used to detect the levels of LDH and creatine phosphokinase in serum. Then, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was adopted to identify myocardial tissue apoptosis, hematoxylin and eosin staining (H&E) was applied to detect myocardial tissue morphology, and enzyme-linked immunosorbent assay (ELISA) was utilized to examine the protein expression level of tumor necrosis factor-alpha (TNF-α) in rat serum. Subsequently, the messenger ribonucleic acid (mRNA) level of TNF-α in myocardial tissues was measured *via* fluorescence quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) method, and the activity of NF-κB was detected by electrophoretic mobility shift assay (EMSA).

RESULTS: Compared with those in group A, apoptotic cells in group B and group D were notably increased ($p<0.05$). At 3 days after administration with rosiglitazone (3 mg/kg), apoptotic cells were markedly decreased ($p<0.05$). H&E staining results manifested that 3 mg/kg rosiglitazone prominently improved myocardial tissue morphology in rats. The protein level of TNF-α in serum, the mRNA expression level of TNF-α in myocardial tissues, and the activity of NF-κB in group C treated with rosiglitazone were lower than those in group A ($p<0.05$).

CONCLUSIONS: Rosiglitazone can alleviate myocardial injury in septic rats by suppressing the TNF-α expression and this process is associated with the regulation on the NF-κB signal transduction pathway.

Key Words:
Rosiglitazone, Inflammatory response, Myocardial injury, NF-κb, TNF-α.

Introduction

Sepsis was defined as host immune response caused by injury or infection by the American College of Chest Physicians and the Society of Critical Care Medicine in 1991, which has been widely accepted[1]. It can lead to excessive inflammatory responses, trigger anti-inflammatory, and pro-inflammatory mechanisms, cause organ injury and the secondary infection, although it is conducive to clearing infection and repairing tissues[2].

Furthermore, sepsis is able to induce myocardial cell injury, whose mechanism is that high levels of inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) are produced through inflammatory responses, leading to the drastically drop of Ca^{2+} concentration in the sarcoplasmic reticulum of cells during myocardial contraction, thus further resulting in dysfunction of myocardial contraction. In the meantime, there is a synergy between IL-1β and TNF-α, which will increase nitric oxide synthase (NOS) and the corresponding metabolites that inhibit the myocardium[3]. It has been reported in previous studies that TNF-α leads to the activation of synovial cells in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway. Then, the activated NF-κB enters the nucleus to coordinate gene expressions of

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inflammatory mediators and cytokines. Among them, the expression levels of TNF-α, IL-1β, and other primary pro-inflammatory mediators are extremely significantly increased[4]. They, in turn, activate the NF-κB pathway, thus producing cascade amplification effect and causing irreversible myocardial injury[5]. Previous studies have indicated that the NF-κB signaling pathway has great significance to the treatment of the sepsis-induced myocardial injury. Therefore, the NF-κB signaling pathway was investigated in this study to explore its correlation with myocardial injury.

Materials and Methods

Experimental Consumables and Reagents

Rosiglitazone tablets and procaine injection used in this experiment were purchased from Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test kit from Roche (Novus, Littleton, CO, USA), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) kit from Chuangwei Century (Beijing, China), TNF-α enzyme-linked immunosorbent assay (ELISA) kit from Yantai Ceres Co., Ltd. (Yantai, China), Light Shift electrophoretic mobility shift assay (EMSA) kit from Pierce (Rockford, IL, USA), nucleoprotein extraction kit from Thermo Fisher Scientific (Waltham, MA, USA) and Bradford protein quantitation kit from Shanghai Sangon Biotech (Shanghai, China). Primers were synthesized by Suzhou GENEWIZ (Suzhou, China).

Establishment of the Rat Model of Sepsis

A total of 60 healthy adult female Sprague-Dawley (SD) rats weighing 180-220 g and aged 6-8 weeks old were provided by the Shandong Laboratory Animal Center. This work was approved by the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology Animal Center. They were kept in SPF-level animal rooms in separate cages under room temperature controlled at (22±2) °C, the humidity of 50-60%, a 12 h light/dark cycle and they had free access to food and water.

The rat model of cecal ligation and perforation (CLP) is the most widely used model to study sepsis and septic shock. In this research, the CLP method was applied to construct the rat model of sepsis. The specific steps of CLP are as follows: (1) 4 mL/kg 0.1% pentobarbital sodium was injected intraperitoneally for anesthesia. (2) rats were fixed on the experimental table in the supine position, an about 1.5-cm long incision was made along the ventral midline, and then the cecum was ligated at the root. (3) The cecum was needled 3 times to form cecum fistula. Each time, the cecum should be penetrated and the cecum was sutured into the abdominal cavity. In sham operation group, the abdomen of the rats was cut open and closed and cecum was not ligated or perforated. All the rats were placed in a warm bed immediately after the operation and replaced in the cage after normal activities were resumed. The rats in rosiglitazone group were given rosiglitazone (12 g/kg) at one week after operation. After gavage the day of the operation in sham operation group and model group, fever, increased heart rate of 50%, doubled respiratory frequency, as well as manifestations such as vertical hair and fatigue appearing in rats in model group, indicated successful modeling.

Animal Grouping and Drug Intervention

A total of 60 SD rats were randomly divided into 4 groups, namely, group A (sepsis model group, n=15), group B (sham operation group, n=15), group C (sepsis model + 3 mg/kg rosiglitazone, n=15) , and group D (sham operation group+3 mg/kg rosiglitazone, n=15), respectively. After the sepsis model was successfully established, the rats were administered with 3 mg/kg rosiglitazone by gavage, with a gavage volume of 1 mL, once a day for a total of 3 days.

Sample Collection

After 3 days of drug intervention, 4 mL/kg 0.1% pentobarbital sodium was injected intraperitoneally for anesthesia, 4 mL blood was extracted from the abdominal aorta and serum was taken by centrifugation at 1000 rpm using a high-speed refrigerated centrifuge. Subsequently, after the rats were killed at one time by cervical dislocation method, the thoracic cavity was opened, and myocardial tissues were taken and stored in a refrigerator at –80°C after the residual blood was washed in normal saline.

Detection of Lactate Dehydrogenase (LDH) and Creatine Phosphokinase in Rat Serum

Blood was collected from the abdominal aorta through the cannula and centrifuged at 1000 rpm/min for 10 min to prepare serum. The levels of LDH (Beyotime, Shanghai, China) and creatine phosphokinase in rat serum were measured using LDH and ELISA kits.

Detection of Myocardial Tissue Morphology Via Hematoxylin and Eosin (H&E) Staining

Sections were deparaffinized in xylene for 5-10 min and in substitutive fresh xylene for another 5-10 min. Then, the sections were soaked in absolute ethanol for 5 min, in 90% ethanol for 2 min, in 80% ethanol for 2 min, in 70% ethanol for 2 min, and in distilled water for 2 min. After that, the sections were stained with hematoxylin staining solution for 10 min, socked in running water to remove redundant staining solution for about 10 min, and washed again with distilled water (for a few s). Thereafter, the sections were stained with eosin staining solution for 2 min and soaked in 70% ethanol for 10 s, in 80% ethanol for 10 s, in 90% ethanol for 10 s, and in absolute ethanol for 10 s. Afterwards the sections were transparentized in xylene for 5 min and in substitutive fresh xylene for another 5 min. Finally, the sections were sealed using neutral gum and observed under a microscope.

Detection of Apoptosis of Myocardial Tissues Via TUNEL Staining

Paraffin-embedded sections were prepared and placed in a transparent liquid for 8 min after deparaffinization and hydration. Then, the dried slides were added with 50 μL TUNEL staining solution dropwise after the slides were dried, washed with distilled water, added with 50 μL converter-POD dropwise, and rinsed with distilled water. Secondly, the sections were counterstained by hematoxylin, repeatedly washed with distilled water, and dehydrated using 75%, 95%, and 100% alcohol at gradient concentration. Ultimately, the sections were sealed using neutral gum after transparentization with xylene. The prepared sections were added with a drop of glycerin and observed under a microscope to identify the apoptosis rate.

Detection of Changes in the Protein Level of TNF-α in Serum Via ELISA

Blood (4 mL) was taken from the abdominal aorta, while serum and supernatant were taken by centrifugation at 1000 rpm using a high-speed refrigerated centrifuge. After specific anti-mouse TNF-α monoclonal antibody was diluted, 50 μL analysis solution was added to each well to coat the enzyme-linked reaction plate, and 50 μL standards and controls were added and mixed for 1 min to the enzyme-linked reaction plate for 2 min. Then, the coating liquid was absorbed, followed by rinsing with clear water for 5 times. Thereafter, the reaction plate was inverted, the liquid was absorbed with paper and the reaction well was dried in the air. Subsequently, 100 μL enzyme-labeled specific anti-mouse TNF-α polyclonal antibodies were added to each well for 2 h of reaction at room temperature, and secondary antibody solution was sucked out. Substrates were diluted after thorough washing with distilled water. After dilution, 100 μL diluents were added for 30 min of reaction at room temperature, after which 100 μL stop buffer was added to terminate the reaction. After the reaction was terminated, the optical density (OD) value of each well was measured at the wavelength of 540 nm using an enzyme-linked detector, and the procedure for each sample was repeated 3 times.

Detection of Changes in the Messenger Ribonucleic Acid (mRNA) Expression Level of TNF-α in Myocardial Tissues Via Quantitative RT-PCR (qRT-PCR)

RT and qPCR were carried out to detect the mRNA expression level of TNF-α in myocardial tissues in the four groups of rats. The tissue samples were removed from the cryopreservation tube, drained and ground into a 5 mL tube using liquid nitrogen. After thorough homogenization using a tissue homogenate machine, the liquid was transferred to a clean imported 1.5 mL Eppendorf (EP) tube. Subsequently, the samples were left at room temperature for 5-10 min, followed by full lysis and centrifugation at 1, 200 rpm/min for 5 min, and the precipitate was discarded. Then, the samples were added with chloroform (chloroform: TRIzol = 200:1), shaken, mixed uniformly, left at room temperature for 15 min and centrifuged at 12,000 rpm/min at 4°C for 15 min. Next, the supernatant phase was sucked into another centrifuge tube, added with isopropanol whose volume was 0.7-1 times that of the supernatant, placed at room temperature for 10-30 min, and centrifuged at 12, 000 rpm/min for 10 min, and the supernatant was discarded. After that, RNAs precipitated at the bottom of the tube, and the centrifuge tube was added with 75% ethanol (75% ethanol: TRIzol=1:1) and gently shaken to suspend the precipitate. Thereafter, centrifugation was performed at 12,000 rpm/min at 4°C for 5 min, the supernatant was discarded as far as possible, and the precipitate was blown dry at a super-clean station for 10-20 min. Finally, 10-50 μL dd H_2O treated with diethyl pyrocarbonate (DEPC) was added to dissolve the precipitate, and the concentration was measured using a One-

Drop micro-volume spectrophotometer. Reverse transcription (RT) reaction was conducted based on the system with 4.5 μL RNase free dH_2O, 2 μL 5×RT reaction buffer, 0.5 μL random primers, 0.5 μL Oligo dT, 0.5 μL reverse transcriptases, and 2 μL RNAs. Complementary deoxyribonucleic acid (cDNA) samples were divided into three parts and the total cDNA sample in each group was diluted 20 times. 3 μL cDNAs were taken for PCR amplification. The amplification level of the target gene was verified by 5% agarose gel electrophoresis. With LabWorks 4.0 image acquisition and analysis software, quantification and data processing were carried out. To obtain reliable data, the procedure was repeated 3 times for each group of samples. In this research, the $2^{-\Delta\Delta Ct}$ method was applied to analyze the changes in the relative expression levels of target genes. The primer sequences used are shown in Table I.

Detection of the NF-κB Activity in Myocardial Tissues Via EMSA

Myocardial tissues (100 mg) were cut into sections and placed into an EP tube. Then, the tissue sections were centrifuged after washed by phosphate-buffer saline (PBS) for 2 times, the supernatant was removed, and the precipitate was taken. After that, 0.5 mL lysis buffer precooled at 4°C was added, the probe of the ultrasonic instrument was inserted under the buffer level and the power supply was turned on to crush cells for 4 times in total, with 30 s each time. Subsequently, homogenate tissues received ice bath for 10 min and shaken for 3 times in the process, after which the cell suspension was centrifuged at high speed for 10 min, and the supernatant was removed. The concentration was determined by the Bradford method. First, the protein solution was added into the properly placed well plate. Second, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1.0 mg/mL bovine serum albumin (BSA) solution were prepared, and 100 μL BSA solution at each concentration was added to the 1.5 mL EP tube and evenly mixed with 900 μL PBS added. Then, 50 μL BSA at each concentration was placed into the well plate with protein solution, respectively, and each well was added dropwise with 200 μL Coomassie brilliant blue, after which the solution was let stand for 10 min, and the OD value of the solution in each well was measured with a microplate reader. Biochemical markers were adopted in the probe sequences used. The sequences are shown below: F: 5'-AGTTGAGGGACTTTCCCAGGC-3' and R: 3'- TCAACTCCCCTGAAAGGGTCCG-5'. The probe was labeled in the reaction system with 5 μL enzyme-free water, 2 μL 1.75 pmol/μL probes to be labeled and 1 μL 10× T4 polynucleotide kinase buffer. After probe labeling, sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis was adopted for identification, with Glyceraldehyde 3-phosphate dehydrogenase (GADPH) as an internal reference, followed by membrane transfer after electrophoresis, fixation and X-ray exposure, and development.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) was applied and the results were expressed as mean ± SD (standard deviation). Statistical differences between groups were detected *via* the *t*-test. $p<0.05$ suggested that there was a statistical difference and $p<0.01$ showed that there was a statistically significant difference.

Results

Detection Results of LDH and Creatine Phosphokinase in Rat Serum

The levels of LDH and creatine phosphokinase in serum represented the degree of myocardial injury in rats. The serum levels of LDH and creatine phosphokinase in group A were significantly higher than those in group B and group D ($p<0.05$). Compared with those in group A, the levels of LDH and creatine phosphokinase in rat serum in group C were remarkably reduced, showing statistically significant differences. The above results indicate that 3 mg/kg rosiglitazone markedly alleviates the myocardial injury in sepsis-induced model rats (Figure 1).

Table I. Primer sequences.

Gene name	Forward/reverse	Primer sequence
TNF-α	F	5'-GAACGACGTAGCCATTGTGAAG-3'
	R	5'-TGAGAAGTTGTTGAGTGGGGACT-3'
GAPDH	F	5'-CAGAGCCTCGCCTTTGCCGATC-3'
	R	5'-GGCCTCGTCGCCCACATAGG-3'

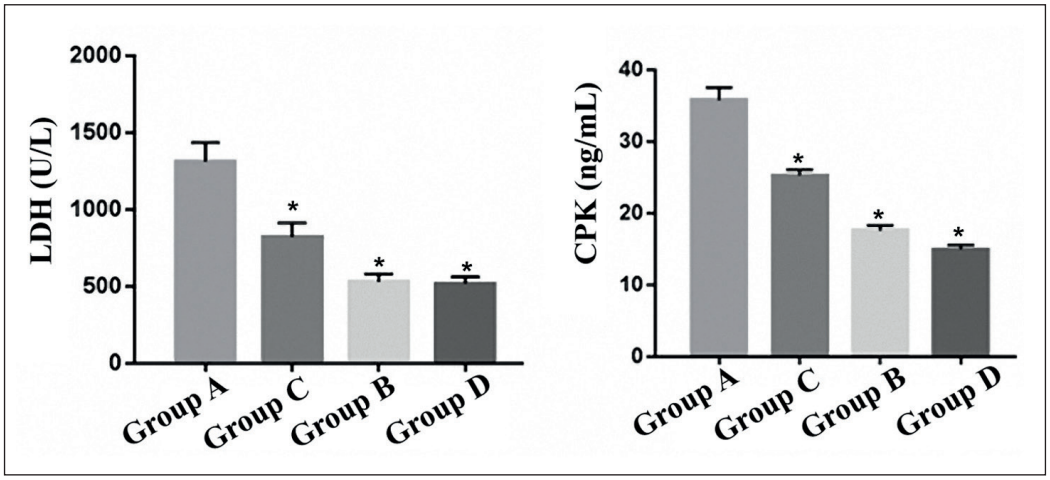

Figure 1. Changes in the levels of LDH and creatine phosphokinase in rat serum. *p<0.05 vs. group A.

Morphological Changes in Myocardial Tissues of Rats Detected Via H&E Staining

Compared with group A with sepsis surgery, group B and group D had regularly arranged myocardial cells with normal size and shape, as well as clear boundaries, evenly stained myocardial tissue fibers and nuclei, and neatly arranged and tightly connected fibers. Compared with group C, group A had loose and disordered myocardial fibers, a significantly widened myocardial cell gap, unevenly stained myoplasm, some degenerated and swollen muscle fibers, interstitial edema, red blood cell leakage and neutrophil infiltration. It can be seen that 3 mg/kg rosiglitazone notably improves myocardial tissue morphology in rats (Figure 2).

Apoptosis Level of Myocardial Tissues Detected by TUNEL Staining

At 72 h after the sepsis model was established, the TUNEL staining area in group A was larger than that in group B (p<0.05), suggesting that

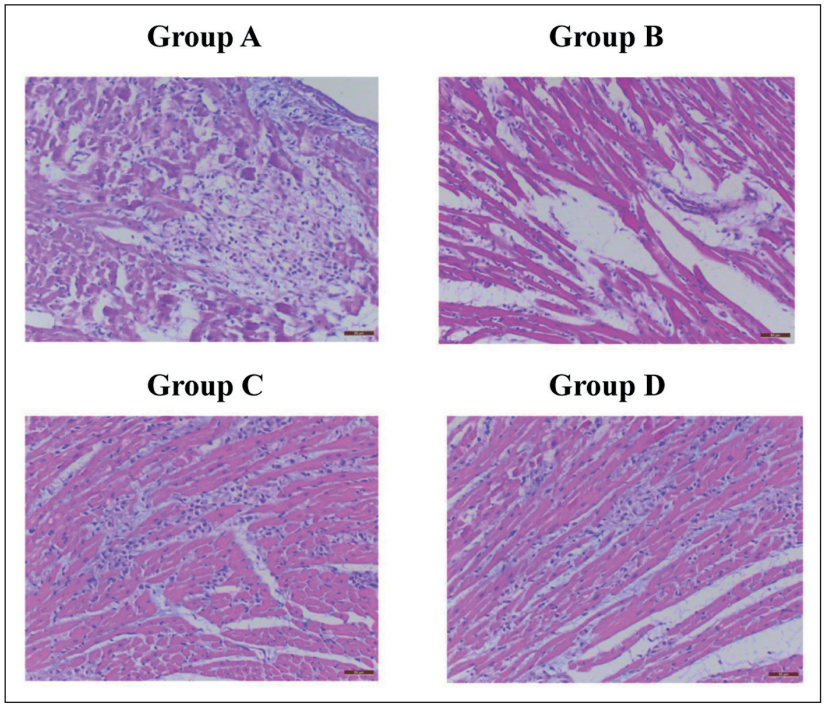

Figure 2. Morphological changes in myocardial tissues in rats detected via H&E staining (magnification: 100×).

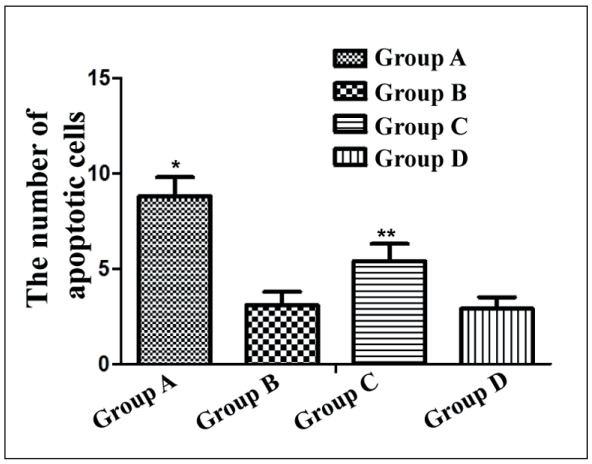

Figure 3. Apoptosis level of myocardial tissues detected by TUNEL staining (n=10). *p<0.05 *vs.* group B, and *p<0.01 *vs.* group A.

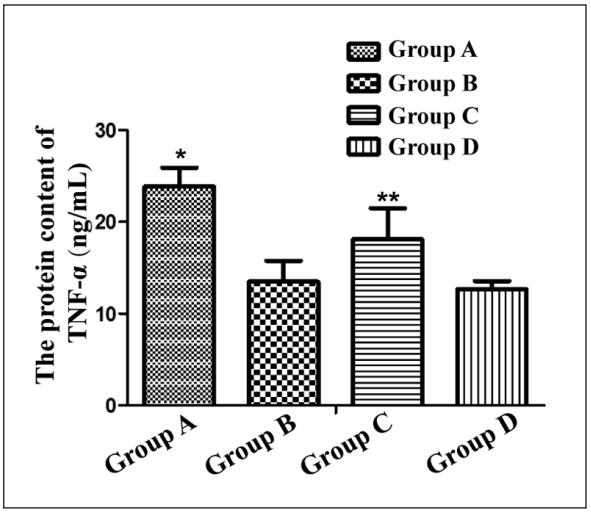

Figure 4. Change in the protein level of TNF-α in rat serum (n=15). *p<0.05 vs. group B, and *p<0.01 *vs.* group A.

sepsis model induces myocardial tissue apoptosis in rats. However, the staining area in group C was smaller than that in group A without treatment, indicating that 3 mg/kg rosiglitazone prominently relieves myocardial tissue apoptosis in sepsis-induced model rats (p<0.05) (Figure 3).

Change in the Protein Level of TNF-α in Rat Serum

At 72 h after the sepsis model was established, the serum protein level of TNF-α in group A was markedly higher than that in group B and group D (p<0.05). Compared with group A, group C had an evidently decreased protein level of TNF-α in rat serum and the difference was statistically significant. It can be seen that 3 mg/kg rosiglitazone remarkably reduces the TNF-α protein level in serum of sepsis-induced model rats (Figure 4).

Change in the mRNA Expression Level of TNF-α in Myocardial Tissues

QRT-PCR results revealed that the mRNA expression level of TNF-α in myocardial tissues of rats in group A was markedly higher than that in group B and group D (p<0.05), while the expression level in group C was lower than that in group A (p<0.05) (Figure 5).

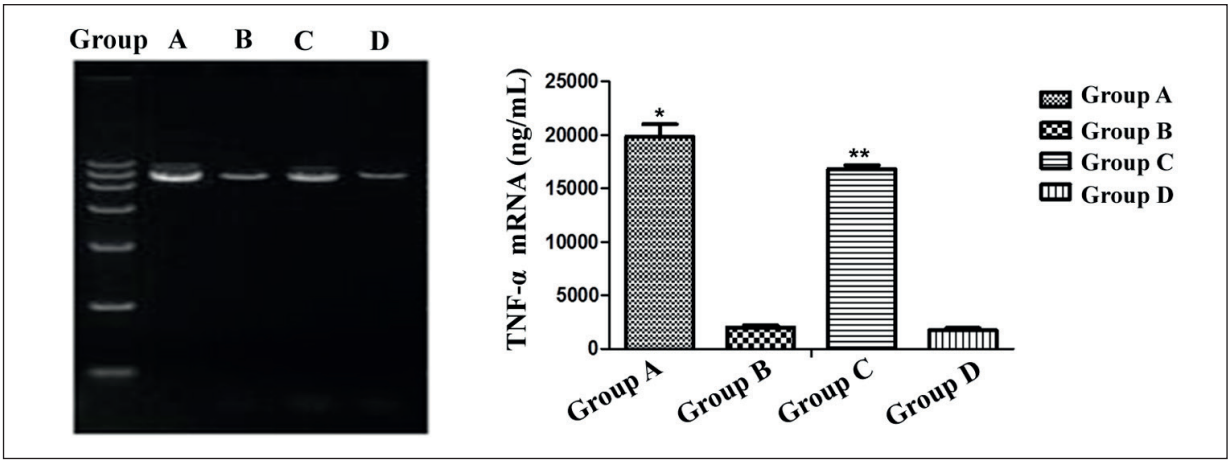

Figure 5. Change in the mRNA expression level of TNF-α in myocardial tissues (n=5). No. 1-4 swimming bands represent the mRNA expression level of TNF-α in group A, group B, group C, and group D, respectively. *p<0.05 *vs.* group B, and *p<0.01 *vs.* group A.

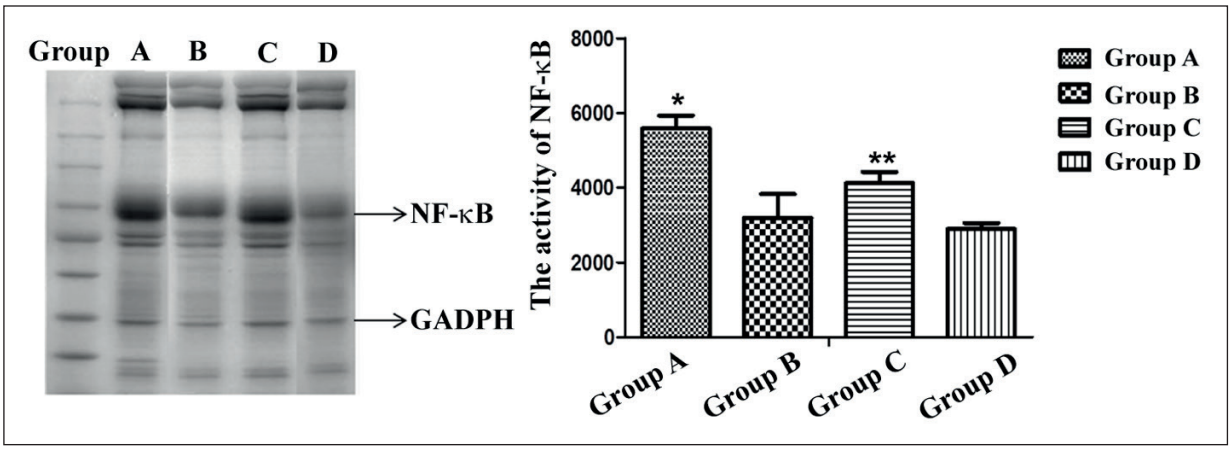

Figure 6. EMSA results of myocardial tissues of the four groups of rats (n=5).*p<0.05 *vs.* group B, and *p<0.01 *vs.* group A.

NF-κB Activity in Myocardial Tissues Examined Via EMSA

According to the EMSA results, compared with that in group B and D, the activity of NF-κB in myocardial tissues of the rats in group A was significantly increased. Compared with that in group A, the NF-κB activity of the rats in group C declined remarkably (p<0.01), suggesting that 3 mg/kg rosiglitazone prominently reduces the enhanced NF-κB activity in myocardial tissues of sepsis-induced model rats (Figure 6).

Discussion

It was found from the results of this study that rosiglitazone suppressed the expression of TNF-α and weakened the NF-κB activity, so as to lower the degree of sepsis-induced myocardial injury caused by inflammatory cytokines. Therefore, it can be concluded that rosiglitazone can intervene in sepsis-induced myocardial injury.

As a transcription factor, NF-κB exerts crucial effects in such physiological processes as immune responses, inflammatory responses, as well as cell growth, survival and development. NF-κB is of great significance for human health due to its role in various malignant diseases, including autoimmune, inflammatory responses, rheumatism, atherosclerosis, enteritis, multiple sclerosis, and malignant tumors after mutation and activation[6]. Previous studies have shown that the NF-κB activity is related to inflammatory responses. According to Matsumori et al[7], myocarditis is accompanied by enhanced NF-κB activity, NF-κB blockers can alleviate the symptoms of myocarditis, and the gene expressions of TNF-α and inducible nitric oxide synthase in myocardial tissues are reduced. In 2018, Ma et al[8] found that Calycosin alleviates caerulein-induced acute pancreatitis by inhibiting the inflammatory response and oxidative stress via the p38 MAPK and NF-kappaB signal pathways in mice. Piao et al[9] discovered that recombinant pyrin domain protein attenuates allergic inflammation by suppressing NF-kappaB pathway in asthmatic mice. Scholars[10,11] also indicated that model animals of surface asthma have higher NF-κB activity.

In addition to TNF-α, there are many inflammatory signaling factors involved in the NF-κB pathway. As a common pro-inflammatory factor, Toll-like receptor (TLR) will activate the MyD88-dependent pathway when binding to ligands to form a complex, and the complex binds to IRAK4 and IRAK1 to phosphorylate and dissociate IRAK1, thereby binding to TRAF6. After activating TRAF6, IRAK1 will bind to transforming growth factor kinase-1 activated by β protein through the mediation by transforming growth factor-β kinase-1, ubiquitylate κB inhibitory proteins, releasing and activating NF-κB. After activation, transposition appears, thus initiating the transcription of related genes[12,13]. Additionally, the TLR signal transduction pathway also includes the MyD88-independent pathway[12]. In this pathway, TLR4 induces adaptor proteins containing TLR homologous regions to bind to β-interferon to increase the gene expressions of IFN and IFN-β, thereby activating the NF-κB pathway[14,15]. In 2009, Kaczorowski et al[16] have

observed that LPS produced in the body during sepsis leads to an increase in the TLR4 level, resulting in a synergistic effect between MD-2 and CD14 and activation of NF-κB to achieve rapid migration to the nucleus. The produced excessive inflammatory cytokines damage tissues and organs[15]. In addition to mediating myocardial injury caused by inflammatory responses, research has also found that NF-κB may be activated to regulate myocardial cell apoptosis[16]. Specifically, Fas, TNF-α, FasL, and other genes that regulate myocardial cell apoptosis initiate transcription and activate the NF-κB pathway. All of the above myocardial cell apoptosis-related genes contain κB sites. In 2013, Roger et al[17] reported that macrophages were found in *Klebsiella pneumoniae* sepsis model. If MIF is absent, they will lead to decreases in the TLR4 expression level, NF-κB, and TNF, thus reducing the ability of hosts to resist infection. Moreover, sepsis will activate renin-angiotensin system to produce angiotensin II that functions in endothelial cells of the vascular wall, which regulates the generation of NF-κB and endothelial NOS, thus increasing inflammatory responses and probably resulting in microcirculation and endothelial dysfunction[18].

Rosiglitazone has been used to treat inflammation such as myocardial injury in recent years. Pan et al[19] reported that rosiglitazone impedes *Porphyromonas gingivalis*-accelerated atherosclerosis by downregulating the TLR/NF-kappaB signaling pathway in atherosclerotic mice. Chen et al[20] discovered rosiglitazone protects rat liver against acute liver injury associated with the NF-kappaB signaling pathway. These studies suggest that rosiglitazone may play a regulatory role in NF-κB related to a variety of important physiological processes. Therefore, rosiglitazone may still have great potential in medical treatment to be discovered.

Conclusions

Rosiglitazone can alleviate myocardial injury in septic rats by suppressing the TNF-α expression and this process is associated with the regulation on the NF-κB signal transduction pathway.

Conflict of Interests

The Authors declared that they have no conflict of interests.

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