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Intermedin1-53 protects cardiac function in rats with septic shock via inhibiting oxidative stress and cardiomyocyte apoptosis

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Abstract. – OBJECTIVE: To investigate the protective effect of intermedin1-53 (IMD1-53) on cardiac function in rats with septic shock and its underlying mechanism.

MATERIALS AND METHODS: Twenty-four male Sprague-Dawley (SD) rats were randomly assigned into three groups, namely the control group (NC group), septic shock group (ET group) and IMD1-53 treatment group (IMD group), with 8 rats in each group. Levels of hemodynamic indicators, blood glucose, lactate acid, CK-MB (creatine kinase-MB) and cTnI (cardiac troponin I) in rats were determined. Cardiac tissues of rats were collected for TUNEL (terminal dexynucleotidyl transferase (TdT)-mediated dUTP nick end labeling) staining. Protein levels of caspase-3, caspase-9, Bax, Bcl2, iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) in cardiac tissues were detected by Western blot. Moreover, activities of SOD (superoxide dismutase), CAT (catalase) and MDA (malondialdehyde) in myocardial homogenate were determined, thereby exploring the effect of IMD1-53 on oxidative stress and cardiomyocyte apoptosis in rats with septic shock induced by endotoxin.

RESULTS: Lower levels of mean arterial blood pressure (MABP), maximum rate of left ventricular diastolic pressure (+LVdp/dtmax) and left ventricular systolic pressure (LVSP) were observed in ET group than those of NC group ($p < 0.05$). Levels of lactic acid, blood glucose, CK-MB and cTnI in ET group were remarkably increased than those of NC group ($p < 0.05$). Moreover, activities of SOD and CAT in myocardial homogenate of ET group were remarkably reduced in comparison with those of NC group ($p < 0.05$). Protein levels of caspase-3, caspase-9, Bcl-2, Bax, iNOS and COX-2 in ET group were all remarkably elevated than those of NC group ($p < 0.05$). The above indicators were all significantly improved in IMD group than those of ET group ($p < 0$ 05).

CONCLUSIONS: IMD1-53 can protect cardiac function in rats with septic shock via inhibiting oxidative stress and cardiomyocyte apoptosis.

Key Words:
IMD1-53, Oxidative stress, Cardiomyocyte apoptosis, Septic shock.

Introduction

Septic shock is a serious medical condition, which is secondary to infection, severe trauma, burns and major surgeries. Severe septic shock may progress to multiple organ dysfunction syndrome (MODS), and even death. Due to its high mobility and mortality, septic shock has been well-recognized worldwide[1]. In the United States, the incidence of septic shock has been astonishingly increased. Currently, there are over 1 million new cases of septic shock annually, and the mortality rate is up to 20-40%[2,3]. Septic shock in children should also be focused on. Reports have demonstrated that the mortality rates of septic shock in children from the United States, United Kingdom and developing countries who are cared for in the Pediatric Intensive Units (PICU) are 10%, 17% and 50%, respectively. The rapid progression and high mortality rate of septic shock pose great challenges to clinical treatment[4,5]. Researches on septic shock have been advanced in recent years. Myocardial injury is a common complication of sepsis shock, accounting for 40% of sepsis shock cases. Myocardial injury will exaggerate the disease condition, which is the leading cause of early death in about 20% of septic shock patients[6-9]. Therefore, it is of great significance in investigating the pathogenesis of myocardial injury induced by septic shock[10-12].

Intermedin (IMD) is a new member of the calcitonin gene related peptide (CGRP) family, which was initially found in the teleost fish by Roh et al[13]. Subsequent researches confirmed that IMD is widely expressed in hypothalamus, heart and kidney of mammals[14]. IMD can be degraded

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into IMD1-53, IMD1-47 and IMD8-47, of which, IMD1-53 is the major active fragment[15]. Studies have shown that IMD1-53 exerts a protective effect on cardiac function after ischemia-reperfusion. Zhao et al[16] found that IMD1-53 can improve lipid peroxidation damage caused by ischemia-reperfusion, thus elevating cardiomyocyte survival. Yang et al[17] found that IMD1-53 pretreatment can reverse cardiac function after ischemia-reperfusion injury in rats by reducing activities of myocardial lactate dehydrogenase (LDH) and malondialdehyde (MDA)[18]. Another report showed that IMD1-53 protects cardiac function induced by ischemia-reperfusion via increasing expressions of anti-apoptotic proteins and reducing mitochondrial release. Accumulating studies[19] have already showed that IMD1-53 is capable of protecting cardiac function induced by ischemia-reperfusion through inhibition of oxidative stress and cardiomyocyte apoptosis. However, there are no reports underlying the effect of IMD1-53 on cardiac function after septic shock.

Materials and Methods

Construction of Experimental Rats

Twenty-four male SD rats (Model Animal Research Center of Nanjing University, Nanjing, China) were housed for one week and randomly assigned into 3 groups, with 8 rats in each group. Rats in NC group were intraperitoneally injected with 2 ml/kg saline. Rats in ET group were intraperitoneally injected with 10 mg/kg lipopolysaccharide (LPS). Rats in IMD group were intraperitoneally injected with 1 ml of IMD1-53 for 3 consecutive days, followed by intraperitoneal injection of 10 mg/kg LPS. This investigation was approved by the Animal Ethics Committee of Weifang Medical University Animal Center. All reagents were purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA).

Determination of Cardiac Function and Blood Pressure

After injection of LPS for 12 h, rats were intraperitoneally injected with sodium pentobarbital for anesthesia. The catheter was pre-washed with 500 U/mL heparin saline and then inserted into the left ventricle through the left common carotid artery. 10 min after the insertion, stable hemodynamic indicators were taken by Powerlab polyphysiograph (AD Instruments Shanghai Trading Co., Ltd, Shanghai, China). Furthermore, blood sample of rat was harvested for serum exaction. Levels of blood glucose, lactate acid, CK-MB and cTnI were then detected. Myocardial tissue homogenate was prepared after collection of rat heart. Meanwhile, dry and weight ratio of left ventricle was calculated.

Determination of Blood Glucose, Lactate Acid, CK-MB and cTnI

Blood samples of rats were centrifuged at 3000 r/min for 10 min. Supernatant was collected for detecting levels of glucose, lactic acid, CK-MB and cTnI by an automatic biochemical analyzer (Beckman Coulter, Brea, CA, USA).

Detection of MDA, SOD and CAT Activities

Tissue homogenate was prepared with 0.1 g ± 0.05 g of myocardial tissue ground in 200 μL of phosphate-buffered saline (PBS). The final dose of homogenate was adjusted to 10% by adding 700 μL of PBS. Homogenate was then centrifuged at 3500 r/min for 10 min. MDA, SOD and CAT activities were detected based on the instructions of relevant commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

TUNEL Staining

Tissue slides were prepared using the TUNEL labeling kit (Yeasen, Shanghai, China) according to the manufacturer's instructions. Briefly, slides were washed with PBS and blocked with hydrogen peroxide solution. After the slides were permeabilized in Trixon-100, they were incubated with TUNEL solution for 90 min. Negative controls were incubated with TdT labeled solution. Finally, slides were stained with DAPI for the following observation of apoptotic cells.

Western Blotting

The total protein was extracted by radioimmunoprecipiation assay (RIPA) lysate. The concentration of each protein sample was determined by a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 50 μg of total protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk, followed by the incubation of specific primary antibodies (caspase-3, caspase-9, Bcl-2, Bax, iNOS and COX-2, diluted in 1:1000)

overnight. Next, membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical product and service solutions (SPSS19.0, SPSS Inc., Armonk, NY, USA) software were used for statistics analysis. Category variables were expressed as numbers and percentages. Continuous variables were shown as mean ± standard deviation. The Student-Newman-Keuls test was used to compare the data between two groups, and x^2-test was used to compare categorical variables. A one-way ANOVA followed by Least Significant Difference (LSD) was conducted to test the significance between groups. $p < 0.05$ indicated the difference was statistically significant.

Results

Behavior Features of Rats

Rats in NC group presented great hair glossiness, good viability and sensitive stimulation. After 20 min of LPS injection, viabilities and stimulus response of rats in ET group were weakened. Less movement, gregarious reaction and slow response of rats were observed in ET group after 1 h of LPS injection. 3 h later, rats in ET group presented less glossiness of hair and less eating. More seriously, some rats were convulsive with a weak response to the stimulus after injection of LPS for 12 h. Rats in IMD group were injected with IMD1-53 three days prior to LPS injection. No significant differences in behavior features were observed between IMD group and NC group before LPS injection. However, viabilities and stimulus response of rats in IMD group were weakened after 6 h of LPS injection. 12 h later, rats in IMD group presented less eating.

IMD1-53 Improved Cardiac Function in Rats With Septic Shock

No significance in rat heart rate among the three groups was found (all $p > 0.05$, Figure 1A). Compared with those in NC group, MABP, +LVdp/dtmax and LVSP in rats of ET group decreased by 29.7%, 31.3% and 40.8%, respectively (all $p < 0.05$, Figure 1B-D). MABP, +LVdp/dtmax and LVSP in rats of IMD group increased by 26.6%, 34.3% and 26.5%, respectively when compared with those of ET group (all $p < 0.05$), suggesting that IMD1-53 can significantly improve cardiac function in rats with septic shock.

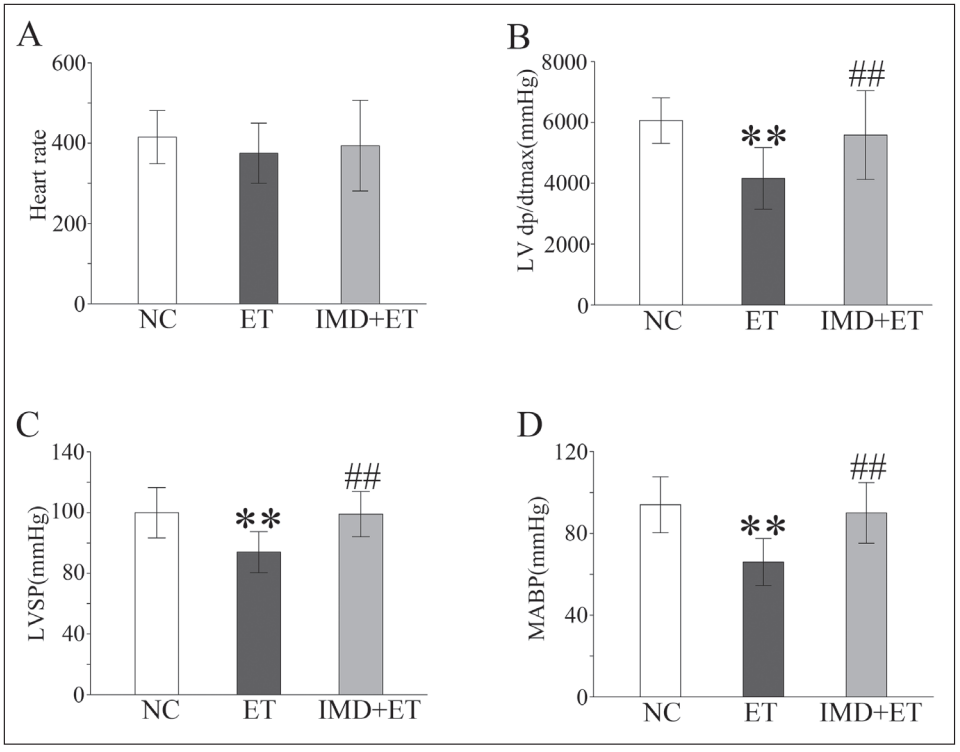

Figure 1. Comparison of vital signs and physiological indicators of rats in different groups. ***A,*** Comparison of rat heart rate in each group. ***B,*** Comparison of rat +LVdp/dtmax in each group. ***C,*** Comparison of rat LVSP in each group. ***D,*** Comparison of rat MABP in each group.

Changes of Serum Glucose, Lactate Acid, CK-MB and cTnI in Rats With Septic Shock

Lactic acid contents in rats of ET group were increased to 171% compared with those of NC group. However, lactic acid contents in rats of IMD group were decreased by 24.2% ($p = 0.011$) in comparison with those of ET group, suggesting that IMD1-53 significantly improved the hyperlactemia in rats with septic shock (Figure 2A). Blood glucose levels of rats in ET group were decreased by 26.5% compared with those of NC group ($p < 0.05$). While blood glucose levels of rats in IMD group were increased to 150% in comparison with those of ET group ($p = 0.062$, Figure 2B). Higher contents of CK-MB and cTnI in rats of ET group were found than those of NC group and IMD group ($p < 0.05$, Figure 2C-D). Moreover, dry and weight ratios of left ventricle in rats of ET group were remarkably lower than those of NC group and IMD group ($p < 0.05$, Figure 2E), indicating that there was myocardial tissue edema in rats of ET group.

Changes of MDA, SOD and CAT Activities in Myocardial Tissues

Our results showed that lower SOD and CAT activities were observed in rats of ET group than those of NC group ($p < 0.05$), suggesting that the myocardial antioxidant activity was decreased. However, SOD and CAT activities in rats of IMD group were remarkably elevated than those of ET group ($p < 0.05$, Figure 2F-G). Our data also indicated that MDA contents in myocardial tissues of ET group were remarkably increased than those of NC group and IMD group ($p < 0.05$, Figure 2H). The data suggested that IMD1-53 could inhibit the oxidative stress in myocardial tissues induced by septic shock.

Protein Expressions in Myocardial Tissues

Higher expressions of iNOS and COX-2 in myocardial tissues were observed in ET group compared with those of NC group (Figure 3A-B). Protein expressions of Bax, caspase-3 and caspase-9 in ET group were also increased in comparison with those of ET group ($p < 0.05$, Figure 3C-F). However, protein expressions of iNOS, COX2, Bax, caspase-3 and caspase-9 in IMD group were remarkably reduced compared with those of ET group ($p < 0.05$, Figure 3A, C and E). Bcl-2/Bax ratio was also significantly decreased in ET group than that of NC group and IMD group ($p < 0.05$, Figure 3G). These results suggested that IMD1-53 inhibits cardiomyocyte apoptosis and oxidative stress in rats with septic shock.

IMD1-53 Inhibited Cardiomyocyte Apoptosis

There was no significant cardiomyocyte apoptosis in rats of NC group detected by TUNEL staining (Figure 4A). However, cardiomyocyte apoptosis was remarkably increased in ET group

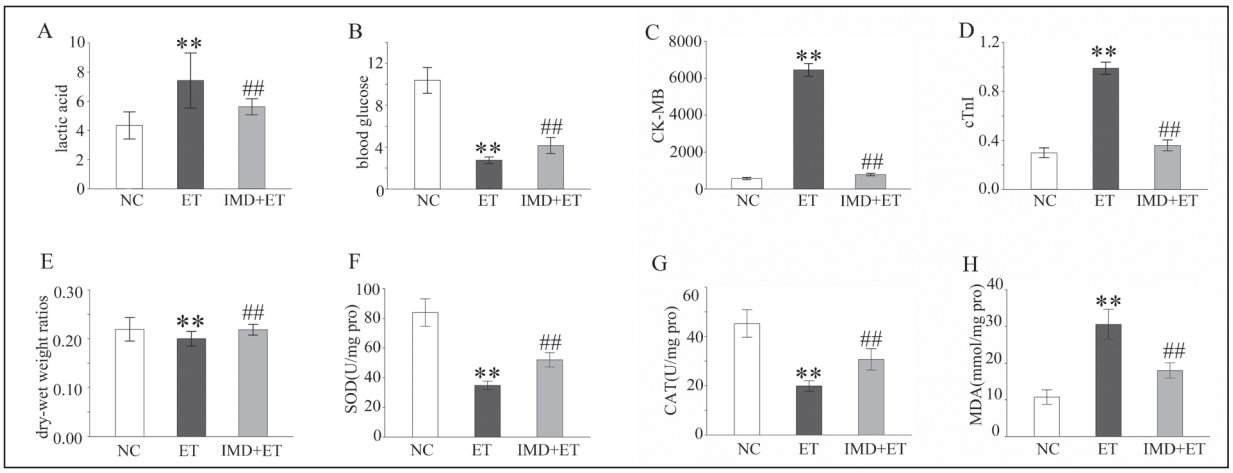

Figure 2. Comparison of serum indicators, dry and wet ratio of left ventricle and activities of SOD, CAT and MDA in different groups. ***A***, Comparison of rat serum lactic acid in each group. ***B,*** Comparison of rat blood sugar in each group. ***C,*** Comparison of rat serum CK-MB in each group. ***D,*** Comparison of rat serum cTnI in each group. ***E,*** Comparison of dry and wet ratio of left ventricle in each group. ***F,*** Comparison of SOD activities in cardiac tissues of rats in each group. ***G,*** Comparison of CAT activities in cardiac tissues of rats in each group. ***H,*** Comparison of MDA activities in cardiac tissues of rats in each group.

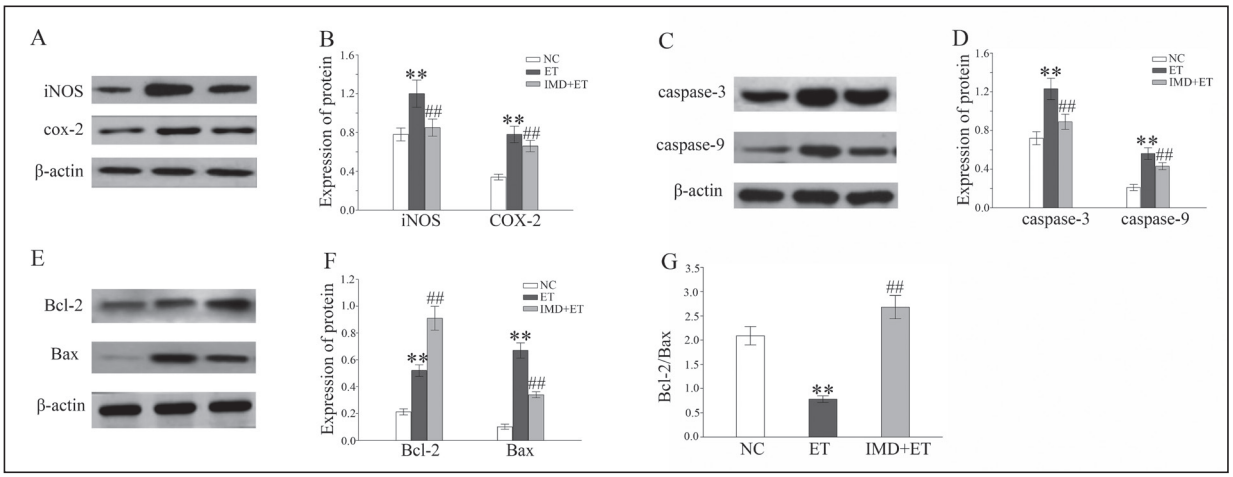

Figure 3. Protein expressions of iNOS, COX-2, Bcl-2/Bax, caspase-3 and caspase-9 in cardiac tissues of rats in different groups. ***A,*** Expressions of iNOS and COX-2 in cardiac tissues of rats. ***B,*** Comparison of expressions of iNOS and COX-2 in each group. ***C,*** Expressions of caspase-3 and caspase-9 in cardiac tissues of rats. ***D***, Comparison of expressions of caspase-3 and caspase-9 in each group. ***E,*** Expressions of Bcl-2 and Bax in cardiac tissues of rats. ***F,*** Comparison of expressions of Bcl-2 and Bax in each group. ***G,*** Comparison of Bcl-2/Bax in cardiac tissues of different groups.

compared with that of NC group (Figure 4B), which was significantly decreased in IMD group ($p < 0.05$, Figure 4C-D).

Discussion

Common animal models of septic shock include the cecal ligation and puncture (CLP), colon ascendens stent peritonitis (CASP) and intravenous injection of bacteria or endotoxin[20-23]. In this investigation, rat septic shock model was constructed by intraperitoneal injection of LPS, which is the most stable and commonly used animal model. Studies[24] have shown that more than 95% of sepsis shock is caused by bacteria, 50% of which are Gram-negative bacteria. LPS is the outer membrane component of Gram-negative bacteria, which is widespread in cells with a strong virulence. Clinical data have shown that

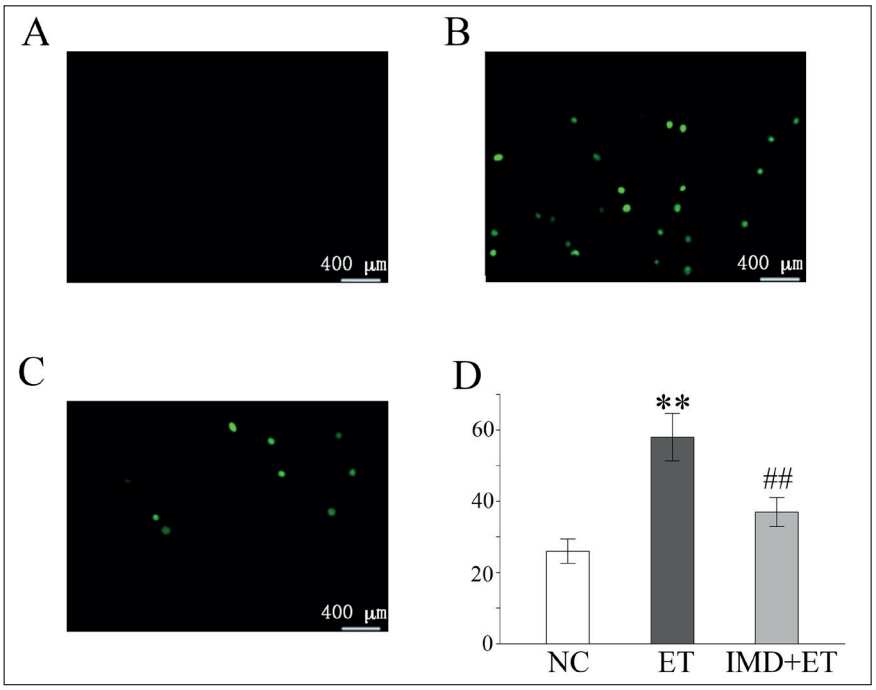

Figure 4. TUNEL staining and apoptosis rate of cardiac tissues of rats. ***A,*** TUNEL staining of cardiac tissues in NC group. ***B,*** TUNEL staining of cardiac tissues in ET group. ***C,*** TUNEL staining of cardiac tissues in IMD group. ***D,*** Apoptosis rate of cardiomyocyte in each group.

manifestations of myocardial damage in patients with septic shock are mainly reduced myocardial systolic and diastolic function, cardiac output changes and decreased ejection fraction. In recent years, immune, biochemical and hemodynamic indicators have been introduced into the diagnostic criteria for myocardial damage in septic shock. Our results showed that the general conditions of rats were progressively deteriorated after LPS injection, including weakened vitality and stimulus response, gregarious reaction, shortness of breath, less glossiness of hair, less eating, and even convulsion. Elevations of blood lactate acid, CK-MB and cTnI, as well as reductions in blood pressure and blood glucose in ET group all indicated the successful construction of rat septic shock model. Furthermore, decreased hemodynamic indicators in ET group indicated severe heart failure in rats with septic shock. IMD1-53 treatment remarkably improved behavior features and cardiac function in rats with septic shock, suggesting that IMD1-53 could protect cardiac function damaged by septic shock.

Inflammatory factors released after sepsis shock lead to multiple systemic dysfunction and structural damage. Studies have found that TNF-α, IL-1, IL-6, endothelin-1, adhesion molecules, NO, etc. are involved in the process of myocardial infarction induced by septic shock. Moreover, a large number of oxygen free radicals (OFRs) are released[25], which are involved in the pathogenesis and progression of septic shock[26,27]. Accumulation of reactive oxygen species (ROS) results in inadequate production of mitochondrial ATP (adenosine triphosphate), which eventually leads to multiple organ dysfunction[28]. MDA is the major product of lipid peroxidation, which reflects OFR level and severity of tissue damage. SOD catalyzes the disproportionation reaction of superoxide radical and protects cell membrane through eliminating OFR attack. Under normal circumstances, MDA and SOD are kept in balance so that the body can prevent from oxidative stress. The present work found that MDA levels in myocardial tissues of ET group were remarkably increased compared with those of NC group. Moreover, IMD1-53 pretreatment can reverse the oxidative stress in myocardial tissues, indicating that IMD1-53 is capable of protecting cardiac function damage induced by septic shock.

Apoptosis is the leading cause of cardiac dysfunction in septic shock. Cardiomyocyte apoptosis has been clearly observed in endotoxin-induced septic cardiomyopathy in animal models[29]. After injection of endotoxin (10 mg/kg) into rat ventricular cardiomyocytes for 4 h, caspase-3 expression was significantly increased[30]. Activated caspase-3 can also regulate phospholamban (PLB) by activating protein phosphatase 2A (PP2A), resulting in reduced uptake of calcium, thus damaging myocardial contractility[31]. Administration of z-FA.fmk (a ubiquitous caspase-3 inhibitor) to mice with septic shock reduced caspase-3 activity and decreased cardiomyocyte apoptosis in myocardial tissues. Notably, the inhibition of caspase-3 significantly improved cardiac dysfunction induced by septic shock[32]. This evidence indicated that apoptosis is involved in the development of septic shock cardiomyopathy. Additionally, Bcl-2 and Bax are capable of regulating apoptosis. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein. Studies[33,34] have shown that a significant decrease in the ratio of Bcl2/Bax refers to severe cell apoptosis. In this work, increased protein levels of caspase-3, caspase-9 and Bax, and decreased ratio of Bcl2/Bax in rats of ET group, were all reversed by IMD1-53 treatment. Further TUNEL staining confirmed that IMD1-53 remarkably inhibited cardiomyocyte apoptosis, which was consistent with the previous study[35]. Therefore, we speculated that IMD1-53 can improve cardiac function damage in rats with septic shock by inhibition of cardiomyocyte apoptosis.

Taken together, our report first observed that exogenous IMD1-53 treatment can significantly improve cardiac function in rats with septic shock by inhibiting oxidative stress and myocardial apoptosis, providing a new basic for improving cardiac function damage induced by septic shock.

Conclusions

We showed that IMD1-53 treatment can significantly improve cardiac function in rats with septic shock by inhibiting oxidative stress and myocardial apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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