The Protective Effect of Shuxin Shengmai Dan on Myocardial Ischemia-Reperfusion Injury in Rats

Xingye Wang
Changchun University of Chinese Medicine
Zhenzhu Hao
Changchun University of Chinese Medicine
Xinghu Piao
Changchun University of Chinese Medicine
Li Sun
Changchun University of Chinese Medicine
Ye Tao
Changchun University of Chinese Medicine
Xiaowei Zhong
Jilin University
Haimiao Yang
Changchun University of Chinese Medicine
Wei Wang
Changchun University of Chinese Medicine
Yicheng Zhao
Changchun University of Chinese Medicine
Bingmei Wang (✉ bingmeiwang1970@163.com)
Changchun University of Chinese Medicine

Research

Keywords: Shuxin Shengmai Dan, MIRI, Inflammation, TLR4, NF-κB

Posted Date: October 9th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-86967/v1

Abstract

Background: The traditional Chinese medicine Shuxin Shengmai Dan (SXSMD) is clinically used to treat angina pectoris. The mechanism of action of SXSMD protection of the heart involves inhibition of inflammation and remains poorly understood. The role of SXSMD in rats with myocardial ischemia reperfusion (IR) and the mechanism of SXSMD action were studied in this research.

Methods: The rats were treated with SXSMD (3.38, 6.76, and 13.52 g/kg/day, p.o.) or Danshen injecta (1.8 mL/kg/day, p.o.) for 15 days, then the coronary arteries were ligated. Cardiac function was evaluated by electrocardiography and hemodynamic measurements. Hematoxylin-eosin (H&E) staining was used to detect pathological changes in ischemic myocardial sections. Transmission electron microscopy (TEM) was used to assess the ultrastructure of cardiomyocytes. The changes in IL-6 and TNF-α in the rat serum were detected by ELISA. The changes in the expression levels of HMGB1, TLR4, MyD88, and NF-κB mRNAs and proteins related to the TLR4/NF-κB pathway in myocardial tissue were detected by qPCR and Western blot, respectively.

Results: Rats with coronary artery ligation had abnormal cardiac function, inflammatory infiltration of myocardial cells, disordered myocardial fiber arrangement, accumulation of mitochondria, and disordered muscle fibers and sarcomeres according to electron microscopy. The levels of the expression of mRNAs and proteins in myocardial tissue of the SXSMD group were decreased compared with those in the MIRI group. The serum levels of IL-6 and TNF-α were decreased. SXSMD treatment can inhibit the inflammatory response and downregulate the TLR4/NF-κB pathway in cardiomyocytes.

Conclusion: SXSMD protects the rats from myocardial ischemia-reperfusion injury in the MIRI model by downregulating the TLR4/NF-κB pathway to inhibit inflammation.

Background

MIRI.

Myocardial ischemia-reperfusion injury (MIRI) is caused by atherosclerotic depositions on the inner wall of the coronary arteries that cause stenosis or occlusion of the arterial lumen to restrict the blood and oxygen supply of myocardial cells. If the ischemic event is prolonged or repeated with or without reperfusion, local tissue ischemia or irreversible tissue damage are induced and eventually will lead to myocardial infarction or even sudden death [1]. These events are clinically manifested as coronary heart disease (CHD). CHD predominantly occurs in people over 40 years of age, and the incidence rate in men is higher than that in women. A global report in 2020 indicated that more than 54 million deaths are due to the diseases, and 32% (approximately 17 million) of deaths can be attributed to cardiovascular diseases (coronary heart disease and cerebrovascular disease) [2].The mechanism of MIRI and its management have been extensively investigated; however, additional studies are needed. Known mechanisms of MIRI are intracellular calcium overload, oxidative stress, inflammation, apoptosis, endoplasmic reticulum stress, mitochondrial dysfunction, myocardial metabolic dysfunction, autophagy,

etc. The inflammatory response is linked to all stages of the MIRI process, and an increase in neutrophils plays an important role in MIRI. Neutrophils can be activated by related cytokines, complement, and various proinflammatory mediators released by cardiomyocytes: tumor necrosis factor (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), etc. Reactive oxygen species can induce the recruitment of neutrophils that release a number of inflammatory factors, which in turn lead to an increase in the area of myocardial infarction to induce damage and dysfunction of coronary vascular endothelial cells. Various cytokines or inflammatory mediators can act alone or synergistically to cause irreversible damage to the myocardium [3]. At present, the methods of CHD treatment include drug therapy, interventional surgery, and other types of management, which can prevent platelet aggregation, stabilize atherosclerotic plaque, and improve blood supply to the heart. However, clinical applications of these methods are limited due to antiplatelet drug resistance, toxic side effects, poor myocardial tissue perfusion, and restenosis after stenting [4]. Traditional Chinese medicine can provide unique solutions to these problems.

Shuxin Shengmai Dan.

At present, the efficacy of traditional Chinese medicine in treatment of various diseases is increasingly recognized internationally [5]. Relevant reports on the treatment of MIRI with Chinese herbal compound prescriptions have determined that the effect of the Danshen-Gegen decoction is mediated by the PKCε/mKATP pathway to protect rats from myocardial injury caused by isoproterenol [1]. The Huoxue Wentong formula improves myocardial infarction in rats by inhibiting oxidation and phosphorylation of calmodulin-dependent protein kinase II [6]. Shuxin Shengmai Dan (SXSMD) is composed of 13 medicinal materials. Some monomer components of Chinese medicinal materials in the formulation (danshensu, ginsenosides Rb3 and Rg1, ginkgolide B, etc.) have anti-inflammatory, antioxidant, antibacterial, and anticancer effects. Danshensu has a protective effect on MIRI and inhibits H9c2 cardiomyocyte apoptosis via Akt and ERK1/2 phosphorylation [7]. Danshensu improves MIRI by activating the Sirt1/FoxO1/Rab7 signaling pathway [8]. Ginsenoside Rb3 is the active ingredient of *Radix panacis quinquefolii* that can protect cardiomyocytes from ischemia-reperfusion injury by activating the PERK/Nrf2/HMOX1 antioxidant signaling pathway [9]. Ginsenoside Rg1 binds to RhoA, downregulates the activity of the RhoA signaling pathway, and improves MIRI in rats by inhibiting myocardial apoptosis and regulating energy metabolism [10]. *Ginkgo biloba* extract (EGb761) protects against MIRI by inhibiting the mitochondrial caspase-dependent pathway [11]. Ginkgolide B protects the heart from MIRI by regulating the A20-NF-κB pathway [12]. SXSMD has a beneficial effect in the treatment of angina pectoris and myocardial ischemia, however, the mechanisms of protection of the heart from MIRI remain unclear and require investigation.

TLR4/NF-κB signaling pathway.

The Toll-like receptor (TLR) signaling pathway can induce the production of inflammatory factors [13]. When tissues are damaged, TLR4, a member of the TLR family, is activated and interacts with the TIR domain adapter molecules MyD88 and TRIAP/MAL, which in turn stimulate MyD88 interaction with IRAK

protein kinases. Then, IRAK4 and IRAK1 are sequentially phosphorylated; IRAK1 is phosphorylated and dissociates from MyD88 to interact with and activate TRAF6 (alternatively, TRAF6 can be activated by TRIF). Then, TRAF6 activates TAK1, and TAK1 activates IKK leading to the activation of NF-κB-dependent nuclear transcription. NF-κB can induce the transcription of many proinflammatory factors and chemokines, such as IL-1, IL-6, and TNF-α. Therefore, protection against MIRI is mediated by the TLR4/NF-κB signaling pathway, which has been extensively investigated in a number of studies. Biochanin A can reduce the levels of IL-1β, IL-18, IL-6, and TNF-α in the serum, inhibit the expression of related proteins TLR4, MyD88, NLRP3, ASC, and caspase-1, and inhibit the phosphorylation of IkB-α and NF-κB to suppress inflammation through the TLR4/NF-κB signaling pathway and thus protect the myocardium [14]. Luteolin protects cardiomyocytes from ischemia-reperfusion injury via the TLR4/NF-κB/NLRP3 pathway [15]. Salidroside inhibits inflammation and protects rat cardiomyocytes by downregulating the TLR4/NF-κB pathway [16]. Valsartan preconditioning protects the myocardium from ischemia-reperfusion injury via the TLR4/NF-κB signaling pathway [17]. This study initially investigated the mitigation of MIRI by Shuxin Shengmai Dan and potential molecular and biological mechanism by comparison with Danshen injection (DSI).

Methods

Preparation of SXSMD.

SXSMD is composed of 13 medicinal materials, including: *Radix panacis quinquefolii, Radix salvia miltiorrhiza, Ganoderma, Carthami flos, Radix notoginseng, Chuanxiong, Crataegi fructus, Ginkgo folium, Trionycis carapax, Dendrobii caulis, Schisandrae chinese fructus, Cassiae semen*, and *Nelumbinis folium.* The batch numbers of the thirteen Chinese herbal formula granules were 20031493, 19096434, 19116164, 19106094, 19076054, 20026034, 19076224, 19010813, 19037364, 19126784, 19116754, 19066234, and 19037454, respectively (Supplementary Fig. 1). Most of the active ingredients of traditional Chinese medicine are poorly defined, thus, thin-layer chromatography (TLC) is used to qualitatively identify the dispensed granules of traditional Chinese medicine. The TLC identification of the formula granules is performed according to the standard of Chinese Pharmacopoeia (version 2015). All data on herbal materials, dispensed granules, and quality control of SXSMD were supplied by Tianjiang Pharmaceutical Co., Ltd (China).

Experimental animals.

All animal experiments were approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine (2020239). Male Sprague-Dawley (SD) rats (17–20 weeks of age) were purchased from Liaoning Changsheng biotechnology Co., Ltd. (China) and reared in a standard experimental animal laboratory with ad libitum access to food and water.

Animal grouping.

The Control, Sham, MIRI, MIRI + DSI, and MIRI + SXSMD groups were set up according to the two-factor model and treatment, each group included 8 animals.

Drug administration.

The daily dosage of rat treatment was calculated according to the conversion factor of the equivalent dose of the drug between rats and humans. The equivalent dose of SXSMD is 3.38 g/kg, which is equivalent to adult clinical medication. To determine whether the protective effect of SXSMD on MIRI is dose-dependent, various doses were used: 3.38 g/kg (L), 6.76 g/kg (M), and 13.52 g/kg (H). SXSMD was used as granules dissolved in distilled water. In the experiment, an equal volume of gavage (15 mL/kg) was used. Rats in each group were gavaged once a day with the corresponding doses for 15 days. The positive control group was treated with DSI purchased from Zhengda Qingchunbao Pharmaceutical Co., Ltd (China). According to calculations, the daily dose of DSI in rats was 1.8 mL/kg diluted with normal saline to 15 mL/kg and administered continuously for 15 days. The control group was treated with normal saline at a dose of 15 mL/kg for 15 days.

Electrocardiogram (ECG) and hemodynamic monitoring.

Before the operation, electrocardiogram of the rats was recorder, and animals with abnormal electrocardiogram were excluded. An 8 gauge needle was inserted subcutaneously in a rat limb and the electrodes were connected; NEG (white) was connected to the right upper limb, GROUND (green) was connected to the right lower limb, and POS (black) was connected to the left lower limb (Fig. 2a). Carotid artery was catheterized with a catheter prefilled with heparin; an incision along the midline of the neck was performed and the subcutaneous muscle and blood vessel layers were separated using a blunt tool; a catheter was inserted into the left ventricle and fixed in place. The Power Lab data acquisition module was used to analyze and monitor the changes in left ventricular hemodynamics throughout the process: rat electrocardiogram (ECG), left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), maximum rate of increase in left ventricular pressure (+ dp/dtmax), and maximum rate of decrease in left ventricular pressure (-dp/dtmax).

Rat models.

MIRI model induction: Rats were anesthetized by intraperitoneal injection of 0.9% pentobarbital sodium (45 mg/kg). Electrocardiogram was monitored, and rats were intubated with a ventilator. The chest skin was longitudinally cut, and tissue and muscle layers were separated using a blunt tool; the pleura was punctured and the heart was exposed; a retractor was used to gently and quickly squeeze the heart out, and the left atrial appendage and the pulmonary artery cone were located. The left anterior descending (LAD) coronary artery is located at the junction. The coronary arteries were ligated with a 6 – 0 suture by tying a long slip knot, and the heart was returned into the chest. The changes in ECG were observed. The ST segment level was significantly elevated and arched upwards. After 30 min, the suture was removed to restore blood flow at the ligation site and the heart was reperfused for 120 min. The coronary arteries of rats in the Sham group were threaded in a similar manner without ligation. In the Control group, only

ECG and hemodynamic changes were monitored (Fig. 1). MI model induction: Rats were intragastrically administered for 15 days. Isoproterenol (1 mg/kg) was injected intraperitoneally on day 13, and the heart was acquired on day 15.

Hematoxylin-eosin staining (H&E).

The pathological changes in the heart structure and heart tissue were investigated. Formaldehyde-fixed heart tissue specimens were embedded in paraffin, and 5 μm sections were prepared according to the standard operating procedures and stained with H&E. The images were acquired by Leica ICC50 HD (Leica,Germany). Analysis of each specimen was performed using an average of 3 fields of view.

Mitochondrial ultrastructure detection.

The sample of left ventricular infarct boundary area of the heart from each group (1 mm*1 mm*2 mm) was selected, fixed in 4% glutaraldehyde for 2 h or longer, and fixed in 1% osmic acid for 1 ~ 2 h; the samples were washed with PBS for 5 min (3 times) and subjected to dehydration, infiltration, embedding, and ultrathin sectioning. Transmission electron microscope (TEM) (Hitachi, Japan) was used to detect the ultrastructural changes of the heart tissue. Analysis of each specimen was performed using an average of 3 fields of view.

Quantitative RT-PCR analysis.

Total RNA was extracted from frozen myocardial tissue samples (50 mg) using the Trizol method, and a Nanodrop 2000c spectrophotometer (Thermo, Germany) was used to detect the A260/280 values of the resulting RNA solution to determine the total RNA concentration.

A NovoScript Plus all-in-one first strand cDNA synthesis supermix kit (Novoprotein, China) was used to synthesize cDNA. The reaction system (20 μl) included template RNA, 2 × NovoScript Plus first strand cDNA synthesis supermix, gDNA eraser, and RNase-free water; the samples were gently mixed, centrifuged, and incubated.

A NovoStart SYBR qPCR supermix plus kit (Novoprotein, China) was used for the assay. The samples contained 2 × NovoStart SYBR qPCR supermix plus, upstream and downstream primers, template, and RNase-free water. The samples in PCR (total reaction volume of 20 μl) were processed by the PCR amplification program: 95ºC, 1 min; 95ºC 20 s, 60ºC 20 s, and 72ºC 30 s for 40 cycles. The program is based on the dissolution curve starting at 60ºC and ending at 95ºC with 1 min holding time. After the reaction, the calculations are performed based on the measured dissolution and amplification curves and the Cq values. The $2^{-\Delta\Delta t}$ method was used to compare the Cq values of the target gene and the internal reference gene GAPDH to calculate the relative expression levels of target mRNAs. The primer sequences were synthesized by Bioengineering Co., Ltd (China).

Serum IL-6/TNF-α ELISA.

Blood was collected from the abdominal aorta of the rats and incubated for 30 min at 4 °C. The separated serum was placed in a tube and tested using a rat IL-6/TNF-α ELISA kit (JYMBIO, China). OD at 450 nm was detected by a Multiskan GO microplate reader (Thermo, Germany). The standard curve was constructed based on the OD values of the standards and the standard curve equations (IL-6: y = 13.595x + 4.2974; TNF-α: y = 75.695x-14.778). The concentrations of IL-6 and TNF-α in each sample were calculated based on the corresponding equations.

Western blot analysis.

The total protein of the myocardial tissue samples was extracted, and a BCA protein assay kit (Biyuntian, China) was used to determine protein concentration according to a standard curve (y = 0.737x + 0.0989). Stacking and separating gels were prepared; the samples were resolved by electrophoresis and transferred onto a membrane. The PVDF membrane (Millipore, USA) was blocked using 5% nonfat milk and incubated on a circular shaker at room temperature. The following primary antibodies were used: HMGB1 (Cell Signaling Technology, USA), TLR4 (ProteinTech, USA), MyD88 (Cell Signaling Technology, USA), NF-κB p65 (Abcam, UK), and GAPDH (Bioss, China). Antibodies were diluted 1:1,000 and incubated at 4 °C overnight. After washing with TBS and TBST, secondary horseradish peroxidase-labeled goat anti-rabbit (IgG) antibodies at 1:1,000 dilution was incubated on a shaker at 4 °C, and the membrane was washed again. An ultrasensitive ECL chemiluminescence kit (Biyuntian, China) and a FUSION FX Spectra system (VILBER, China) were used to develop and detect the target signals and quantify the protein expression.

Statistical analysis.

All analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation (SD). One-way analysis of variance (one-way ANOVA) was used to analyze the normally distributed data. The data that were not normally distributed were analyzed by nonparametric tests, including Friedman or Kruskal-Wallis tests. Variance was considered homogeneous if the P value of the homogeneity of variance test (F) was > 0.1. The test result at $P < 0.05$ were considered different, and the results at $P < 0.01$ were considered significantly different corresponding to statistically significant differences.

Results

Development of the rat MIRI model.

A rat MIRI model was established to determine the changes in cardiac electrophysiologal parameters and evaluate the protective effect of SXSMD on the heart. Coronary artery ligation was used to establish the rat MIRI model. Changes in electrocardiogram (Supplementary Fig. 2a) clearly indicate that within 30 min after ligation, the ST segment is significantly elevated and arched upwards demonstrating that the heart has insufficient blood and oxygen supply. The blockade indicates that myocardial ischemia is successful.

After 30 min ligation, the blood supply was restored, and the ST segment was gradually decreased (Supplementary Fig. 2a) indicating that the rat MIRI model was successfully established.

Effect of SXSMD on myocardial electrophysiology in the rat MIRI model.

ECG before operation in each group produced similar waveforms (Supplementary Fig. 2b). After 20 min of LAD ligation-induced ischemia, the changes in the ST segment in each group indicated the presence of myocardial ischemia; however, the degree of the changes was slightly different. The ST segment of the MIRI + SXSMD-H group was significantly decreased at the same time point. The changes in the ST segment at two time points IV and V after restoration of the blood supply were consistent with myocardial ischemia and reperfusion. The ECG waveform changes in the MIRI + SXSMD-L group after restoration of the blood supply for 30 min indicated that the heart function is restored. The ST segment waveform changes in the Sham group indicated that the operation caused some damage to the heart. In summary, SXSMD-L can improve cardiac function after myocardial ischemia-reperfusion injury in rats, and SXSMD-H can improve myocardial blood supply to a certain extent during myocardial ischemia.

Effect of SXSMD on cardiac hemodynamics in the rat MIRI model.

The changes in rat heart hemodynamics were monitored to assess rat heart function and evaluate the protective effect of SXSMD. After coronary ligation in the animal MIRI model (Fig. 2c, g), LVSP was decreased, LVEDP was increased, and the pressure difference was increased in the MIRI group indicating the presence of myocardial ischemia; +/-dp/dt_{max} was decreased indicating a decrease in cardiac function. The +/-dp/dt_{max} value in the MIRI + SXSMD-M group was significantly different from that of the MIRI group indicating that SXSMD can improve heart function. During reperfusion in the animal MIRI model (Fig. 2d ~ f), LVSP and LVEDP in the MIRI group were decreased. There were no differences in blood pressure between the groups; however, LVEDP in each treatment group had a downward trend. Reperfusion improved heart function in the animal MIRI model (Fig. 2h ~ j) in all treatment groups. After 30 min of reperfusion, cardiac function was improved in the MIRI + DSI group; after 60 min of reperfusion, cardiac function was significantly improved in the MIRI + SXSMD-M group. Thus, SXSMD has a significant protective effect in the rat MIRI model 60 min after ischemia-reperfusion (IR), and the effect of SXSMD is better than that of DSI; the protective effect of DSI can be detected 30 min after IR in the rat MIRI model.

Effect of SXSMD on the morphology and structure of the myocardial tissue in the rat MIRI model.

The myocardial tissue morphology (400x magnification) was assessed by H&E staining, and the protective effect of SXSMD was evaluated in the rat MIRI model. The results indicate that cardiomyocytes in the Control group were normally arranged and uniform in size with normal cell morphology (Fig. 3a). Unlike the Control group, the Sham group was characterized by more regular arrangement of cardiomyocytes, absence of inflammatory infiltration and muscle fiber decomposition, and normal cell morphology (Fig. 3b). In the MIRI group, cardiomyocytes were arranged irregularly with considerable inflammatory infiltration and decomposition and dissolution of muscle fibers; cell morphology was

variable with swelling, deformation, and abnormal nuclei (Fig. 3c). In the DSI group, irregularity of myocardial cell arrangement was reduced compared with that in the MIRI group; inflammatory infiltration and a few myocardial fiber breaks were detected; cell morphology was relatively intact, and nuclear morphology and distribution were relatively normal (Fig. 3d). In the SXSMD group, the irregularity of myocardial cell arrangement was reduced compared with that in the MIRI group; inflammatory infiltration and muscle fiber rupture and dissolution were reduced; cell morphology was relatively intact, and nuclear morphology and distribution were relatively normal (Fig. 3e ~ g). Therefore, SXSMD can improve the pathological changes in the MIRI myocardial tissue by inhibiting inflammation.

Effect of SXSMD on the ultrastructure of cardiomyocytes in the rat MIRI model.

Transmission electron microscopy (1,500x magnification) of ultrathin sections treated with glutaraldehyde and osmic acid was used to evaluate the protective effect of SXSMD on the myocardial ultrastructure in the rat MIRI model. The results showed that in the Control group, muscle fibers were tightly arranged, sarcomeres were orderly arranged, and mitochondria were normal (Fig. 3h). The muscle fibers and sarcomeres of the Sham group were normal; the number of mitochondria was increased compared with that in the Control group, and the mitochondrial cristae were slightly damaged and deformed (Fig. 3i). In the MIRI group, the arrangement of muscle fibers was disordered, the sarcomeres were irregular, and the mitochondria were swollen with cavitation and deformation. The irregular state was manifested as mitochondrial accumulation (Fig. 3j). The muscle fibers and sarcomeres in the DSI group were orderly arranged; the number of mitochondria was decreased compared with that in the MIRI group, and the mitochondria were swollen and cavitated (Fig. 3k). The arrangement of muscle fibers and sarcomeres in the SXSMD group was more orderly than that in the model group, and the relative number of mitochondria was reduced. Compared with two other groups, the arrangement of muscle fibers and mitochondria in the SXSMD-L group was more orderly, and the mitochondria had more regular morphology and fewer cavities (Fig. 3l ~ n). Therefore, SXSMDS can significantly improve the myocardial ultrastructure in the rat MIRI model.

Effect of SXSMD on the expression levels of myocardial genes in the rat MIRI model.

Inflammation is regulated by multiple pathways: Jak/Stat signaling, NF-κB signaling, TLRs (Toll-like receptors), BCR (B cell receptor signaling), TCR (T cell receptor signaling), etc. The TLR4/NF-κB signaling pathway plays an important role in the regulation of MIRI inflammation. The expression levels of the genes of the TLR4/NF-κB pathway were assayed to determine the mechanism of SXSMD regulation of the inflammatory response after myocardial IR.

The results of real-time PCR showed that relative expression of HMGB1 mRNA in the myocardial tissue of the MIRI group was significantly increased compared with that in the Control group. The relative expression of HMGB1 mRNA in the DSI and SXSMD groups was significantly decreased compared with that in the MIRI group (Fig. 4a). Thus, SXSMD can downregulate the expression of the HMGB1 gene in rat myocardial tissue, and the effect is better than that of the Danshen injection.

The relative expression of TLR4, MyD88, and NF-κB mRNA in the myocardial tissue of the MIRI group was significantly increased compared with that in the Control group. The relative expression of TLR4 mRNA in the DSI and SXSMD groups was significantly decreased compared with that in the MIRI group; a decrease in the SXSMD-H group was the highest. The relative expression of MyD88 mRNA in the DSI and SXSMD groups was significantly decreased. The relative expression of NF-κB mRNA in the DSI and SXSMD groups was significantly decreased (Fig. 4b ~ d). SXSMD can downregulate the expression of the TLR4, MyD88, and NF-κB genes in rat myocardial tissue. There were no significant differences in downregulation between the DSI and SXSMD groups indicating that the two drugs induce similar downregulation of NF-κB gene expression.

The relative expression of IL-6 mRNA in the myocardial tissue of the MIRI group was significantly increased compared with that in the Control group. The relative expression of IL-6 mRNA in the DSI and SXSMD groups was significantly decreased compared with that in the MIRI group. The relative expression of TNF-α mRNA in the DSI and SXSMD groups was significantly decreased (Fig. 4e ~ f). Thus, Danshen injection and SXSMD can induce the downregulation of the expression of the IL-6 and TNF-α genes in rat myocardial tissue.

Therefore, SXSMD can reduce the expression of the pathway-related genes, including HMGB1, TLR4, MyD88, and NF-κB, and the downstream IL-6 and TNF-α mRNA in rat cardiomyocytes to inhibit the inflammatory response in the myocardial tissue in the rat MIRI model.

Effect of SXSMD on the changes in IL-6 and TNF-α in the serum in the rat MIRI model.

To determine the cardioprotective effect of SXSMD in rats and associated changes in the serum, ELISA was used to determine the levels of IL-6 and TNF-α. The IL-6 content in the MIRI group was significantly increased compared with that the Control group; the IL-6 levels in all groups were reduced to a variable degree compared with that in the MIRI group; the reduction was detectable in the DSI group and was significant at various doses in the SXSMD group (Fig. 5a). The TNF-α content in the MIRI group was significantly increased compared with that in the Control group; the TNF-α levels in all groups were reduced to variable degree compared with that in the MIRI group, and this reduction was detectable at various treatment doses in the DSI and SXSMD groups (Fig. 5b). Thus, SXSMD can inhibit the release of inflammatory factors and has a protective effect on the heart of rats.

Effect of SXSMD on the changes in pathway-related proteins in cardiomyocytes in the rat MIRI model.

Western blot was used to detect the expression levels of pathway-related proteins to determine the mechanism of protection of rat heart by SXSMD. Analysis of the protein bands indicated significant changes in the TLR4 protein levels. The signal in the MIRI group was significantly higher than that in the Control group. The level of TLR4 was reduced in the DSI and SXSMD groups. SXSMD-M and SXSMD-H demonstrated the highest reduction indicating that SXSMD can reduce the expression of TLR4 protein. The MyD88 protein signal in the MIRI group was significantly higher, and the signal in the SXSMD-H group was significantly lower indicating that SXSMD can reduce the expression of MyD88 protein.

Expression of NF-κB protein in the MIRI group was significantly higher than that in the Control group, and the levels in the SXSMD-H group were lower indicating that SXSMD can reduce the expression of NF-κB protein (Fig. 5c). Thus, SXSMD can reduce the expression of pathway-related TLR4, MyD88, and NF-κB proteins in rat cardiomyocytes. Therefore, SXSMD can alleviate the inflammation-induced damage to rat myocardium after IR by regulating the TLR4/NF-κB pathway.

Effect of SXSMD on myocardial tissue morphology and ultrastructure in the rat MI model.

To determine whether SXSMD has a protective effect in the MIRI model of myocardial ischemia, another MI model was developed and the morphology and ultrastructure of myocardial cells was assessed. The results of the H&E staining indicated that the MI group was characterized by irregular arrangement of cardiomyocytes, large area of inflammatory infiltration, muscle fiber decomposition and dissolution, swelling and deformation of the cells, and abnormal nuclear morphology compared with those in the Control group, (Supplementary Fig. 3a ~ b).The irregularity of myocardial cell arrangement was reduced, inflammatory infiltration was reduced, muscle fiber rupture and dissolution was relatively reduced, cell morphology was relatively complete, and nuclear morphology and distribution were relatively normal in the SXSMD-M/H groups compared with those in the MI group (Supplementary Fig. 3d ~ f). The SXSMD-L group performed poorly according to histological analysis. The results of TEM in the MI group indicated that the arrangement of muscle fibers was disordered, the sarcomere was expanded, and numerous mitochondria accumulated between the muscle fibers and had cavitation, swelling, and deformation compared with those in the Control group (Supplementary Fig. 3h); the muscle fibers and sarcomeres in the DSI group were orderly arranged, the number of mitochondria was decreased, and cavitation was less common compared with those in the MI group (Supplementary Fig. 3i); the muscle fibers and sarcomeres in the SXSMD-L/H group were arranged more orderly than those in the model group; the relative number of mitochondria was reduced, and the mitochondria had lower cavitation (Supplementary Fig. 3j ~ l). Thus, SXSMD can protect the myocardium in the stage of myocardial ischemia in the rat MI model and can improve the morphology and ultrastructure of the myocardium.

Discussion

This experimental study mainly investigated the protective effect of SXSMD in the rat MIRI model. According to the literature, rats or mice are usually used to establish the MIRI models. The blood flow to the heart is interrupted by ligating LAD, and then the blood supply is restored after a certain time. Zhaoqi H. et al. used c57BL6/J mice to establish a MIRI model to study the protective effect of microRNA-374a targeting the MAPK6 pathway [18]. Xuehui Z. use ICR mice to establish a MIRI model to study inhibition of the JNK and p38 MAPK by *Rosa rugosa* flavonoids to reduce myocardial ischemia-reperfusion injury [19]. Yang D. et al. developed an SD rat MIRI model by ligating LAD for 1 h and subsequent reperfusion for 24 h [20]. A model involving a surgical intervention is expected to cause considerable damage to the animals. Additionally, differences in ligation time of LAD may cause heart failure and death [21]. Considering the difficulty of surgery and animal mortality, this study used rats to establish the MIRI model. Ligation of LAD in rats can accurately block the blood supply to the local myocardial tissue of the

rat heart resulting in ischemia and hypoxia of the myocardial tissue. This model can simulate the onset of myocardial ischemia or angina pectoris in clinical patients. After 30 min of ligation, the ligating suture was removed to restore the blood supply, which corresponds to the effects of a medication or rest. MIRI usually occurs a few minutes or even hours after the blood flow is restored; hence, the changes in rats were assessed within two hours after the ischemic event.

The study by Yang. et al. used a PowerLab system to record and analyze the changes in left ventricular pressure to assess the protective effect of asiatic acid on cardiac function. Their results showed that LV dp/dtmax and dp/dtmin in the MI/R group were significantly lower than those in the Sham group. After asiatic acid treatment, LV dp/dtmax and dp/dtmin were significantly higher than those in the MI/R group [20]. Our study used a PowerLab 8/35 physiological recorder and Labchart 7.0 software to monitor the hemodynamics of the rat heart and cardiac electrophysiology and to analyze and process the data. The rat MIRI model after SXSMD pretreatment can be effective. The +/-dp/dtmax in the MIRI group was significantly lower than that in the Control group, and the +/-dp/dtmax was significantly increased after SXSMD pretreatment indicating that the protective effect on the heart is similar in the case of SXSMD and asiatic acid. After 15 days of pretreatment, the waveform changes can be detected according to the data of ECG suggesting functional recovery of the rat heart.

Rui Z.et al. studied the structure of cardiomyocytes by H&E staining and TEM to investigate the protective effect of ginkgolide B (GB) on MIRI heart. After ischemia and reperfusion, the results of H&E staining demonstrated irregular arrangement of myocardial cells, tissue necrosis, and substantial levels of myocardial interstitium. Detection of inflammatory cells was associated with fibrotic scar formation. GB can significantly improve histological damage; the effect was manifested by regular arrangement of muscle cells and reduced inflammatory infiltration. TEM showed that myocardial fibers were disrupted and mitochondria were swollen and degenerated in the I/R group. In the 8 mg/kg GB group, some mitochondrial cristae were damaged and lost. In the 16 mg/kg GB group, the damaged mitochondria showed mild cristae loss and swelling. In the 32 mg/kg GB group, there was only a small mitochondrial swelling [12]. In our study, H&E staining of the cardiac samples of rats showed that SXSMD reduced inflammatory infiltration and maintained normal cell morphology. The ultrastructure of cardiomyocytes was assessed by TEM; the data indicated that SXSMD can maintain the order of myofilament fibers and sarcomeres, inhibit mitochondrial accumulation, and maintain normal shape of the mitochondria.

DSI was selected as a positive control in this experiment. SXSMD contains traditional Chinese medicine Radix salvia miltiorrhiza. A number of studies have shown that Radix salvia miltiorrhiza and its active ingredients can protect the myocardium during MIRI. Danshensu can inhibit apoptosis of H9c2 cardiomyocyte via Akt and ERK1/2 phosphorylation and has a protective effect on MIRI of the heart [7]. Danshensu can activate the Sirt1/FoxO1/Rab7 signaling pathway and enhance the antioxidant defense system to improve apoptosis and promote endogenous reactive oxygen species-mediated myocardial ischemia reperfusion injury [8]. DSI is one of the traditional Chinese medications derived from *Salvia miltiorrhiza*; hence, Danshen injection was selected as the positive control drug.

SXSMD has a significant mitigating effect on the inflammatory response in MIRI. To determine the mechanism of drug action, the classic inflammation-related TLR4/NF-κB signaling pathway was assayed. Bai Y. et al. determined that biochanin A attenuates myocardial ischemia-reperfusion injury via the TLR4/NF-kappaB/NLRP3 signaling pathway [14]. HuaSheng D. et al. determined that the HMGB1-TLR4 axis participates in myocardial ischemia-reperfusion injury by regulating myocardial cell apoptosis [22]. Quercetin is an active ingredient of the traditional herbal medicines Radix notoginseng, Crataegi fructus, and Ginkgo folium. Liya D. et al. demonstrated that quercetin reduces myocardial ischemia-reperfusion injury by downregulating the HMGB1-TLR4-NF-κB signaling pathway [23]. Therefore, we hypothesized that quercetin is one of the possible effective components of SXSMD involved in the protection of the heart by the TLR4/NF-κB pathway. Danshensu is one of the ingredients of traditional Chinese medicine Radix salvia miltiorrhiza. A study by Xu, H. et al. showed that danshensu can protect the nerves from cerebral ischemia-reperfusion in rats via cooperative regulation of the TLR4/NF-κB pathway, and these effects are associated with the anti-inflammatory and antioxidant pathways [24]. Therefore, danshensu may be one of the active components of SXSMD that inhibits inflammatory response and protects against MIRI. Ginsenoside Rg1 is one of the active ingredients of Radix panacis quinquefolii and Radix notoginseng. A study of Luo, M. et al. on myocardial dysfunction (SIMD) caused by sepsis showed that ginsenoside Rg1 can improve the cardiac function of mice, reduce lipopolysaccharide-induced apoptosis and inflammation, and restore impaired cardiac function. The compound may act by blocking the TLR4/NF-kappaB/NLRP3 pathway [25]. Thus, ginsenoside Rg1 may regulate the TLR4/NF-κB pathway to inhibit inflammation and protect cardiomyocytes from MIRI, and this compound is one of the potential active components of SXSMD. Studies of Hu Y. et al. and Li, X. et al. demonstrated that ginkgolide A (GA), ginkgolide B (GB), ginkgolide K (GK), and the mixture of diterpene lactones (GDL) from ginkgo can downregulate the TLR4/NF-kappaB signaling pathway to inhibit inflammation and reduce cerebral ischemic damage [26, 27]. GA, GB, GK, and GDL are the main components of traditional Chinese medicinal materials derived from *Ginkgo biloba*. Therefore, we suggest that GA, GB, GK, and GDL may be the active components of SXSMD. Our results indicate that SXSMD can reduce the expression of HMGB1, TLR4, MyD88, and NF-κB and the downstream levels of IL-6 and TNF-α mRNA and protein in myocardial tissue. Thus, possible active ingredients of the traditional Chinese medicine SXSMD with protective effects on the heart in the rat MIRI model include quercetin, danshensu, ginsenoside Rg1, GA, GB, GK, and GDL.

A rat MI model was developed using isoproterenol to determine whether tested drugs can protect the rat heart during ischemic events [1]. The drug absorbed after administration enters circulation and reaches the left ventricle to cause ventricular ischemia. This model is unstable due to problematic drug absorption; the success rate is not high, and the reproducibility is poor; thus, only myocardial sections and ultrastructure of cardiomyocytes in the MI model were used. The results of H&E staining and TEM indicate that the morphology and structure of cardiomyocytes in the rat MI model can be improved. Thus, SXSMD can effectively protect the heart during ischemic events. This result is consistent with the ECG phenotype (Fig. 2, d) of the SXSMD-H group in the MIRI model and demonstrates that SXSMD can protect the heart from myocardial ischemia and reperfusion.

This study initially investigated the mechanism of action of Chinese herbal formulation in protection of the heart from myocardial ischemia reperfusion injury and assessed the effect in the MIRI model of inflammation. SXSMD downregulates the TLR4/NF-κB pathway, inhibits inflammation, and protects the heart from ischemia-reperfusion. Additional studies are needed to determine whether SXSMD inhibits inflammation via other pathways or acts via other mechanisms, such as oxidative stress and apoptosis.

Conclusion

SXSMD can improve myocardial electrophysiology and cardiac function in the rat MIRI model. SXSMD can reduce the mRNA expression levels of the HMGB1, TLR4, MyD88, and NF-κB and downstream IL-6 and TNF-α genes of the key TLR4/NF-κB pathway thereby inhibiting the inflammatory response in the myocardial tissue. SXSMD can reduce the expression of pathway-related TLR4, MyD88, and NF-κB proteins in myocardial tissue and reduce the levels of IL-6 and TNF-α in the serum. Thus, SXSMD can significantly improve the morphological structure and ultrastructure of myocardial cells in rats by inhibiting inflammation.

Abbreviations

CHD: coronary heart disease; DSI: danshen injecta; ECG: electrocardiogram; H&E: hematoxylin-eosin staining; HMGB1: high mobility group protein B1; HPLC: high performance liquid chromatography; IL-1: interleukin-1; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; IR: ischemia-reperfusion; LAD: left anterior descending; LVEDP: left ventricular end diastolic pressure; LVSP: left ventricular systolic pressure; MI: myocardial ischemia; MIRI: myocardial ischemia-reperfusion injury; MyD88: myeloiddifferentiationfactor88; NF-κB: nuclear factor kappa-B; SXSMD: Shuxin Shengmai Dan; TEM: transmission electron microscope; TLC: thin-layer chromatography; TLR4: toll-like receptor 4; TNF-α: tumor necrosis factor-α.

Declarations

COMPETING INTERESTS

The authors declare no interests of competing.

FUNDING

This research was supported by the Natural Science Foundation of Jilin Province (grant number: 20180201086YY to LS and 20200404085YY to WW)

AUTHORS' CONTRIBUTION

BMW, YCZ and LS designed the study. XYW, ZZH and XHP participated in the MIRI&MI Rats model work, and RT-PCR analysis. LS and YT participated in the WB and ELISA work. XWZ, HMY and WW conducted

the analysis. YCZ and XYW drafted manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

Thanks for the TEM support from Laboratory Teaching Center of Basic Medicine, Jilin University. Thanks for the SXSMD components quality control support from Tianjiang Pharmaceutical Co., Ltd (China).

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine (2020239).

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

References

1. Wong, S.M., et al., *Myocardial post-conditioning with Danshen-Gegen decoction protects against isoproterenol-induced myocardial injury via a PKCepsilon/mKATP-mediated pathway in rats.* Chin Med, 2011. **6**(1): p. 7.
2. Virani, S.S., et al., *Heart Disease and Stroke Statistics-2020 Update: A Report From the American Heart Association.* Circulation, 2020. **141**(9): p. e139-e596.
3. Kalogeris, T., et al., *Ischemia/Reperfusion.* Compr Physiol, 2016. **7**(1): p. 113-170.
4. Sun, M.Y., et al., *Effect and Safety of Guanxinning Tablet () for Stable Angina Pectoris Patients with Xin (Heart)-Blood Stagnation Syndrome: A Randomized, Multicenter, Placebo-Controlled Trial.* Chin J Integr Med, 2019. **25**(9): p. 684-690.
5. Liu, H. and Z.G. Ma, *Analysis on Situation of Traditional Chinese Medicine Development and Protection Strategies in China.* Chin J Integr Med, 2020.
6. Liu, T., Q. Wang, and K. Yao, *Huoxue Wentong Formula ameliorates myocardial infarction in rats through inhibiting CaMKII oxidation and phosphorylation.* Chin Med, 2020. **15**: p. 3.
7. Yin, Y., et al., *Cardioprotective effect of Danshensu against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation.* Eur J Pharmacol, 2013. **699**(1-3): p. 219-26.
8. Sun, D.W., Q. Gao, and X. Qi, *Danshensu Ameliorates Cardiac Ischaemia Reperfusion Injury through Activating Sirt1/FoxO1/Rab7 Signal Pathway.* Chin J Integr Med, 2020. **26**(4): p. 283-291.

9. Sun, J., et al., *Ginsenoside Rb3 protects cardiomyocytes against hypoxia/reoxygenation injury via activating the antioxidation signaling pathway of PERK/Nrf2/HMOX1.* Biomed Pharmacother, 2019. **109**: p. 254-261.
10. Li, L., et al., *Ginsenoside Rg1 Ameliorates Rat Myocardial Ischemia-Reperfusion Injury by Modulating Energy Metabolism Pathways.* Front Physiol, 2018. **9**: p. 78.
11. Shen, J., et al., *Ginkgo biloba extract (EGb761) inhibits mitochondria-dependent caspase pathway and prevents apoptosis in hypoxia-reoxygenated cardiomyocytes.* Chin Med, 2011. **6**: p. 8.
12. Zhang, R., et al., *Cardioprotection of Ginkgolide B on Myocardial Ischemia/Reperfusion-Induced Inflammatory Injury via Regulation of A20-NF-kappaB Pathway.* Front Immunol, 2018. **9**: p. 2844.
13. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-20.
14. Bai, Y., et al., *Biochanin A attenuates myocardial ischemia/reperfusion injury through the TLR4/NF-kappaB/NLRP3 signaling pathway.* Acta Cir Bras, 2019. **34**(11): p. e201901104.
15. Zhang, X., et al., *The protective effect of Luteolin on myocardial ischemia/reperfusion (I/R) injury through TLR4/NF-kappaB/NLRP3 inflammasome pathway.* Biomed Pharmacother, 2017. **91**: p. 1042-1052.
16. Zhu, L., et al., *The cardioprotective effect of salidroside against myocardial ischemia reperfusion injury in rats by inhibiting apoptosis and inflammation.* Apoptosis, 2015. **20**(11): p. 1433-43.
17. Yang, J., et al., *Valsartan preconditioning protects against myocardial ischemia-reperfusion injury through TLR4/NF-kappaB signaling pathway.* Mol Cell Biochem, 2009. **330**(1-2): p. 39-46.
18. Huang, Z.Q., et al., *MicroRNA-374a protects against myocardial ischemia-reperfusion injury in mice by targeting the MAPK6 pathway.* Life Sci, 2019. **232**: p. 116619.
19. Zhang, X., et al., *Rosa rugosa flavonoids alleviate myocardial ischemia reperfusion injury in mice by suppressing JNK and p38 MAPK.* Microcirculation, 2017. **24**(7).
20. Dai, Y., et al., *Asiatic acid protests against myocardial ischemia/reperfusion injury via modulation of glycometabolism in rat cardiomyocyte.* Drug Des Devel Ther, 2018. **12**: p. 3573-3582.
21. Gao, K., et al., *Qishen granules exerts cardioprotective effects on rats with heart failure via regulating fatty acid and glucose metabolism.* Chin Med, 2020. **15**: p. 21.
22. Ding, H.S., et al., *The HMGB1-TLR4 axis contributes to myocardial ischemia/reperfusion injury via regulation of cardiomyocyte apoptosis.* Gene, 2013. **527**(1): p. 389-93.
23. Dong, L.Y., et al., *Quercetin attenuates myocardial ischemia-reperfusion injury via downregulation of the HMGB1-TLR4-NF-kappaB signaling pathway.* Am J Transl Res, 2018. **10**(5): p. 1273-1283.
24. Xu, H., et al., *Synergistic neuroprotective effects of Danshensu and hydroxysafflor yellow A on cerebral ischemia-reperfusion injury in rats.* Oncotarget, 2017. **8**(70): p. 115434-115443.
25. Luo, M., et al., *Ginsenoside Rg1 attenuates cardiomyocyte apoptosis and inflammation via the TLR4/NF-kB/NLRP3 pathway.* J Cell Biochem, 2020. **121**(4): p. 2994-3004.

26. Hu, Y.Y., et al., *Ginkgolide B reduces neuronal cell apoptosis in the hemorrhagic rat brain: possible involvement of Toll-like receptor 4/nuclear factor-kappa B pathway.* J Ethnopharmacol, 2011. **137**(3): p. 1462-8.
27. Li, X., et al., *Ginkgo diterpene lactones inhibit cerebral ischemia/reperfusion induced inflammatory response in astrocytes via TLR4/NF-kappaB pathway in rats.* J Ethnopharmacol, 2020. **249**: p. 112365.

Figures

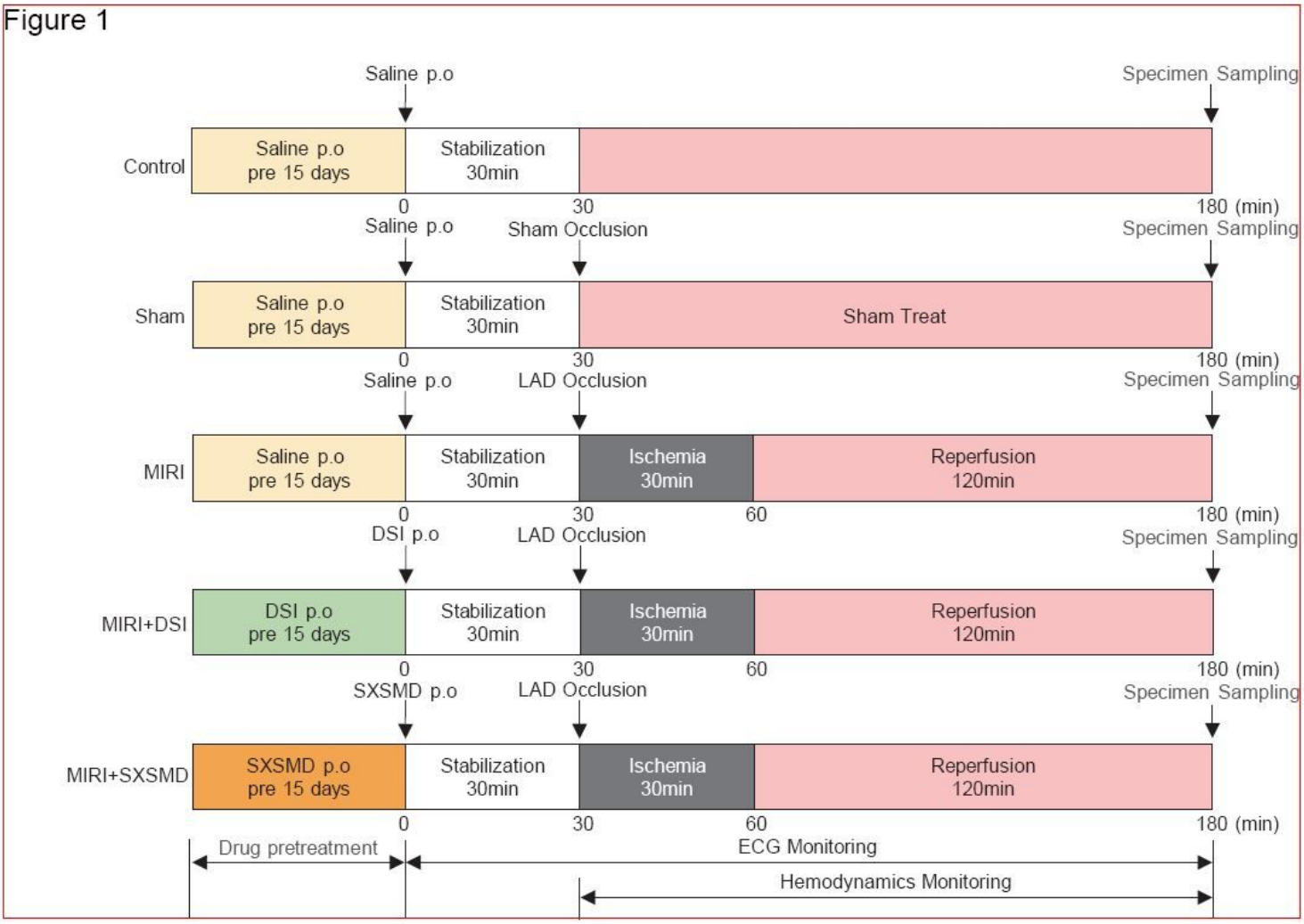

Figure 1

The experimental procedures of in vivo MIRI rat model.

Figure 2

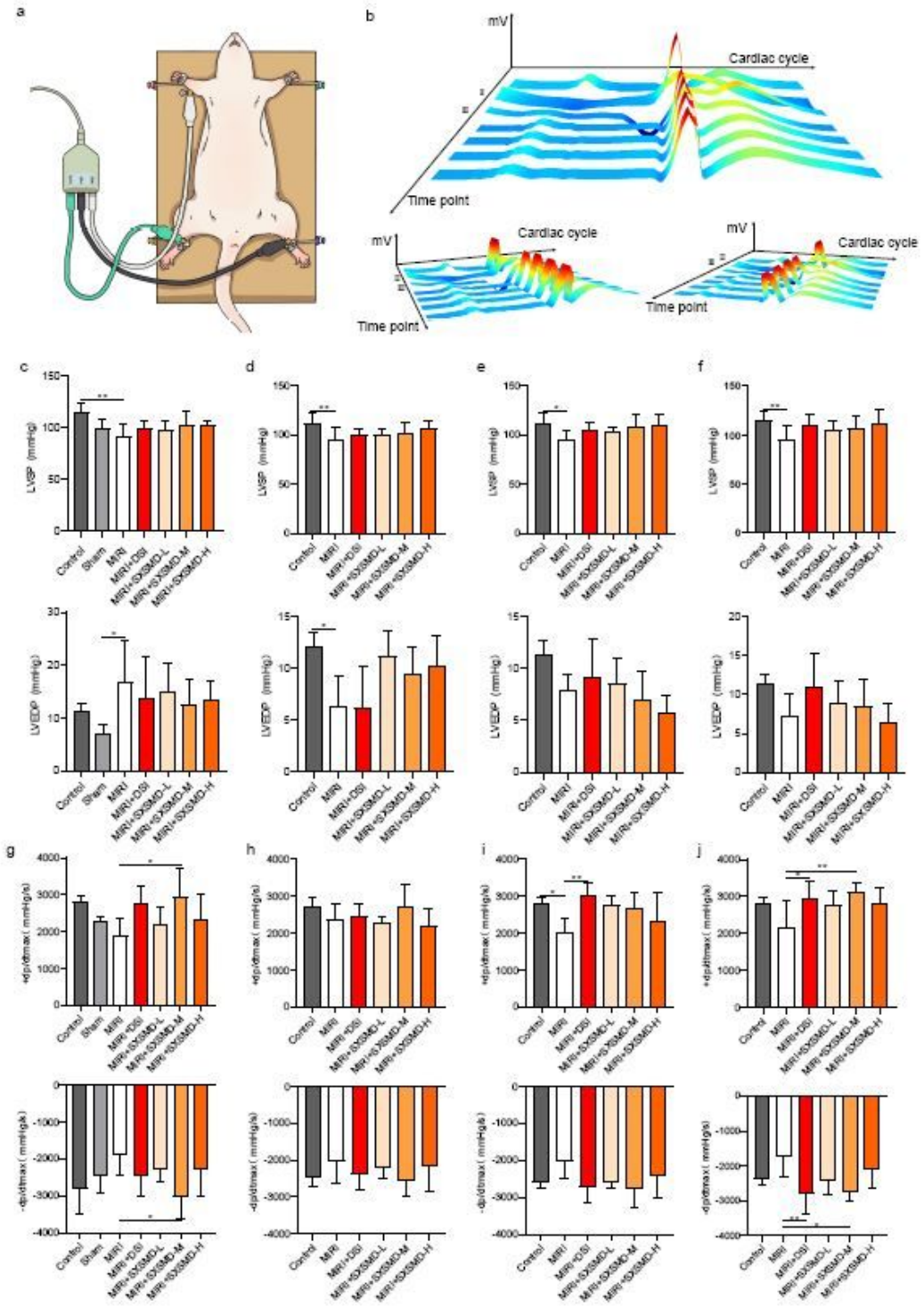

Figure 2

The effects of SXSMD on cardiac electrophysiology and hemodynamics in MIRI rats. (a) Electrodes for measuring rat ECG pattern diagram; (b, c) ECG waveform at seven time points. Time point II represents LAD Occlusion; Time point III represents ischemia after LAD Occlusion for 20 minutes. (c-f) Left ventricular pressure changes in rats, including the maximum value (systolic blood pressure) and

minimum value (diastolic blood pressure); (g-j) Left ventricular pressure changing rates in rats. *P<0.05, **P<0.01 compared to the MIRI group. Values are presented as mean□SD (n=8 per group)

Figure 3

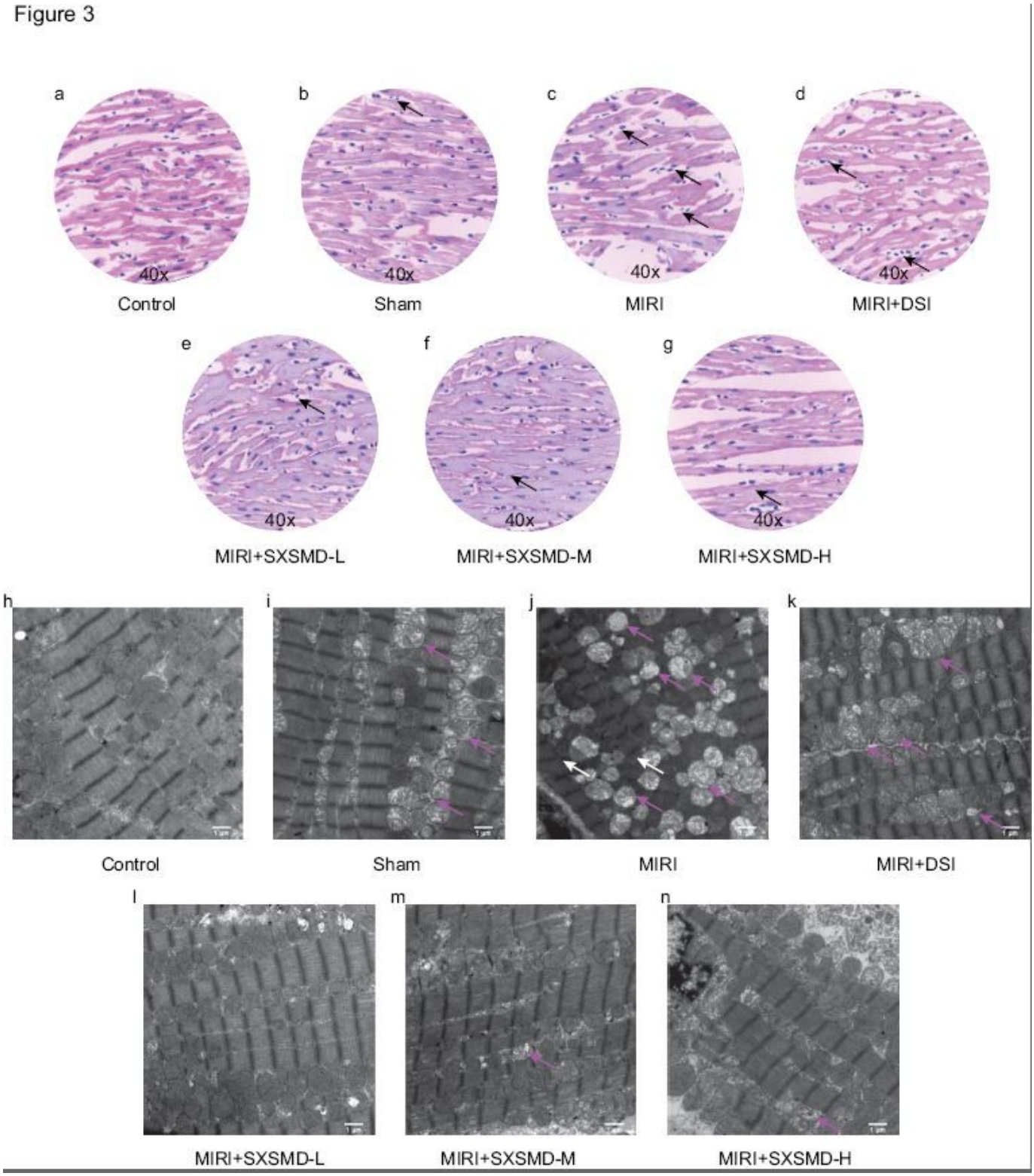

Figure 3

The effects of SXSMD on the morphology and ultrastructure of myocardial cells in MIRI rats. (a~g) H&E staining (40x) of the ischemia and reperfusion area. Black arrows indicate the typical inflammatory cells and inflammatory infiltrates in the myocardium. (h~n) TEM myocardial ultrastructure (1.5kx). Purple

arrows indicate typical abnormal mitochondria, white arrows represent disordered muscle fibers and irregular sarcomere. Scaling is supported as 1 μm.

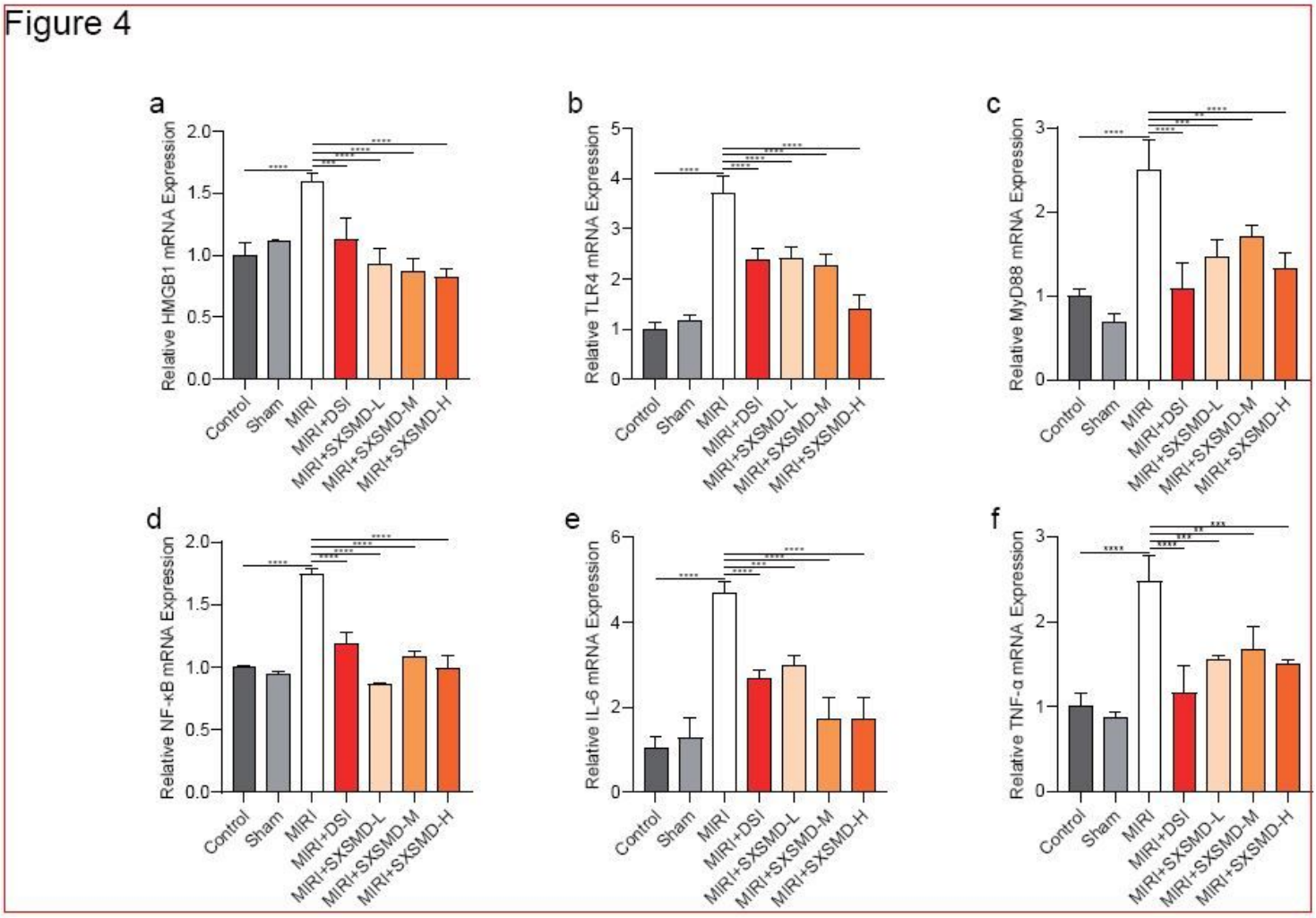

Figure 4

The effects of SXSMD on the mRNA expression level of myocardial cells in MIRI rats. **P<0.01, ***P<0.001, ****P<0.0001 comparing to the MIRI group. Values are presented as mean□SD (n=3 per group)

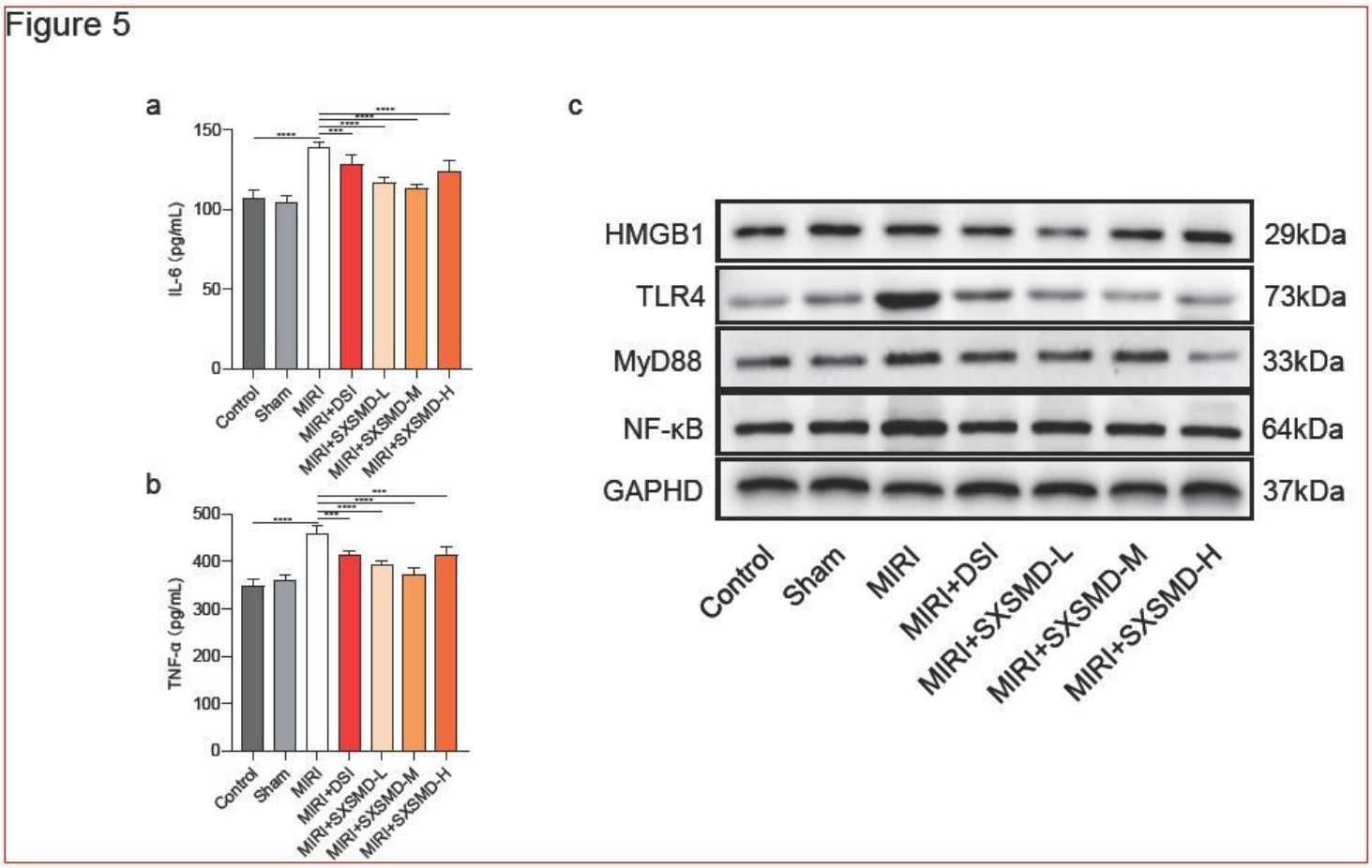

Figure 5

The effects of SXSMD on MIRI rat cardiomyocyte protein levels and serum inflammatory factors. ***P<0.001, ****P<0.0001 compared to the MIRI group. Values are presented as mean□SD (n=3 per group)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformation1.pdf