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Effect of serum high mobility group box 1 protein on immune function and autophagy level of myocardial cells in rats with sepsis

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Abstract. – OBJECTIVE: To investigate the effects of serum high mobility group box 1 protein (HMGB1) on immune function and autophagy level of myocardial cells in rats with sepsis.

MATERIALS AND METHODS: Cecal ligation and perforation (CLP) was used to establish rat sepsis models. A total of 60 SD rats were selected and randomly divided into blank control group (BCG, n=20), sham group (SG, n=20) and cecal ligation and perforation group (CLPG, n=20). Enzyme-linked immunosorbent assay (ELISA) was used to detect the serum HMGB1 level in sepsis rats. The expression levels of inflammatory factors in rats were detected, and the ratio of CD4+/CD8+ T cells was detected by flow cytometry. Western blot was used to detect the expression of autophagy-related protein microtubule-related protein 1 light chain 3 (LC3), Beclin-1 and apoptosis-related protein B lymphoblastoma-2 (Bcl-2), and cTnT protein, respectively.

RESULTS: The level of serum HMGB1 in the CLPG was significantly higher than that in the BCG and the SG (*p*<0.05). Compared with BCG and the SG, the CLPG had lower peripheral blood T lymphocyte proliferation response, lower IL-6 and IL-10 levels, and lower CD4+/CD8+T lymphocyte ratio (*p*<0.05). Bcl-2 and cTnT in the CLPG and SG were higher than those in the BCG. LC3-11 and Beclin-1 expression in the CLPG were higher than those in the BCG and SG (*p*<0.05). After HMGB1 interference in the CLPG, CD4+/CD8+T and Bcl-2 were significantly increased, while the other indicators were significantly decreased (*p*<0.05). The level of serum HMGB1 is directly related to the severity of sepsis.

CONCLUSIONS: The increase of serum HMGB1 level in sepsis has a significant impact on cellular immune dysfunction. Sepsis can effectively activate myocardial autophagy, and the level of autophagy shows an increasing trend.

Key Words:

Serum high mobility protein B1, Sepsis, Immune function, Myocardial cell autophagy level.

Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) caused by infection, with clinically confirmed bacteria or highly suspected focal infections[1]. Although sepsis is caused by infection, its development and progression follow its own pathological processes and rules. Therefore, sepsis is essentially the body's response to infectious factors.

High mobility group box-1 protein[2] (HMGB1) was discovered by Goodwin et al[2] in 1973. It was named for its fast migration in polyacrylamide gel electrophoresis (PAGE)[3]. HMGB1 is highly conserved and has 99% homology in mammals. It is a single-chain polypeptide containing 215 amino acid residues. Its N-terminus is rich in positively charged lysine, and the C-terminus is rich in negatively charged aspartic acid and glutamic acid (also called acidic tail). HMGB1 is ubiquitous in mammalian tissue cells and plays a vital role in the pathogenesis of many inflammatory diseases, especially in sepsis and endotoxemia. HMGB1 targeted therapy research has attracted wide attention[4,5].

In the past, people paid more attention to the function of HMGB1 as a nuclear protein. However, in addition to its important role in immune and inflammatory processes, researchers have found in recent years that HMGB1 can also affect cell autophagy as a regulator factor[6]. Autophagy is a programmed way of cell survival. Cells engulf self-cytoplasmic proteins or organelles and coat them into vesicles. Then, they were fused with lysosomes to form autolysosomes. Finally, all of the inclusions were degraded. Cells thereby meet their own metabolic needs and renew certain organelles[7]. At the same time, autophagy can also selectively or non-selectively degrade pathogens.

In this paper, sepsis rat models were established to study the effects of serum HMGB1 on

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immune function and myocardial cell autophagy in sepsis rat models, in order to provide a theoretical basis for further research and new insights and methods for treatment.

Materials and Methods

Animals

A total of 60 SD rats were selected as study subjects. All of the rats were provided by the Institute of Disease Control and Prevention of Jiangsu Province [Certificate No.: SYXK (Su) 2018-0004]. They were randomly divided into blank control group (BCG, n=20), sham group (SG, n=20) and cecal ligation and perforation group (CLPG, n=20 animals). This study was approved by the Ethics Committee of People's Hospital of Rizhao.

Modeling

The CLP technique was used to establish rat sepsis models. A 5 cm incision was made along the midline of the abdomen, and the cecum was ligated at the 1/2 end in the vascular arch. No. 7 scalp acupuncture was used to prevent pinhole closure. Then, the cecum was put back to the abdominal cavity. The abdominal incision was sutured with 10-0 silk thread layer by layer. Immediately after the operation, 50 ml/kg equilibrium liquid was subcutaneously injected for anti-shock. Rats in the SG had only laparotomy, abdomen closure and resuscitation, without cecum ligating or perforating. Each rat was fed with 20 g food at 8 a.m. and had free access to water. Blood and tissue samples were collected respectively for testing. Each group was drenched 0.5 h before surgery, while the SG and the MG were given the same volume of normal saline. Each group was drenched at the same time every day at a volume of 10 ml/kg.

Detection Indicators and Methods

Enzyme-linked Immunosorbent Assay[8] (ELISA) in Detection of Serum Related Factor Expression Levels

Serum was prepared before sacrificing the rats. HMGB1, IL-6, and IL-10 were detected by ELISA. Blood was taken from the vena cava and was left to stand in a greenhouse for 4 hours, then centrifuged at 3000 r/min for 10 minutes at 4°C. Then, the serum was collected and divided. The relative expression of protein factors Beclin-1 and LC3-11 was detected by ELISA kit. Finally, the microplate reader was used to measure the absorbance value (A) of each sample at a wavelength of 450 nm. Ensure that there were no water droplets on the plate and no air bubbles in the drip holes in this step. After subtracting the A value of the TMB blank coloration hole from the A of all standards and samples, the standard curve was drawn with the standard concentration as the abscissa and the value of A after zero as the ordinate, and the actual concentration of each sample was calculated. HMGB1 ELISA kit was purchased from Wuhan Moshake Biotechnology Co., Ltd. (kt21150, article number 69-21150). T-lymphocyte ELISA kit was purchased from Wuhan Moshake Biotechnology Co., Ltd. (BA21524, article number 69-21524). IL-2 ELISA kit was purchased from Wuhan Moshake Biotechnology Co., Ltd. (kt99933, article number 69-99933).

Flow Cytometry in Detection of the Ratio of CD4+/CD8+T Cell [9]

Splenic lymphocyte suspension (50 µl) was added to the corresponding detector tubes, and then diluted CD4-FITC and CD8-PE were added. The mixture was incubated in a refrigerator at 4°C for 30 minutes in the dark. Phosphate-buffered saline (PBS; 1 ml) was added to each tube and centrifuged 1500 r/min at 4°C for 5 min, with a centrifugal radius of 20.8 cm. The supernatant was discarded, and excess water in the wall of the tube was dried upside down on the absorbent paper. 200 µl 2% PFA was added, dissolved and stabilized in each tube, then the CD4+ and CD8+T lymphocyte ratio was detected.

Western Bblotting[10] in Detection of the Epression of Autophagy-Related Proteins LC3-11, Beclin-1 and B Lymphoma-2 (Bcl-2), Troponin (cTnT)

The myocardial tissue of rats was taken, and the cell suspension was prepared. The suspension was irradiated and washed twice with cold PBS. Cell lysis buffer was added to lyse it at 4°C for 20 min, and then centrifuged at 1200 r/min for 20 min. The supernatant was collected to extract total cell protein. Totally 20 g of sample was taken and boiled to deformation. After that, Tricine-SDS-PAGE electrophoresis was performed. The membrane was transferred and then sealed with 5% skim milk powder at 37°C for 2 h, incubated at 4°C overnight. The membrane was washed, secondary antibody was added, and incubated at

37°C for 1 h, developing with enhanced chemiluminescence (ECL) method. The gray value of the target band was analyzed, and the relative expression of protein was expressed by the gray value ratio between the target band and β-actin band.

Interference Test

In the CLPG, 10 cecal ligation and perforation rats were randomly selected, and HMGB1 antagonist (Ammonium glycyrrhizinate) was added. All the indicators above were detected at 4, 8, and 12 hours after injection.

Statistical Analysis

In this study, SPSS18.0 software (Bizinsight (Beijing) Information Technology Co., Ltd.) was used for statistical analysis on collected data. GraphPad Prism 6 software was applied to draw all the pictures in this research. Chi-square test was used to compare the enumeration data. The measurement data were expressed as mean ± standard deviation. The *t*-test was used for analysis between the two groups, and analysis of variance was used for comparison between multiple groups. Pearson correlation analysis was used for the relationship between variables. When $p<0.05$, there was statistical difference.

Results

ELISA in Detection of Expression Levels of Serum Related Factor

The level of serum HMGB1 in the CLPG was significantly increased, and the HMGBl level in the CLPG was significantly higher than that in the BCG and the SG ($p<0.05$). The inflammatory factors IL-6 and IL-10 in the CLPG were significantly higher than those in the BCG and the SG ($p<0.05$). The ratio of CD4+/CD8+T lymphocytes in the CLPG were lower than those in the BCG and the SG, and all differences were statistically significant ($p<0.05$; Figure 1).

Expression of Beclin-1, LC3-11, and Apoptosis-Associated Protein Bcl-2 and cTnT in Each Group of Rats

Compared with the BCG and the SG, cTNT in the CLPG was significantly increased ($p<0.05$). Compared with the BCG and the SG, Bcl-2 in the CLPG was significantly decreased ($p<0.05$). Both LC3-11 and Beclin-1 expression were higher than those of the BCG and SG, with statistical significance ($p<0.05$; Figure 2).

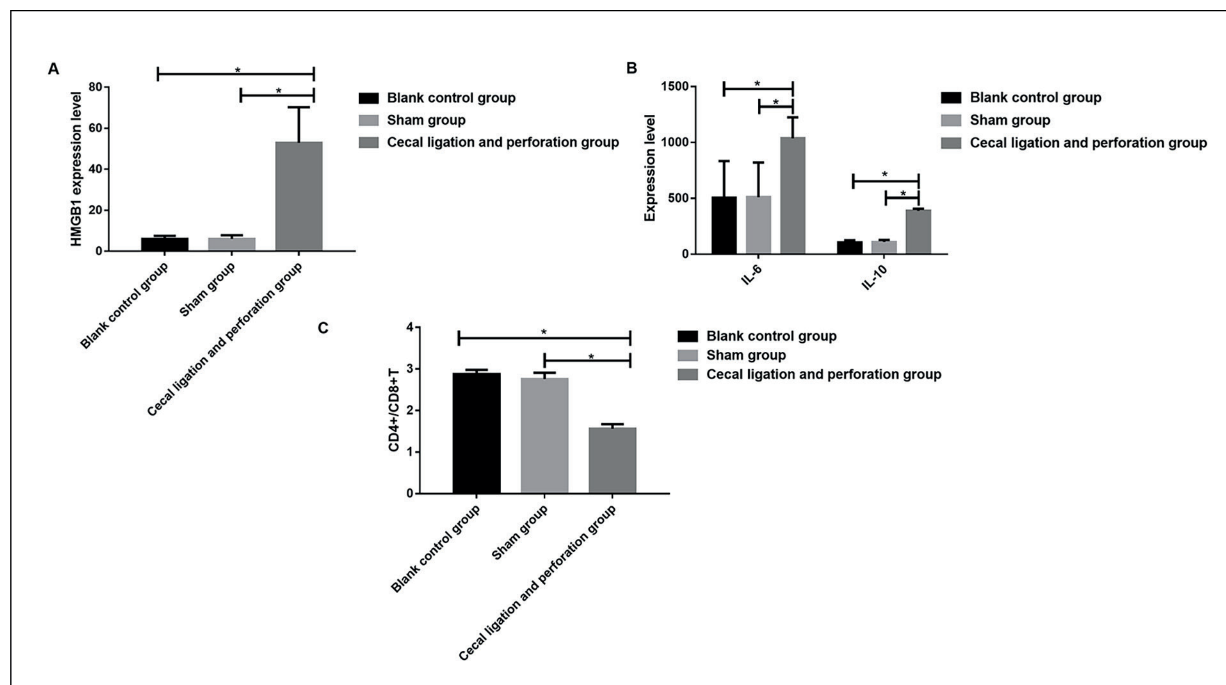

Figure 1. ELISA in detection of expression levels of serum related factor. **A**, The expression level of HMGB1 in the serum of the CLPG was significantly higher than that in the BCG and the SG. **B**, The expression levels of IL-6 and IL-10 in the serum of the CLPG were significantly higher than those in the BCG and the SG. **C**, The ratio of CD4+/CD8+T lymphocytes in the serum of the CLPG was lower than those in the BCG and the SG. *Means $p<0.05$.

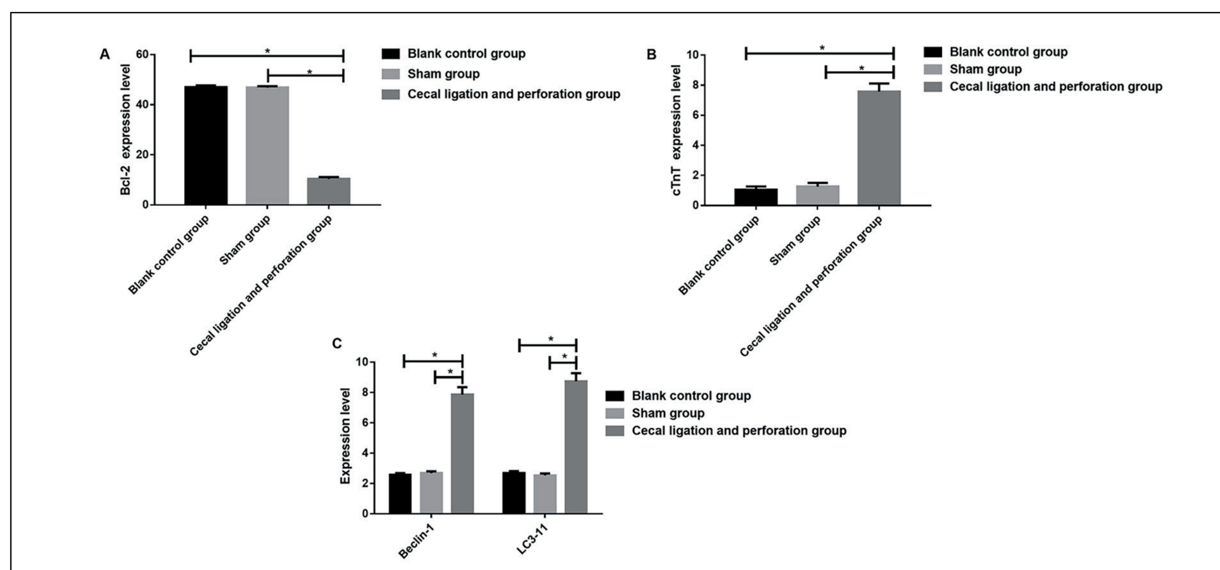

Figure 2. Expression of Beclin-1 and LC3-II and apoptosis-related protein Bcl-2 and cTnT in each group of rats. **A**, The expression level of Bcl-2 in the CLPG was significantly higher than that in the BCG and the SG. **B**, The cTnT expression level in the CLPG was significantly higher than that in the BCG and the SG. **C**, The expression of LC3-11 and Beclin-1 in the CLPG were higher than those in the BCG and the SG. *Means $p<0.05$.

Interference Test

By using HMGB1 antagonist in the CLPG, the level of HMGB1 in the body was disturbed. The results showed that the level of HMGB1 expression in the interference group was significantly lower than that in the CLPG ($p<0.05$). The expression levels of IL-6 and IL-10 were significantly higher than those in the CLPG ($p<0.05$). The ratio of CD4+/CD8+T lymphocytes in the interference group increased, which was significantly higher than that in the CLPG ($p<0.05$). The expression level of Bcl-2 in the interference group was significantly higher than that of the CLPG ($p<0.05$). The cTnT expression level of the interference group was significantly lower than that of the CLPG ($p<0.05$). The expression levels of Beclin-1 and LC3-11 were significantly lower in the interference group than those in the CLPG ($p<0.05$; Figure 3).

Discussion

Inflammatory factors play vital roles in the pathogenesis of sepsis, systemic inflammatory response and multiple organ dysfunction. They are also the inducing factors in cell apoptosis. Both pro-inflammatory and anti-inflammatory factors produce a marked effect on the entire process of sepsis. The results of this study showed that the inflammatory factors IL-6 and IL-10 increased notably, and the CD4+/CD8+T lymphocyte ratio in the CLPG were lower than those in the BCG and the SG. IL-6 is mainly secreted by macrophages and is an important transmitter of early sepsis. It plays an important part in the acute period of injury physiology. However, the excessive production and continuous increase of IL-6 after injury would increase the prevalence and mortality of sepsis[11]. IL-10 is produced by TH2 lymphocytes, which inhibits the production of inflammatory factors by activated macrophages. Although IL-10 is an anti-inflammatory factor, it is significantly elevated in patients with sepsis. The duration of excessive rise is related to the severity of sepsis and predicts a poor prognosis[12].

High mobility group box is named for their high mobility in polyacrylamide gel electrophoresis, including three families, HMGA, HMGB, and HMGN. There are three members in the HMGB family, namely HMGB1, HHMGB2, and HMGB3. HMGB1 is a typical non-histone in the nucleus. For a long time, people focused on its nuclear functions, including participation of the construction and stabilization of nucleosomes, regulation of gene transcription, and involvement in the recombination, repair and replica-

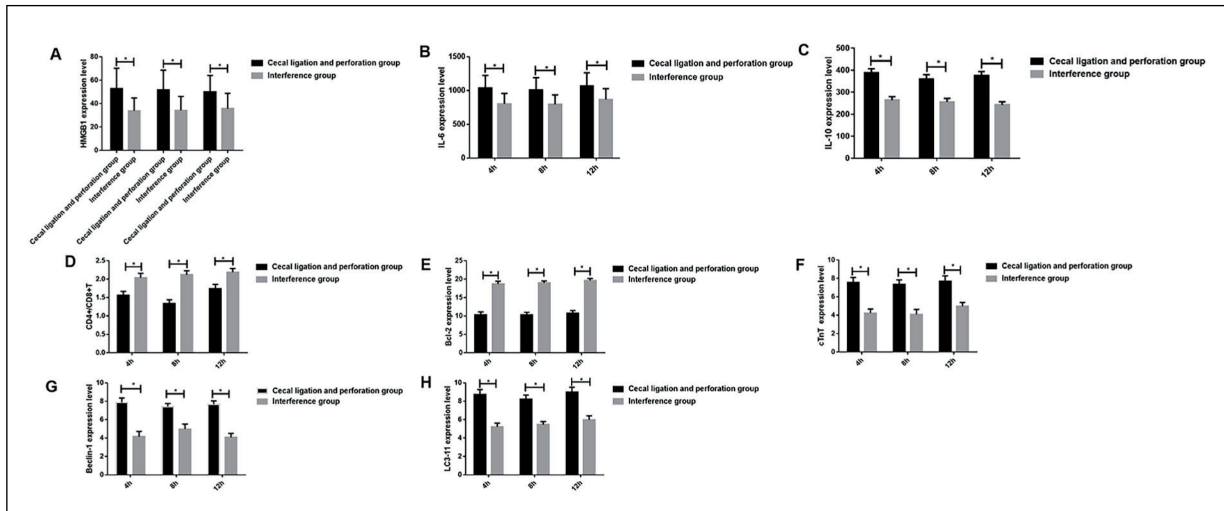

Figure 3. Detection indicators after interference test in CLPG. **A**, The expression level of HMGB1 in the interference group was significantly lower than that in the CLPG. **B**, The expression level of IL-6 in the interference group was significantly higher than that in the CLPG. **C**, The expression level of IL-10 in the interference group was significantly higher than that in the CLPG. **D**, The ratio of CD4+/CD8+T lymphocytes in the interference group was increased, which was significantly higher than that in the CLPG. **E**, The expression level of Bcl-2 in the interference group was significantly higher than that in the CLPG. **F**, The expression level of CTnT in the interference group was significantly lower than that in the CLPG. **G**, The expression level of Beclin-1 in the interference group was significantly lower than that in the CLPG. **H**, The expression level of LC3-11 in the interference group was significantly lower than that in the CLPG. *Means $p<0.05$.

tion of DNA. In 1999, Wang et al[13] found that HMGB1 could be released to the outside of the cell. It mediates inflammatory responses and is an important inflammatory mediator of sepsis. Some studies[14,15] have indicated that extracellular HMGB1 is an important inflammatory mediator and is involved in the pathogenesis of many diseases such as sepsis, endotoxemia, arthritis, acute pancreatitis, and pneumonia. At present, the research of HMGB1 targeted therapy has attracted wide attention. Effective HMGB1 antagonists are the key to HMGB1 targeted therapy. Our study is the first comprehensive analysis of HMGB1 on the immune function and autophagy levels of myocardial cells in septic rats.

The results of this study exhibited that HMGB1 was highly expressed in the sepsis model rats. At the same time, the autophagy factor proteins Beclin-1 and LC3-11 were also significantly higher in the sepsis rat models than those in the BCG and the SG. This result suggests a potential relationship between HMGB1 and cell autophagy. The study found that HMGB1 had different effects on autophagy and stress in different organs and tissues. HMGB1 knockout mice died shortly after birth, indicating that HMGB1 played an important role in maintaining life[16]. Mice with knocked out HMGB1 in pancreatic[17], liver[18,19], heart[19,20] and bone marrow[21] were all able to survive. They had no defects such as lethal hypoglycemia and energy metabolism in the natural growth state without the stressor. However, these mice showed different manifestations of autophagy and cell survival when confronted with different stressors. Mice with pancreatic HMGB1 knockout were more sensitive to aseptic inflammation, and their autophagy was downregulated when stimulated by lipopolysaccharide[17]. Huebener et al[19] first knocked out HMGB1 of hepatocytes and myocardial cells of mice with abundant mitochondria. They found that the structure and function of mitochondria, the organ function and long-term survival were not affected. Kitahara et al[20] stated that in mice with HMGB1 knockout, the myocardial infarction area was reduced, and more myocardial cells were preserved through autophagy, the myocardial contractility was thus protected, and cardiac function was improved. Yanai et al[21] knocked out macrophages HMGB1 of mice and found that HMGB1 could protect mice by promoting autophagy and reduce the endotoxemia and bacterial infection induced by lipopolysaccharide or Listeria. monocytogenes.

Conclusions

To sum up, combined with the results of the study, the level of serum high mobility protein B1 level is directly related to the severity of sepsis, and the increase in serum high mobility group protein B1 level in sepsis has a significant effect on the immune disorders of body. Sepsis can effectively activate myocardial autophagy, and the level of autophagy has an increasing trend.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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