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PROTECTIVE EFFECT OF SANSHEN WEIXIN CAPSULA ON CARDIAC FUNCTION OF RATS WITH HEART FAILURE FOLLOWING MYOCARDIAL INFARCTION

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ABSTRACT

The aim of this study was to prepare and characterize puerarin nanosuspension to enhance the dissolution rate and oral bioavailability of this drug. Nanosuspension was prepared by the anti-solvent precipitation method. The particle size and zeta potential of nanosuspension were 195 nm (± 8 nm) and -12.5 mV (± 1.2 mV), respectively. The morphology of nanosuspension was found to be flaky-shaped by scanning electron microscopy (SEM) observation. The X-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC) analysis confirmed that the nanosuspension was in the amorphous state. The flow-through cell was considered to be the most robust dissolution method for the nanoparticulate system. The dissolution rate of nanosuspension was significantly increased by reducing the particle size. The dissolution testing in water showed that the nanosuspension exhibited significantly a higher dissolution rate with 71.9% drug dissolved in 5 min as compared to the raw puerarin (15.2%).

KEYWORDS

heart failure, cardiac function, autophagy, Sanshen Weixin Capsula

1. INTRODUCTION

Currently, a variety of drugs can be used for myocardial ischemia, however, there still remains a need to develop drugs for the treatment and prevention of heart failure(HF) induced by myocardial infarction. We developed Sanshen Weixin Capsula (Sanshen) as a new drug for the treatment of HF according to the principle of traditional Chinese medicine. Sanshen consists of *Radix Ginseng Rubra, Radix Salvia Miltiorrhiza* and *Radix Scrophulariae*, and plays a role of benefiting vital energy and activating blood circulation. This study will investigate the effects of Sanshen on cardiac function of rats with HF induced by myocardial infarction (MI).

2. METHODS AND METERIALS

2.1 Animal preparation

Male Wistar rats (N = 48, age 8 weeks, weighing 250-300 g) were purchased from the Center of Laboratory Animal Science, Jilin University, Changchun, China. They were housed four/cage in a controlled environment (23±1 ℃; 45%-50% relative humidity; fixed 12/12 h light/dark cycle, lights on at 08:00 am) with food and water adlibitum. All procedures were performed in accordance with the National Institute of Health's Guide for the Use and Care of Laboratory Animals and were approved by the Committee on Animal Care and Use of the Jilin University.

After one week acclimation, animals had their body weights measured at first and then replication model of HF following MI according to literature method [1,2]. As follows, rats were anesthetized with inhalation of ether, then fixed on their backs, and shaved the fur of the center of the chest. An incision of the skin and intercostal muscles was made between the third and fourth ribs. A thoracotomy was performed and the pericardium was opened, which left the heart adequately exposed. To induce myocardial infarction, the left anterior descending (LAD) coronary artery was ligated 1.5-2mm from the aortic root between the pulmonary cone and the left auricular appendage with silk sutures. Then the muscles and skins were sutured, the thoracotomy must be finished within 30 seconds. The sham-operated animals underwent the same procedure except that the silk suture was placed around the left coronary artery without being tied.

After the surgery, all animals were injected with penicillin (100,000U/ rat/day, intramuscular injection) for five days to prevent infection. Nine rats with induced MI died during the operation whereas none rats in the sham group died of infection. The operative mortality was 18.75%. Three days later, animals underwent echocardiography (GE vivid-Ⅱ, USA). Animals were randomized based on confirmation of MI and demonstration of similar MI size based upon fractional shortening (FS). Animals whose FS less than 45% to be selected into the experiment [3].

One week after the surgery, animals with MI following LAD ligation received daily treatment of double-distilled water (the HF group), Ramipril (the HF + Ramipril group), or Sanshen (the HF + Sanshen group) by gavage (1mL/100g body weight) for consecutively five weeks, while sham-operated animals (the Sham group) were administered double-distilled water once daily for five weeks. These doses were calculated according to the conversion table of animal doses to human equivalent doses based on body surface area(Sevilla, et al., 2004) [4]. Based on a study, the equivalent doses of Ramipril and Sanshen should be 1 mg/kg/ day and 0.5 g/kg/day, respectively [5].

2.2 Sanshen preparation

Sanshen is developed by our own research team. We selected the genuine regional herbs, implementation of modern pharmaceutical technology.The specific preparation of method is as follow: *Radix Ginseng Rubra* 200g, *Radix Salvia Miltiorrhiza* 400g and *Radix Scrophulariae* 200g crushed to a coarse

powder respectively. *Radix Ginseng Rubra* and *Radix Scrophulariae* were extracted 2 hours every time and three times with sixfold amount of 60% ethanol, filtered the extract and recoverd ethanol,then concentrated to thick paste with a relative density of 1.3 at 60℃, however, *Radix Salvia Miltiorrhiza* was extracted with 80% ethanol and the remaining steps as above. The residue of these three herbs of ethanol extraction was extracted 1 hours every time and three times with water, filtered the extract and concentrated to thick paste with a relative density of 1.3 at 60℃. The above mentioned thick paste was mixed and drying under reduced pressure, then preparing the capsula preparation after crush and add suited starch.

2.3 Echocardiography measurements

Transthoracic echocardiography was performed at 3 days post-MI and after drug treatment for 5 weeks. Animals were anesthetized by inhalation of ether, then fixed on their backs with their fur shaved. The following parameters were measured: left ventricular end-diastolic dimension (LVEDD), left ventricular end-systolic dimension (LVESD), left ventricular ejection fraction (LVEF) and fractional shortening (FS). All parameters were measured using the leading-edge method of the American Society of Echocardiography from three consecutive cardiac cycles, and the average was used for data analysis. Measurements were performed by an experienced technician who was blinded to the group identity.

2.4 Hemodynamic measurements

Following drug treatment for five weeks, a terminal surgical procedure was performed to evaluate left ventricular pressure and contractile properties as described previously [6]. Animals were anesthetized with 20% Urethane (1200 mg/kg,intraperitoneal injection), and the right carotid artery was exposed. A microtip pressure transducer catheter (2Fr, Millar Instruments, USA) was introduced via the right carotid artery into the left ventricle. The left ventricular end-systolic pressure (LVESP), left ventricular end-diastolic pressure (LVEDP), and the maximum rising and dropping rates of left ventricular pressure (±dp/dt) were measured using a biological data acquisition & analysis system (TME, BL-420F, CHN).

2.5 Tissue preparation

After hemodynamic measurements were performed, the heart was lavaged with normal saline. The atria and the right ventricular free wall were excised; the ventricles were rinsed with normal saline then dissected and weighed. The weights of the ventricles were normalized to the body weight and used as an index of ventricular hypertrophy. All left ventricular specimens were cut into three sections in cross-section below the ligation. The section nearly ligation was frozen in liquid nitrogen, and then stored at −80℃ for Western bloting analysis. The section away from the ligation was fixed in 10% formalin, dehydrated and then embedded in paraffin for subsequent hematoxylin-eosin (HE) staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining, and immunohisto- chemistry analysis [7]. The middle one was used for 2, 3, 5-triphenyl tetrazolium chloride(TTC) staining.

2.6 Assessment of infarct size

The middle section was cut into 2-mm thick slices that were incubated in 1%TTC (Sigma, USA) in phosphate buffered saline (PBS) for 10 min at room temperature, followed by fixation in 10% formalin for 60min. The slices were then digitally scanned (i800, Microtek, USA) and the images were analyzed using the medical image analysis system(TME, BI-2000, CHN). Infarct area was identified as an area unstained by TTC and infarct size was expressed as the ratio of infarct area to the whole left ventricular area.

2.7 Histological analysis

The pathological changes of left ventricular (LV) were examined by HE staining under optical micros- cope（Olympus, BX50, Japan）. Images of cross-section of the left ventricular were obtained by digitally scanner (i800, Microtek, USA).

2.8 Detection of TUNEL-Positive Cardiomyocytes

TUNEL staining was performed using the Apoptosis in situ Detection Kit (Roche, USA). Deparaffinized sections(2.5 μm thick) were washed with distilled water and treated with protein digestion enzyme for 15min at 37℃. After washing with 3 changes of 0.01mol/L PBS, sections were treated with terminal deoxynucleotidyl transferase (TdT) solution, incubated with 3% hydrogen peroxide for 5min to block endogenous peroxidase activity, and then treated with peroxidase-conjugated antibody for 10min at room temperature. After washing with 0.01mol/L PBS, nick end-labeling was visualized by immersing the sections in 3,3'-diaminobenzidine (DAB) solution with 0.006% hydrogen peroxide and counterstaining with hematoxylin. As a negative control, tissue sections were incubated with TdT buffer that did not contain the enzyme. As a positive control, tissue sections were treated with DNase I prior to treatment with TdT.

To assess the distribution of TUNEL-positive cardiomyo- cytes in the left ventricular wall, the segments of the right lateral border area adjacent to the interventricular septum and the left lateral border area adjacent to the left ventricular posterior wall were selected. For each of the segments, color video images of 3 separate fields were captured randomly and digitized using an ×400 objective on a microscope （Olympus, BX50, Japan ）. We determined the ratio between cardiomyocytes with TdT-stained nuclei (TUNEL-positive cardiomyocytes) and the total number of cardiomyocytes within the infarct area in the 3 fields. TUNEL-positive cardiomyocytes were carefully distinguish- ed from non-cardiomyocytes, such as macrophages.

2.9 Immunohistochemical Staining

The immunohistochemical staining for MAP-LC3β and Ub used paraffin sections that were 2.5 μm thick. After the sections were deparaffinized, epitope unmasking was achieved by treated with microwave irradiation (500W) for 10min in 0.01mol/L citrate buffer (pH 6.0). Intrinsic peroxidase activity was inhibited by the addition of 3% hydrogen peroxide, and nonspecific binding was blocked with normal goat serum. The primary antibodies MAP-LC3β (Santa Cruz, USA) and Ub (Santa Cruz, USA) were diluted 1:50 and incubated with the tissue sections for 60min at room temperature. The secondary antibody was incubated with the tissue sections for 30min at room temperature. Sections were then stained with DAB solution for 10min at room temperature. Between each step, the sections were washed with 0.1mol/L PBS. The sections were then counterstained with hematoxylin.

2.10 Western blotting

Extracted total proteins from myocardial tissue using lysis buffer. Protein concentrations were determined by Bradford Protein Assay (Beyotime, CHN). Aliquots of protein (50 μg) were added to loading buffer, heat denatured by boiling for 10 min, separated by SDS-Polyacrylamide gel electroph- oresis, and transferred to polyvinylidene fluoride membranes (Millipore, USA). Membranes were blocked with 5% non-fat milk (1.5h), and incubated with primary antibodies specific for β-actin (Proteintech, USA), MAP-LC3β (Santa Cruz, USA), SQSTM1/P62 (Santa Cruz, USA) overnight at 4 ℃. The signals were developed with horseradish peroxidase conjugated specific secondary antibodies and the ECL system (Thermo, USA). Images were obtained by a biological imaging system (Synoptics, SYGN2/7001, UK).

2.11 Statistical Analysis

Statistical analysis were conducted using SPSS 17.0 software. Data are expressed as the mean ± standard deviation. Group differences were evaluated using LVEF, FS reduction were significantly attenuated by the Ramipril and Sanshen treatment compared with the HF group (P<0.01). The echocardi- ography parameters did not differ significantly between the drug treated groups (Table 2).

Table 1: Animal characteristics of the experimental groups at sacrifice

Group	n	BW(g)	HW/BW(g/100g)	LVW/BW(g/100g)	Infarct size(%)
A	9	329.63±20.17	0.32±0.03	0.22±0.02	—
B	8	301.71±18.67	0.42±0.03*	0.29±0.03**	41.3±5.5
C	9	315.24±19.85	0.34±0.02#	0.26±0.03#	26.0±4.2#
D	9	320.36±21.28	0.37±0.03#	0.25±0.03#	22.9±3.8#

A: Sham group, B: HF group, C: HF+Ramipril group, D : HF+Sanshen group. *P＜0.05, **P＜0.01, comparing with group A; #P＜0.05, comparing with group B.

2.12 Hemodynamic data

Hemodynamic analysis revealed that LVESP and the ±dp/dt were significantly lower in the HF group compared with the Sham group (Table 3). The LVEDP was significantly higher in the same groups. These data confirmed the induction of severe HF in rats following LAD ligation. Treatment with Ramipril and Sanshen significantly improved these hemodynamic variables (P<0.05-0.01). There were no significant differences between the drug treated groups.

2.13 Histology

Compared with the Sham group,the LV intracavity was significantly expanded but ventricular wall becomes more thinner, Sanshen group attenuated these changes whereas Ramipril group did not (Figure 1 Upper). The HE staining shows that cardiomyocytes became disordered and hypertrophic, fibrous tissues hyperplasiaed surrounding one-way analysis of variance (ANOVA) or Kruskal-Wallis non- parametric test. A value of P<0.05 was considered statistically significant.

3.2 Cardiac remodeling and function

Echocardiography examinations were performed again after drug treatment for five weeks. Compared with the Sham group, LVEDD and LVESD were significantly increased (P<0.01), while LVEF and FS were reduced in the HF group (P<0.01). In addition, the LVEDD, LVESD increase and the cardiac muscle fibers and scar formation can be found in the HF group compared with the Sham group. Importantly, these deteriorations can be reversed by Sanshen rather than Ramipril (Figure 1 Lower).

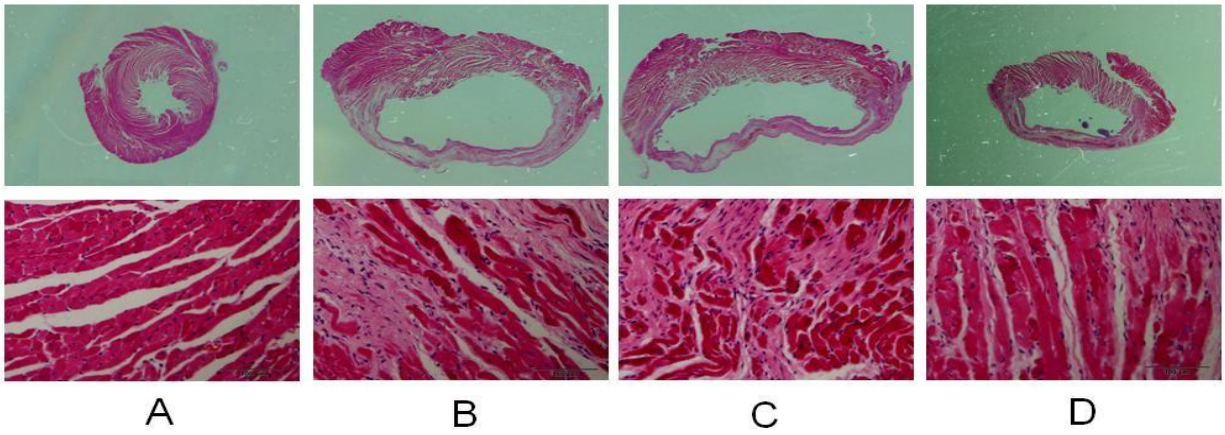
3.3 TUNEL-Positive Cardiomyocytes

In HF group, the percentage of TUNEL-positive cardiomyo- cytes was significantly increased in the left and right lateral border areas (P<0.01). The percentage of TUNEL-positive cardiomyocytes was significantly reduced in the same areas in HF + Ramipril group and HF + Sanshen group comparedwith HF group (P<0.01) (Figure 2).

Table 2: Echocardiography parameters of the experimental groups at sacrifice

Group	n	LVEDD(mm)	LVESD(mm)	LVEF(%)	FS(%)
A	9	5.8±0. 8	2.6±0. 4	91±10	55±7
B	8	8.2±0.9**	6.8±0. 8**	42±6**	17±4**
C	9	7.3±0. 9##	5.4±0. 7##	59±7##	26±5##
D	9	6.9±0. 8##	5.0±0. 6##	61±8 ##	27±5##
A	9	5.8±0. 8	2.6±0. 4	91±10	55±7
B	8	8.2±0.9**	6.8±0. 8**	42±6**	17±4**
C	9	7.3±0. $9^{\#\#}$	5.4±0. $7^{\#\#}$	$59±7^{\#\#}$	$26±5^{\#\#}$
D	9	6.9±0. $8^{\#\#}$	5.0±0. $6^{\#\#}$	$61±8^{\#\#}$	$27±5^{\#\#}$

A: Sham group, B: HF group,C: HF+Ramipril group, D: HF+Sanshen group. **P＜0.01, comparing with group A; # #P＜0.01, comparing with group B.



A: Sham group, B: HF group, C: HF+Ramipril group, D : HF+Sanshen group. Upper, The cross-section; Lower, HE staining, the right lateral border area adjacent to the interventricular septum (×200).

Figure 1: The cross-section of left ventricular and HE staining of animals in each group.

3. RESULTS AND DISCUSSIONS

3.1 Animal Characteristics

No death was recorded over the treatment period. Although the body weights (BW) were attenuated in the HF group compared with the Sham group, the difference did not reach statistical significance. The heart weights (HW and LV weights （LVW）, indexed to BW was lower in two treated animal groups when compared with HF animals (P<0.05- 0.01). Infarct size was significantly reduced in two active treatment groups (P<0.05) (Table 1).

3.4 Immunohistochemistry and Western Blot Analysis

Ub-positive cardiomyocytes were observed diffusely in the LV lateral border(Figure 3 Upper), whereas LC3-positive cardiomyocytes were distributed minimally at the same segment in HF group (Figure 3 Lower).

Both Ramipril and Sanshen can weaken the distribution of Ub but enhance LC3. Western blot analysis for LC3 and P62 expression in the LV myocardium are shown in Figure 4. Normalized densities for the LC3 signal were significantly greater in HF+Ramipril group and HF+Sanshen group than in HF group (P<0.05-0.01), consistent with the result of immuno- histochemistry. Compared with the HF group, expression of P62 was significantly reduced by Ramipril and Sanshen treatment (P<0.01). Currently, the drugs generally available to treat myocardial ischemia include nitrates (eg, Nitroglycerol), β-adrenergic blocking drugs （ eg, Propranolol） and calcium channel blockers (eg, Verapamil) and so on.

Clinical trials have showed that patients with acute myocardial infarction often developed HF in the follow up period [8,9]. Based on a research, drugs used to treat heart failure have already developed from diuretics (eg, Hydrochlorothiazide), cardiants (eg, Digoxin) to rennin-angiotonin-aldosterone system inhibitors (eg, Captopril, Aliskiren), however, these ones are still hardly ideal [10,11]. In traditional Chinese medicine science,

Qi asthenia and Blood stasis are thought as the basic factors which may contribute to the pathogenesis of heart failure, also named as "palpitation", "severe palpitation" or "phlegm retention" [12]. Therefore, benefiting vital energy and activating blood circulation is the main therapeutic method. As we know, Radix Ginseng Rubra has a role in invigorating Qi and Ningxin, Radix Salvia Miltiorrhizae could work through activating blood circulation to remove stasis and Radix Scrophulariae may contribute to nourishing Yin and clearing lung. Therefore, the new preparation developed by our team Sanshen, composed of all these three ones, could not only support healthy energy but also play a role in governing both the heart and lung by benefiting vital energy and activating blood circulation, implying its potential role in treating HF.

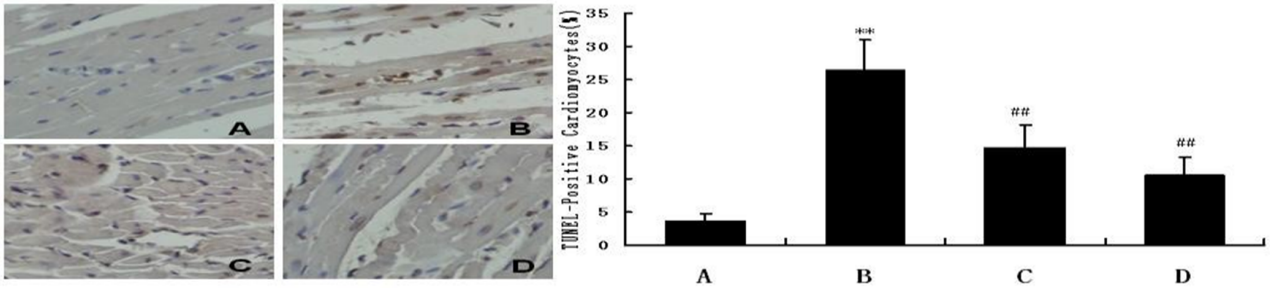


Figure 2: TUNEL staining of LV of animals in each group.

A: Sham group, B: HF group, C: HF+Ramipril group, D: HF+Sanshen group. These images are the right lateral border area adjacent to the interventricular septum (×400). **P＜0.01, comparing with Sham group, ##P＜0.01, comparing with HF group.

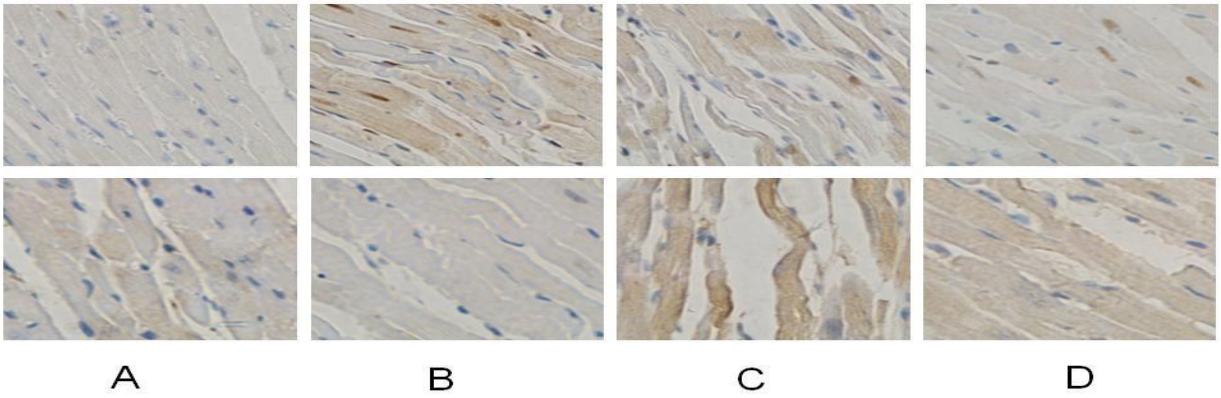


Figure 3: Immunohistochemistry of Ub and LC3.

A: Sham group, B: HF group, C: HF+Ramipril group, D: HF+Sanshen group. Upper, Ub; Lower,LC3. These images are the right lateral border area adjacent to the interventricular septum (×400).

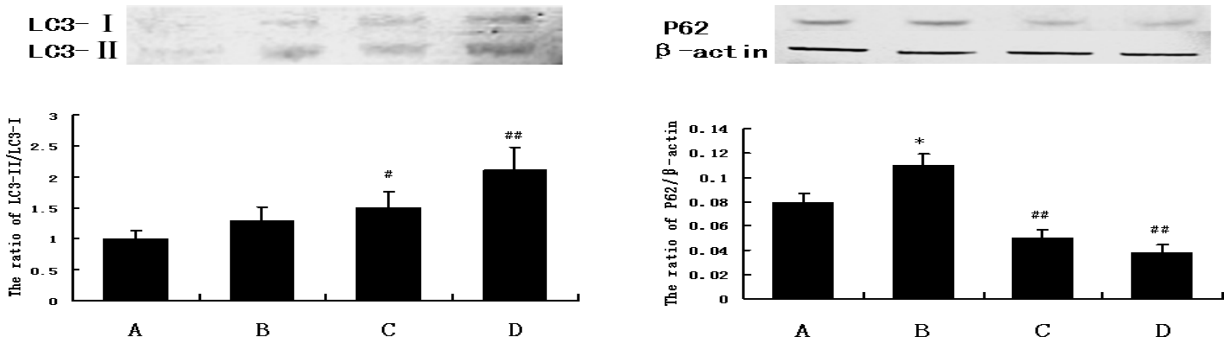


Figure 4: Western blot analysis of LC3 and P62 in left ventricular myocardium.

A: Sham group, B: HF group, C: HF+Ramipril group, D: HF+Sanshen group. *P＜0.05, comparing with Sham group；#P＜0.05, ##P＜0.01, comparing with HF group.

In the current study, the HF model was induced by ligate the LAD of animals. Cardiac function was detected by transthoracic echocardiography at 3 days post-MI, the animals which LVEF less than 45% were considered successful model [13]. After treatment with Sanshen for five weeks, echocardiography and hemodynamic parameters such as LVEDD, LVESD and LVEDP marked decreased whereas LVEF, FS, LVSP and ±dp/dt max increased. These changes indicated that Sanshen had profound effects on ventricular structure, ventricular ejection as well as myocardial contraction and relaxation, implying its protective role in cardiac function. Changes in cardiac function are closely related to ventricular remodeling after myocardial infarction. According to a study, the remolding of ventricular structure and function, resulting in obvious ventricular dilation and geometry change, may finally lead to the impairment of myocardial contraction and relaxation, accompanied by the shift from compensatory myocardial hypertrophy to decompensation of heart failure [14,15]. As our studies showed that Sanshen could reduce the ratio of HW/BW and LVW/BW, decrease left ventricular infarct size and inhibit myocardial hypertrophy; in the meantime the tissue morphology results indicated that this preparation may suppress the dilation of ventricular chamber and attenuate the extent of disorder and hypertrophy of cardiomyocytes, and fibrous tissues hyperplasiaed, therefore Sanshen is probably able to postpone the progression of ventricular remodeling. During the HF process, cardiomyocytes in the lateral border area adjacent to the interventricular septum successively underwent apoptosis, leading to the escalation of infarct size and the hypofunction of myocardial contraction and relaxation. So one could inevitably draw the conclusion that inhibition of the apoptosis of cardiomyocytes is a basis for the pathophysiology of prevention and cure of heart failure [16]. TUNEL staining results showed that Sanshen was able to decrease the apoptosis rate, indicating that this preparation may exert myocardial protective effects by inhibiting apoptosis. During the

development of HF, previous studies have found that there were abundant deposition of polyubiquitinated proteins and ubiquitin-positive aggregates, implying that the dysfunction of ubiquitin-proteasomes system may be an important pathogenesis of HF [17,18]. In addition, polyubiquitinated proteins may also be process- ed through autophagy in vivo [19,20]. Our study found that Sanshen may attenuate the deposition of left ventricle ubiquitin-positive aggregates, and in the meantime upregulated the level of autophagy, implying that Sanshen may relieve myocardial injury and exert protective effects by degrading polyubiquitinated proteins through autophagy.

4. CONCLUSION

In conclusion, Sanshen, the new preparation developed by our team, may improve the cardiac function of rats with HF through decreasing the cardiac apoptosis, by enhancing the level of autophagy and promoting the degradation of polyubiquitinated proteins.

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