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Original Article

Astragaloside Ⅳ Attenuates Polymicrobial Sepsis-Induced Cardiac Dysfunction in Rats via IKK/NF-κB Pathway*

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ABSTRACT **Objective:** To evaluate the protective effects of Astragaloside Ⅳ (AST) in a rat model of myocardial injury induced by cecal ligation and puncture (CLP). **Methods:** The model of sepsis-induced cardiac dysfunction was induced by CLP. Using a random number table, 50 specific pathogen free grade of Sprague Dawley rats were randomized into 5 groups: the sham group (sham), the model group (CLP, 18 h/72 h) and AST group (18 h/72 h). Except the sham group, the rats in other groups received CLP surgery to induce sepsis. CLP groups received intragastric administration with normal saline after CLP. AST groups received intragastric administration with AST solution (40 mg/kg) once a day. The levels of inflammatory mediators and oxidative stress markers in the serum of the septic rats were determined via enzyme-linked immunosorbent assay (ELISA) at different time point, such as interleukin 6 (IL-6), IL-10, high mobility group box-1 protein B1 (HMGB-1), superoxide dismutase (SOD), and malondialdehyde (MDA). Cardiac function was determined by echocardiography. Moreover, changes in myocardial pathology were evaluated using hematoxylin and eosin staining. The levels of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) were analysed to determine the status of CLP-induced myocardium. In addition, the apotosis of myocardial cells was analysed by terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL). The protein levels of B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), Iκ B kinase α (IKKα), nuclear factor kappa B p65 (NF-κB p65) were detected by Western blot analysis. Moreover, survival rate was investigated. **Results:** AST improved the survival rate of CLP-induced rats by up to 33.3% (P<0.05). The cardioprotective effect of AST was observed by increased ejection fraction, fractional shortening and left ventricular internal diameter in diastole respectively (P<0.01 or P<0.05). Subsequently, AST attenuated CLP-induced myocardial apoptosis and the ratio of Bcl-2/Bax in the myocardium, as well as the histological alterations of myocardium (P<0.01 or P<0.05); the generation of inflammatory cytokines (IL-6, IL-10, HMGB-1) and oxidative stress markers (SOD, MDA) in the serum was significantly alleviated (P<0.01 or P<0.05). On the other hand, AST markedly suppressed CLP-induced accumulation of IKK-α and NF-κB p65 subunit phosphorylation (P<0.01 or P<0.05). **Conclusions:** AST plays a significant protective role in sepsis-induced cardiac dysfunction and survival outcome. The possible mechanism of cardioprotection is dependent on the activation of the IKK/NF-κB pathway in cardiomyocytes.

KEYWORDS Astragaloside Ⅳ, cecal ligation and puncture, myocardial dysfunction

Sepsis is currently defined as a life-threatening host inflammatory response that is the leading cause of death in intensive care units worldwide.[1] It is considered to be related to a cascade of inflammatory factors, bringing about systemic inflammatory response syndrome and multiple organ dysfunction syndromes, which eventually lead to death.[2] Cardiac dysfunction induced by sepsis, which is closely related to increased mortality, usually appears in severe sepsis or septic shock patients. Compared to the 20% mortality in sepsis patients without cardiac dysfunction, sepsis patients with cardiac dysfunction have a 70%–90%

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mortality rate.[3] However, there is no specific therapy for sepsis-induced cardiac dysfunction.

Astragaloside Ⅳ (AST) is one of the major active ingredients extracted from the root of *Astragalus membranaceus*. They have been reported that AST is effective in the treatment of sepsis experimentally.[4] However, the possible beneficial outcomes of AST on cecal ligation and puncture (CLP) induced polymicrobial septic rats have not been previously investigated. Clinic trails showed that Astragalus Injection was effective in improving the activity of mononuclear macrophages of septic patients, which was play an immunomodulatory role in preventing excessive inflammation and immunosuppression.[5] It is well known that *Astragalus membranaceus* is a representative drug of benefiting qi in Chinese medicine, which has been used in China for centuries, plays a progressively important role in clinical treatment of cardiovascular diseases.[6,7] But, the underlying mechanisms in treating polymicrobial sepsis-induced cardiovascular disease are elusive.

In a sepsis study that mediated the induction of pro-survival/anti-apoptotic molecules, it was reported that the nuclear factor kappa B (NF-κB) signaling pathway involves the mechanisms of inflammation.[8] IκB kinase α (IKKα), IκB kinase β (IKKβ) and IκB kinase γ (IKKγ, also known as NF-κB-essential modulator) are together to form the IκB kinase (IKK) complex, which is the key to the activation of the canonical NF-κB signaling pathway.[9] It has been shown that NF-κB is an essential molecule in various inflammatory diseases for the regulation of cell proliferation and motility.[10] In this study, we hypothesize that AST mediates a protective effect against sepsis-induced myocardial injury as well as myocyte apoptosis through the IKKα/NF-κB pathway.

METHODS

Reagents and Animals

AST (E-0146) was obtained from Shanghai Winherb Medical Technology (Tauto Biotch, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kit was purchased from Promiga (USA, lot No. 0000205829). Enzyme-linked immunosorbent assay (ELISA) kits were purchased from Shanghai Westang Bio-tec Co. Antibodies such as p-p65 (3033S), p-IKKα (2679S), GAPDH (2118L), p65 (8142S), IKKα (2682S) were manufactured by Cell Signaling Technology (USA).

Healthy adult male Sprague Dawley (SD) rats (specific pathogen free grade, SCXY (Yue) 2016-0041) weighed 220–250 g were obtained from Southern Medical University. All animal experiments were performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (SYXK (Yue) 2018-0094). Rats were kept in a temperature-controlled room (25.0 ℃±0.2 ℃) under pathogen-free conditions, with a 12-h light/dark photoperiod and 50% humidity. All rats were allowed free access to food and water. The experimental protocol was approved by the Medicine Ethical Committee of Guangdong Provincial Hospital of Chinese Medicine.

Grouping, Modeling, and Treatment

According to the method of random number table, 50 SD rats were randomized into 5 groups: the sham group (sham), the CLP 18 h group, CLP 72 h group, AST 18 h group and AST 72 h group. Except the sham group, the rats in other groups received CLP surgery to induce sepsis. The CLP procedure was performed generally as Rittirsch reported.[11] In brief, abdominal incision was made to performe cecal ligation and puncture after anesthetized. The sham group rats were performed laparotomy without ligation. After suegery, all rats received fluid resuscitation with a 0.9% saline solution, injected subcutaneously (20 mL/kg of body weight). AST (40 mg/kg)[12] was intragastrically administered at 2 h after surgery, then once a day for 18 or 72 h. Meanwhile, the sham group and CLP group intragastrically administered saline. Rats were sacrificed with a lethal dose of anaesthetic at 18 and 72 h after CLP. The surviving rats were subjected to echocardiograph assessment subsequently, the hearts were extracted for histology, ELISA, Western blot or other analysis at 18 and 72 h after surgery.

Echocardiography

The rats were anaesthetized by isoflurane and echocardiographic examinations. They were performed by transthoracic echocardiography with VisualSonics Vevo® 2100 System (VisualSonics Inc, Toronto, Ontario, Canada) at 18 and 72 h after CLP. The left ventricular internal diastolic diameter (LVIDd), fractional shortening (FS) and ejection fraction value (EF) was calculated.

Histologic Analysis

Hearts samples were fixed in 4% paraformaldehyde at 4 ℃ and embedded in paraffin. Tissue sections of the myocardium were sectioned (5 μm) and stained with hematoxylin and eosin (HE) staining. The morphological changes were measured and analyzed by light microscopy at a magnification of 100× and 400×.

TUNEL Staining

The level of myocardial apoptosis was analyzed using a TUNEL assay. The myocardial tissue was stained with TUNEL and 4',6-diamidino-2-phenylindole (DAPI). TUNEL staining was used to detect the number of apoptotic cells. DAPI staining was used to detect the number of the total myocardial cells. TUNEL-positive cells showed green fluorescence and the nuclei of all the cells showed blue fluorescence. The apoptotic index was expressed as the ratio of the number of TUNEL-positive cells to the total number of cells.

ELISA Assays

The levels of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) in the serum were measured by ELISA according to the manufacturers' instructions. Inflammatory factors such as interleukin 6 (IL-6) and high mobility group box-1 protein B1 (HMGB-1), in the serum were evaluated using a commercially available ELISA kit. The serum was collected to evaluate the level of superoxide dismutase (SOD), malondialdehyde (MDA) by ELISA according to the manufacturers' instructions.

Western Blot Analysis

Samples were stored on ice and then homogenized in a lysis buffer. The supernatants of tissue homogenates were separated using dodecyl sulfate, sodium salt (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. After being blocking with 5% skim milk in Tris-HCl buffer solution (TBS) for 2 h at room temperature, the membrane was incubated overnight at 4 ℃ with the primary antibody against Bcl-2, Bax, p-IKKα, IKKα, p-NFκB p65, NFκB p65 and GAPDH. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then visualized by advanced chemiluminescence (Immobilon Western; Millpore, Shanghai, China). The blots were imaged using the Molecular Imager ChemiDoc XRS Gel Imagine System (BioRad, Hercules, CA, USA) and normalized against GAPDH. The final results are expressed as fold changes by normalizing the data to the control values.

Survival Time

The 72 h survival study was conducted to determine whether AST could significantly improve survival in a model of CLP sepsis. The rats in each group had free access to food and water and were kept under pathogen-free conditions.

Statistical Analysis

All data analyses were performed by SPSS 23.0 statistical software (IBM Co. Ltd., Armonk, NY, USA). All data were presented as mean ± standard deviation ($\bar{x} \pm s$). Survival rates were compared using Kaplan-Meier tests. The differences between groups were analyzed using a one-way analysis of variance (ANOVA). Differences in values were considered as statistically significant if $P<0.05$.

RESULTS

AST Improved Survival Rate in Septic Rats

As an extract of *Astragalus membranaceus*, the peak of AST extract was shown in high performance liquid chromatography (HPLC) chromatogram. The purity of AST is more than 98.0%.

As shown in Figure 1, AST administration could dramatically increase the 72 h survival rate of rats that underwent CLP surgery ($P<0.05$). The 72 h survival rate in the sham group was 100%. However, 57.9% mortality was observed in CLP rats at 72 h, the value only reached 33.3% in the AST treatment group.

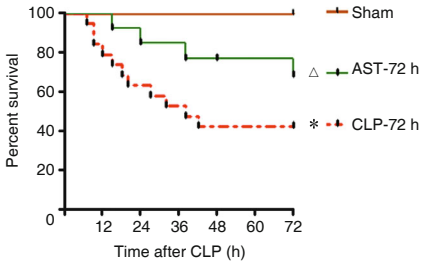

Figure 1. Effect of AST on Survival Rate (*n*=5)

Notes: *$P<0.05$ *vs*. the sham group; △$P<0.05$ *vs*. the CLP 72 h group

AST Improved Cardiac Function in Septic Rats

As shown in Figure 2, CLP surgeries significantly increased the myocardial injury in rats as evidenced by decreasing EF, FS and LVIDd ($P<0.01$ or $P<0.05$). AST treatment dramatically blocked the changes in EF and FS ($P<0.01$ or $P<0.05$).

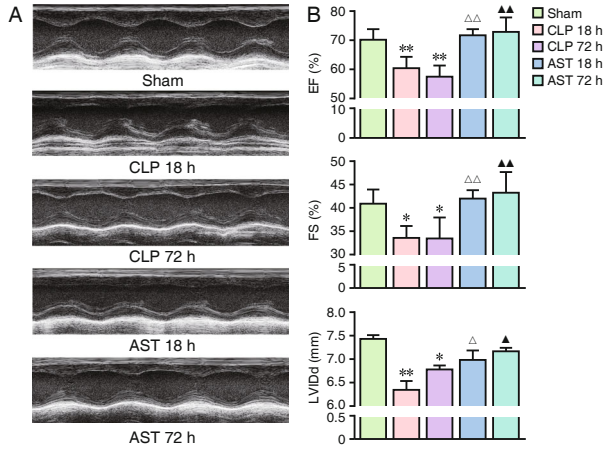

Figure 2. Effect of AST on Myocardial Injury in Rats (n=5, $\bar{x} \pm s$)

Notes: (A) Representative images of M-mode echocardiography. (B) EF: ejection fraction, FS: fractional shortening, LVIDd: left ventricular internal diastolic diameter, CLP: cecal ligation and puncture, AST: Astragaloside Ⅳ; $^{*}P$<0.05, $^{**}P$<0.01 *vs*. the sham group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. the CLP 18 h group; $^{\blacktriangle}P$<0.05, $^{\blacktriangle\blacktriangle}P$<0.01 *vs*. the CLP 72 h group; the same below

AST Protected against Myocardial Injury in Septic Rats

As shown in Figure 3A, increased interstitial edema and decreased spaces was observed after CLP. Cardiac muscle cross striations were not visible. There was an observably infiltration of red blood cells in the CLP 18 h group, and cytolysis of myocardial cell in the CLP 72 h group. In contrast, AST treatment attenuated the histologic injury and had a normal stucture. As shown in Figure 3B, CLP surgery triggered a significantly increase in the serum CK-MB and LDH levels. Conversely, AST treatment progressively decreased the levels of CK-MB and LDH (P<0.01 or P<0.05).

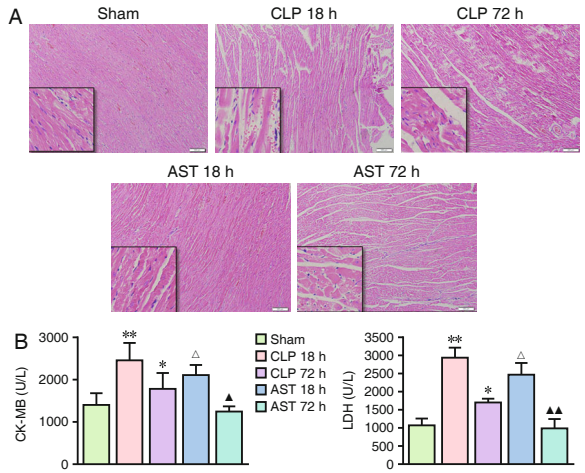

Figure 3. AST Ameliorated CLP-Induced Myocardial Injury (n=5, $\bar{x} \pm s$)

Notes: The scale bar is 200 μm (100×) and 50 μm (400×)

AST Mitigated Myocardial Apoptosis in Septic Rats

As shown in Figure 4, CLP surgery significantly increased the number of apoptosis cells, and AST treatment reduced the TUNEL-positive cells in the heart tissue (all P<0.01).

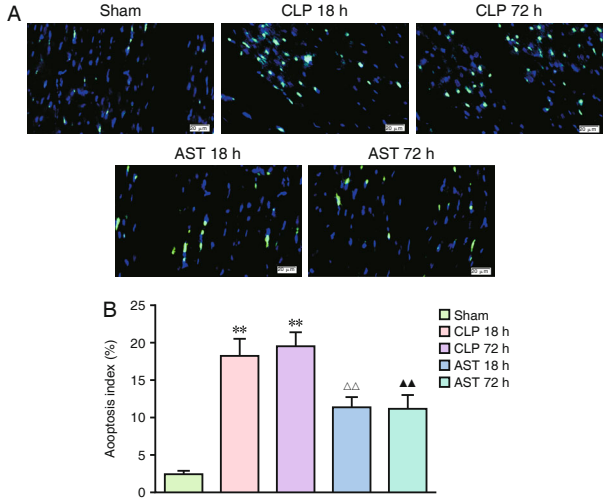

Figure 4. AST Moderate Myocardial Apoptosis in Septic Rats (n=5, $\bar{x} \pm s$)

Notes: The apoptotic cells were depicted by TUNEL (green), and the nuclei were depicted by DAPI (blue); the scale bar is 20 μm (200×)

Effects of AST on Inflammatory Cytokine Production in Serum of Septic Rats

As shown in Figure 5, CLP significantly increased levels of IL-6 and HMGB-1 in the serum. However, AST treatment markedly reduced the levels of IL-6 and HMGB-1 at 72 h after CLP (P<0.01 or P<0.05).

Effects of AST on Oxidative Stress in Serum of Septic Rats

As shown in Figure 5, the level of SOD production in the CLP group was dramatically decreased in comparison to that in the sham group (P<0.01 or P<0.05), while treatment with AST notably enhanced SOD production (P<0.01). The level of MDA increased in the CLP group and lowered after AST treatment (P<0.01 or P<0.05).

Effects of AST on NF-κB Signaling Pathway

As shown in Figure 6, with the prolonged operation time, the CLP group could gradually increase IKKα phosphorylation level (P<0.05 or P<0.01). In contrast, the phosphorylation of NF-κB p65 in CLP group showed the trend of an increase followed by a decline (P<0.05). AST treatment dramatically accelerated the phosphorylation of IKKα (P<0.05 or P<0.01), and inhibited the phosphorylation

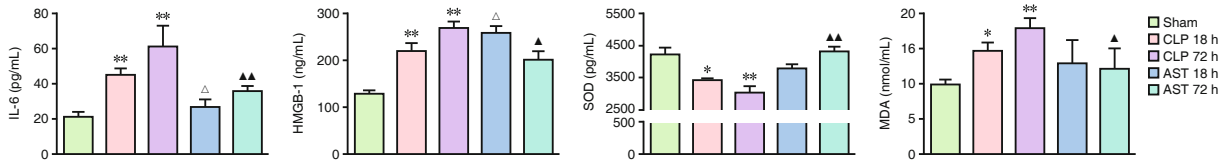

Figure 5. Protective Effect of AST on Septic Rats (n=5, $\bar{x} \pm s$)

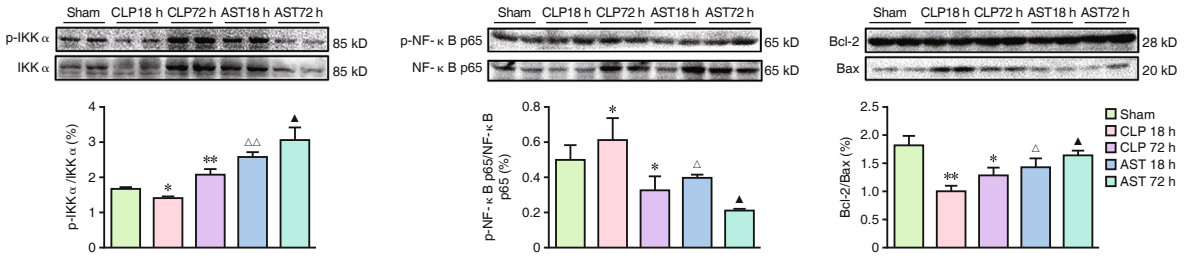

Figure 6. Effect of AST on NF-κ B Signaling Pathway (n=5, $\bar{x} \pm s$)

Note: The typical figures of Western blot

of NF-κ B p65 (P<0.05). Meanwhile, AST treatment up-regulated the ratio of Bcl-2/Bax compared to the CLP-induced septic rats (P<0.05).

DISCUSSION

In this study, we investigated the effects of AST on CLP-induced septic rats and explored the possible molecular mechanisms. We found that AST administration could improve cardiac function, relieve myocardial apoptosis, and decrease the level of inflammatory cytokines and oxidative stress factors in septic rats. Furthermore, we identified the IKK-NF-κ B signaling pathway as one of the possible molecular target of AST on septic rats.

AST is a small-molecule saponin stemmed from *Astragalus membranaceus*. Animal experiment has shown that AST can attenuate cardiac malfunction and maintains the integrity of the myocardial structure in myocardial ischemia and reperfusion injury.[13] The protection of AST is mainly associated with its anti-inflammation and anti-oxidation properties. Our previous study revealed that AST could improve vascular function by regulating the endothelial progenitor cells' biological function.[14] It has been reported that AST is protective against lipopolysaccharide (LPS)-induced septic rats. In addition, with respect to LPS-induced myocardial injury, AST attenuated inflammatory impairment and improved survival rates.[15] Consistent with previous results, we found that AST improved the 72-h survival rate after CLP surgery.

Sepsis is a complex pathophysiologic process, accompanied with the release of large amounts of inflammatory mediators including cytokines and chemokines. This proinflammatory response triggers the release of powerful secondary mediators, such as inflammatory enzymes and reactive oxygen species, which further amplify the inflammatory process.[9] The major proinflammatory cytokines, IL-6, is a major contributor to the development of myocardial dysfunction in sepsis.[16] IL-6, an important pro-inflammatory factor, which is not only directly injure the myocardium but also contribute to the aggravation of inflammation and induce cell apoptosis.[17] HMGB1 has recently been recognized as an important mediator in sepsis, which appears later in the serum as a persistent response.[18] Studies have demonstrated that HMGB1 is elevated in septic patients. In addition, oxidative stress and its consequent excessive reactive oxygen species (ROS) production aggravate lipid peroxidation, and trigger cell apoptosis and inflammatory mediators.[19] The level of SOD is regarded as potent anti-oxidant enzymes that serve as ROS scavengers. MDA is often used as an indicator of oxidative damage.[20] These cytokines have been confirmed to be involved in the molecular pathogenesis of sepsis, which might be the potential targets of AST to exert its effects on improving cardiac dysfunction.

The cardiovascular system plays a key role in the progression of sepsis. A study demonstrated the evidence of myocardial dysfunction during septic syndromes 30 years ago.[21] However, septic myocardial impairment remains a clinical challenge. Heart is a particular organ in which it is difficult to distinguish between the direct septic cardiac effects,

and the cardiac response to changes in sympathetic effects or other neurohumoral activity that occurs during sepsis.[22] There is no unified definition of septic myocardial dysfunction. The researcher believed that myocardial dysfunction is a complex pathophysiology including ventricular systolic and diastolic dysfunction, but a reversible dysfunction of both the left and right sides of the heart.[23] The underlying mechanisms of cardiac failure in sepsis involve autonomic dysregulation (β-adrenergic), inflammatory cytokines mitochondrial dysfunction cell necrosis or apoptosis, and oxidative stress.[24] In the present study, tissue Doppler imaging and perfusion echocardiography were used to recognize myocardial dysfunction during CLP-induced polymicrobial sepsis. As expected, AST treatment dramatically increased myocardial injury induced by CLP, as evidenced by increased EF, FS and LVIDd showed in echocardiography. Moreover, the level of LDH and CK-MB in serum, the biochemical marker for myocardial cells' death, was significantly decreased in the AST treatment group. Meanwhile, cardiac histological changes can be dramaticlly improved after AST treatment.

The NF-κB signaling pathway plays an important role in the mechanisms of inflammation in sepsis studies. The NF-κB signaling pathway activates in two ways, the canonical (classical) pathway and the noncanonical (alternative) pathway.[25] The canonical NF-κB pathway mainly participates in natural immunity and inflammation, while the noncanonical NF-κB pathway is involved in autoimmune diseases. As a well-known component of the noncanonical NF-κB pathway, IKKα negatively regulates the pathway by the complex of IκBα. Lawrence[26] discovered that IKKα-deficient mice can enhance the pro-inflammatory responses of IKKα-deficient macrophages. Shembade[27] showed that IKKα contribute to the negative regulation of the canonical NF-κB pathway. In our study, we demonstrate that the cardioprotection afforded by AST is related to the activation of the IKKα/NF-κB pathway.

Furthermore, AST treatment also significantly decreased myocardial apoptosis, as evidenced by the descending apoptotic index, the increased level of Bcl-2, and decreased level of Bax. Bax, a pro-apoptotic protein, is a major regulatory checkpoint for apoptosis. Bcl-2 is a dominant regulator of apoptosis which can prevent the activation of Bax. Studies have implicated that the ratio of Bcl-2/Bax is a rheostat in the protection against or acceleration of apoptosis. Bcl-2/Bax is considered as an index of apoptotic activation.[28] What is more, TUNEL assay exhibited that the number of apoptotic cells in myocardium was notably decrease after AST treatment.

In conclusion, our study shows the potential therapeutic use of AST in sepsis treatment. AST plays a significant protective role in sepsis-induced cardiac dysfunction and survival outcome. The possible mechanism of cardioprotection is dependent on the activation of the IKK/NF-κB pathway in cardiomyocytes. Our work has provided a more thorough examination for the clinical application of Chinese medicine.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Huang X performed the experiments and wrote the manuscript. Guo LH designed the study, Yin X and Ma SY contributed some experiments and the data analysis. Zhang MZ and Liu B revised the manuscript.

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