

Original Article

Optimized new Shengmai powder (优化新生脉散方) inhibits myocardial fibrosis in heart failure by regulating the rat sarcoma/rapidly accelerated fibrosarcoma/mitogen-activated protein kinase kinase/extracellular regulated protein kinases signaling pathway

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Abstract

OBJECTIVE: Exploring the effect of Optimized New Shengmai powder (优化新生脉散方, ONSMP) on myocardial fibrosis in heart failure (HF) based on rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/extracellular regulated protein kinases (ERK) signaling pathway.

METHODS: Randomized 70 Sprague-Dawley rats into sham ($n = 10$) and operation ($n = 60$) groups, then established the HF rat by ligating the left anterior descending branch of the coronary artery. We randomly divided the operation group rats into the model, ONSMP [including low (L), medium (M), and high (H) dose], and enalapril groups. After the 4-week drug intervention, echocardiography examines the cardiac function and

calculates the ratios of the whole/left heart to the rat's body weight. Finally, we observed the degree of myocardial fibrosis by pathological sections, determined myocardium collagen (COL) I and COL III content by enzyme-linked immunosorbent assay, detected the mRNA levels of COL I, COL III, α -smooth muscle actin (α -SMA), and c-Fos proto-oncogene (c-Fos) by universal real-time, and detected the protein expression of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ETS-like-1 transcription factor (p-ELK1), p-c-Fos, α -SMA, COL I, and COL III by Western blot.

RESULTS: ONSMP can effectively improve HF rat's cardiac function, decrease cardiac organ coefficient, COL volume fraction, and COL I/III content, down-regulate the mRNA of COL I/III, α -SMA and c-Fos, and the protein of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, c-Fos, COL I/III, and α -SMA.

CONCLUSIONS: ONSMP can effectively reduce myocardial fibrosis in HF rats, and the mechanism may be related to the inhibition of the RAS/RAF/MEK/ERK signaling pathway.

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Keywords: myocardial fibrosis; heart failure; sarcoma; rapidly accelerated fibrosarcoma; mitogen-activated protein kinases; extracellular regulated protein kinases; optimized new Shengmai powder

1. INTRODUCTION

Heart failure (HF) is a complex clinical syndrome caused by structural and functional abnormalities of the heart and the end stage of various cardiovascular diseases. Myocardial remodeling is the pathological basis of HF, and myocardial fibrosis is a significant pathological change in myocardial remodeling.^{1,2} Myocardial fibrosis

is the pathologic development of the extracellular matrix of the myocardium, in which cardiac fibroblasts are the core effector cells.³ During the development of HF, cytokines, growth factors, G protein-coupled receptor ligands, osmotic pressure changes, advanced glycation end products, and other intracellular and extracellular stimuli can promote the rat sarcoma (RAS)/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/extracellular regulated protein kinases (ERK) signaling pathway in a cascade form of signaling, accelerate the proliferation, migration, and differentiation of cardiac fibroblasts, increase the production of collagen (COL) I/III in myocardial tissue, and cross-link with elastin remnants, ultimately leading to matrix COL deposition and increased levels of mature COL fibers, resulting in myocardial fibrosis.⁴⁻⁶ In conclusion, inhibiting the activation of the RAS/RAF/MEK/ERK signaling pathway is significant for the prevention and treatment of myocardial fibrosis in HF. Although modern medicine continues to enrich the methods and means of treating HF, further reduction of mortality and re-hospitalization rates and improvement of quality of life in hypertensive patients are still the difficulties of current research. In the clinical practice of HF treatment in China, Traditional Chinese Medicine has been widely added and applied because of its characteristics of increasing exercise tolerance, improving quality of life, and improving cardiac function. The combination of Traditional Chinese Medicine and Western medicine has become an objective and widely accepted medical model for the treatment of HF.

Compared with modern medicine, Traditional Chinese Medicine is effective in treating HF from the perspective of holism and syndrome differentiation and treatment. There is no name for HF in Chinese medicine, but medical practitioners categorize HF into palpitations and edema based on patients' clinical manifestations. Chinese medicine summarizes the pathomechanism of HF as a syndrome of deficiency, mainly due to the deficiency of the heart's *Qi*, blood, *Yin*, and *Yang* leading to a dereliction of its functions, resulting in an imbalance of the balance of the five viscera, a malfunction of *Qi*, blood, and fluids, and the formation of stasis and blood stasis, water-dampness, phlegm and beverages, and other pathologic products.⁷ Optimized New Shengmai powder (优化新生脉散方, ONSMP) is an effective Chinese herbal formula for the clinical treatment of heart failure by Prof. Mao Jingyuan at the First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, which is mainly composed of Huangqi (*Radix Astragali Mongolici*), Dangshen (*Radix Codonopsis*), Ciwujia (*Radix et Caulis Acanthopanax Santicosi*), Danshen (*Radix Salviae Miltiorrhizae*), Biejia (*Carapax Trionycis*), Fuling (*Poria*), Tinglizi (*Semen Lepidii Apetali*), Maidong (*Radix Ophiopogonis Japonici*), Zhiqiao (*Fructus Aurantii Submaturus*). Sovereign drug Huangqi (*Radix Astragali Mongolici*) replenishes vitality to solidify the essence, nourishes the body without over-nutrition, and has the function of moisture; Ministerial

drug Dangshen (*Radix Codonopsis*), Ciwujia (*Radix et Caulis Acanthopanax Santicosi*), and Danshen (*Radix Salviae Miltiorrhizae*) invigorate *Qi* to channel the veins; The adjuvant drug is Biejia (*Carapax Trionycis*) and Fuling (*Poria*) to activate blood and water; Tinglizi (*Semen Lepidii Apetali*) to disperse bitter diarrhea, cold decreases, and flow lung *Qi* to facilitate Control and regulate human blood circulation; Zhiqiao (*Fructus Aurantii Submaturus*) assists all medicines to promote *Qi*, Maidong (*Radix Ophiopogonis Japonici*) nourishes blood and activates the veins to prevent injury to *Yin*, and the two together have the effect of invigorating *Qi* and activating blood and water. Clinical studies have found that ONSMP significantly improves patients' clinical symptoms and increases ejection fraction.⁸ Experimental studies have confirmed that this herbal compound has anti-myocardial fibrosis, cardiomyocyte death, myocardial hypertrophy, and delays myocardial remodeling.^{9,10} Therefore, this study aims to explore whether the ONSMP can inhibit myocardial fibrosis by acting on the RAS/RAF/MEK/ERK signaling pathway, aiming to provide new ideas and new strategies for the prevention and treatment of HF.

2. MATERIALS AND METHODS

2.1. Drugs and reagents

In the ONSMP, Huangqi (*Radix Astragali Mongolici*), Dangshen (*Radix Codonopsis*), Ciwujia (*Radix et Caulis Acanthopanax Santicosi*), Danshen (*Radix Salviae Miltiorrhizae*), Biejia (*Carapax Trionycis*), Fuling (*Poria*), and Maidong (*Radix Ophiopogonis Japonici*) each has 10 g, while Tinglizi (*Semen Lepidii Apetali*) and Zhiqiao (*Fructus Aurantii Submaturus*) each has 6 g. The Chinese Pharmacy of the First Affiliated Hospital of Tianjin University of Traditional Chinese Medicine provided all the herbs. Firstly, ground Danshen (*Radix Salviae Miltiorrhizae*) into fine powder for use, and the remaining drugs were decocted with water 3 times, each time adding 10 times the amount of water. After 1.5 h of decoction, the decoction was combined, filtered, and concentrated into an extract, with an extract rate of 25.21%. Vacuum drying, grinding into a fine powder, and mixing with Danshen (*Radix Salviae Miltiorrhizae*) fine powder. Enalapril (ENP, SFDA approval number: H32022378) was provide by Jiangsu Hengrui Medicine Co., Ltd. (Lianyungang, China).

Hematoxylin and eosin (HE, batch number: G1003) and Masson (G1006) staining solutions were purchased from Wuhan Servicebio Biotechnology Co., Ltd. (Wuhan, China). COL I (batch number: MM-0995R2) and COL III (batch number: MM-0080R2) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Jiangsu Meimian Industrial Co., Ltd. (Yancheng, China). RNA extraction kit (batch number: R1200), 2 × SYBR green polymerase chain reaction (PCR) master mix (batch number: SR1110), Universal real-time PCR (RT-

PCR) Kit (batch number: RP1100), and bicinchoninic acid protein assay kit (batch number: PC0020) were purchased from Beijing Solarbio Biotechnology Co., Ltd. (Beijing, China). For primary antibodies, phosphor-RAS (p-RAS, batch number: AF8509) and phosphor-MEK1/2 (p-MEK1/2, batch number: AF8035), and α -smooth muscle actin (α -SMA, batch number: AF1032) were purchased from Jiangsu Affinity Biosciences Research Center Co., Ltd. (Liyang, China). phosphor-RAF (p-RAF, batch number: ab173539), phosphor-ERK1/2 (p-ERK1/2, batch number: ab201015), phosphor-ETS-like-1 transcription factor (p-ELK1, batch number: ab218133), phosphor-c-Fos protooncogene (p-c-Fos, ab193365), COL I (batch number: ab138492), COL III (batch number: ab184993), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, batch number: ab8245) were purchased from Abcam (Cambridge, UK).

2.2. Animal grouping and treatment

We purchased 70 Sprague-Dawley (SD) rats (male, weighing 180-220 grams, pathogen-free grade) from Beijing Sibeifu Laboratory Animal Technology Ltd. under Production License No. SCXK (jing) 2021-0006. Housed rats in the animal room of the Institute of Radiology, Chinese Academy of Medical Sciences, with a temperature of $(22 \pm 2)^\circ\text{C}$, a humidity of $40\% \pm 5\%$, 12 h light and dark cycles, and free access to water. All experiments complied with the Guide for the Institutional Animal Care and Use Committee. After three days of adaptive feeding, 70 SD rats were divided into the sham group ($n = 10$) and operation group ($n = 60$) by random number table method. Anesthesia was performed by intraperitoneal injection of 1 % pentobarbital sodium (50 mg/kg), orotracheal intubation, and small animal ventilator (model number: SAR-1000, Shanghai Yuyan Scientific Instrument Co., Ltd., Shanghai, China), tidal volume 6 mL, respiratory rate 80 times/min, respiratory ratio 1 : 2. A layer-by-layer thoracotomy was performed between the 3rd and 4th ribs to expose the heart and open the pericardium. Ligated the left anterior descending coronary artery with a 5-0 surgical suture 2 mm below the rat's Left Atrial Appendage, and a drop of lidocaine was placed on the surface of the heart to remove blood from the chest cavity, and closed the chest layer by layer with 2-0 sutures. Finally, turned off the ventilator, the rats were placed on an electric blanket to keep them warm, and they were returned to their cages when they were fully awake. Sham did not ligate the left anterior descending coronary artery, and the other steps were the same.

Ultrasound showed left ventricular ejection fractions (LVEF) $\leq 50\%$ at 8 weeks after operation as the standard for successful modeling. We excluded non-compliant rats and included 50 rats with successful modeling in the experiment. The rats were divided into 5 groups (10 rats in each group) using the random number method, including the model group, the ONSMP group [including low (L), medium (M), and high (H) dose groups], and the ENP group. We calculated the equivalent drug dose of

rat gavage according to the body surface area of the human and rat. The ONSMP-L group was 0.5 times the equivalent dose (4.05 g crude drug/kg), the ONSMP-M group was the equivalent dose (8.1 g crude drug /kg), the ONSMP-H group was 2 times the equivalent dose (16.2 g crude drug /kg), and the ENP group was the equivalent dose (0.9 g/kg). The sham operation and model groups were given the same volume of blank solvent (0.5% CMC-Na) by intragastric administration, and the other groups were respectively given corresponding drugs by intragastric administration, with an administration volume of 1 mL/100 g, once a day, for 4 consecutive weeks.

2.3. Determination of cardiac function and organ coefficient

After administration, 6 rats in each group were randomly selected for cardiac ultrasound again, and the detection indexes included LVEF and left ventricular fractional shortening (LVFS). At the end of the experiment, we opened the chest of the rats, separated the tissues and blood vessels around the heart, took out the heart, and rinsed it with ice saline. Remove excess water with filter paper, weigh the heart with an electronic balance, and calculate the heart weight index (HWI). Continue to separate the right atrium and right ventricle, and weigh the remaining left ventricle to calculate the left ventricular mass index (LVMI).

2.4. Detection of pathological changes in myocardial tissue

We first soaked the left ventricular myocardial tissue in 4% paraformaldehyde for 72 h, then dried it with filter paper, cut the apical myocardial tissue, dehydrated it with ethanol gradient, cleaned it with xylene, embedded it in paraffin wax, and finally cut it into sections with a thickness of 4 μm , stained it with HE and Masson staining, respectively, and observed the pathological changes of the heart under the upright optical microscope (model number: Nikon Eclipse E100, Nikon Corporation, Tokyo, Japan). Use Image J image analysis software to quantitatively analyze the COL fiber area of pathological sections stained by Masson. The collagen volume fraction (CVF) expresses the amount of COL and the severity of fibrosis, that is, $\text{CVF} (\%) = (\text{COL area} / \text{total area of muscle tissue in the center of the visual field}) \times 100\%$.

2.5. ELISA detection of myocardial tissue COL I and COL III content

The aim of this experiment is to detect the levels of COL I and COL III in myocardial tissue. Firstly, 50mg of myocardial tissue was precisely weighed and homogenized with 450mL of phosphate-buffered saline under ice-cold conditions. After homogenization, the mixture was centrifuged using a high-speed frozen centrifuge (model number: ST16R, Thermo Fisher Scientific, Waltham, MA, USA). During the

centrifugation process, the speed was set to 5000 rpm and the centrifugation lasted for 15 min. Following centrifugation, the supernatant was carefully collected, and the levels of COL I and COL III in the supernatant were measured strictly according to the instructions provided in the ELISA kit.

2.6. RT-PCR detection of COL I, COL III, α -SMA, c-Fos mRNA expression levels

We first took 50 mg of fresh rat heart tissue to extract total RNA according to the kit instructions. After the RNA concentration was detected and determined using an Ultra Trace UV-Vis Spectrophotometer (model number: DYCZ-40D, DeNovix, DE, USA), total RNA from all samples was diluted to 200 ng/ μ L and then reverse transcribed into cDNA. The instruments used in the experiment include a PCR machine (model number: DS-11, Thermo Fisher Scientific, Waltham, MA, USA) and a real-time PCR instrument (model number: ABI ProFle LightCycler 9, Roche, Basel, Switzerland). The amplification reaction system used is real-time fluorescent quantitative PCR. Relative quantitative analysis of mRNA was performed after 40 cycles using the $\Delta\Delta$ CT method to detect the relative expression levels of mRNA of relevant genes. Tianjin Boyou Biotechnology Co., Ltd. (Tianjin, China) provided the primers we needed (Table 1).

2.7. Western blot detection of related protein expression

The rat left ventricular heart tissue was added to the lysate containing 1% PMSF and ground, then centrifuged at 4 °C and 14000 r/min for 15 min, and the supernatant was collected. Proteins were first quantified using the bicinchoninic acid protein quantification kit, then added 5 $\times$ protein loading buffer was and finally denatured by boiling. Protein samples were run on 10% electrophoresis gels, transferred to polyvinylidene fluoride (PVDF) membranes, and blocked with a protein-free fast-blocking solution. Wash 5 times with 1 $\times$ Tris Buffered Saline with Tween 20 for 5 min each. Corresponding antibodies such as p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-c-Fos, α -SMA, COL I, COL III, and GAPDH were added, respectively. Incubate overnight at 4 °C, wash three times with 1 $\times$ Tris Buffered Saline with Tween 20 for 5 min each, and incubate with

secondary antibody for 1 h at room temperature. Immerse the PVDF membrane in enhanced chemiluminescence developer solution for 1 min. Expose and develop PVDF membranes using a multifunctional imaging system (model number: NIKON DS-U3, Nikon Corporation, Tokyo, Japan). After using PhotoShop to collect, classify, and decolorize the images, using Image J software to analyze the grayscale values of the protein bands.

2.8. Statistical analysis

Data were analysed using SPSS 21.0 statistical software (IBM Corp., Armonk, NY, USA). Measurement information was expressed as mean $\pm$ standard deviation when it conformed to normal distribution and median (interquartile spacing) when it did not work to normal distribution. When analysing the influencing factors, one-way analysis of variance was used for between-group comparisons when the measures conformed to a normal distribution with a χ^2 variance. If the difference was statistically significant, compare the data two by two using the Bonferroni test. The Kruskal-Wallis *H*-test was used to compare multiple groups when the information did not fit a normal distribution, or the variance was not homogeneous, and the Mann-Whitney *U* test was further used for two-by-two comparisons when the difference was statistically significant. A statistical difference was indicated by $P < 0.05$. Finally, create statistical graphs using excel.

3. RESULTS

3.1. Comparison of cardiac function and organ coefficient of rats in each group

Compared with the sham group, LVEF and LVFS were significantly lower in the model group, whereas HWI and LVMI were dramatically higher ($P < 0.01$). Compared with the model group, LVEF and LVFS were significantly higher, while HWI and LVMI were substantially lower in each treatment group ($P < 0.01$). Compared with the ONSMP-L group, LVEF and LVFS were higher, while HWI and LVMI were lower in the ONSMP-M, ONSMP-H, and ENP groups ($P < 0.01$). Compared with the ONSMP-M group, LVEF and LVFS were significantly higher, while HWI and LVMI were substantially lower in the ONSMP-H and ENP groups ($P < 0.01$). The

Table 1 Primer sequence table

Primer name		Sequence (5'-3')	Size (bp)
COL I	F	GAG AGG TGA ACA AGG TCC CG	153
	R	AAA CCT CTC TCG CCT CTT GC	
COL III	F	AAG GCT GCA AGA TGG ATG CT	95
	R	GTG CTT ACG TGG GAC AGT CA	
α -SMA	F	CTATGAGGGCTATGCCTTGCC	122
	R	GCTCAGCAGTAGTAACGAAGGA	
c-Fos	F	GTCTTCCTTTGTCTTCACCTACCC	384
	R	CCCTGCCTTCTCTGACTGCT	
β -actin	F	GTCGTACCACTGGCATTGTG	180
	R	TCTCAGCTGTGGTGGTGAAG	

Notes: COL I: collagen I; COL III: collagen III; α -SMA: α -smooth muscle actin; c-Fos: c-Fos proto-oncogene; F: forward primer; R: reverse primer.

differences in LVEF, LVFS, HWI, and LVMI between ONSMP-H and ENP groups were not statistically significant. It indicates that the left ventricular systolic function and diastolic function of HF rats are impaired. ONSMP can significantly improve the left ventricular systolic force and improve the cardiac function of HF rats, which is similar to the positive drug ENP (Figure 1).

3.2. Histopathological observation of rat heart

The rat myocardial fibers in the sham group were uniform in size, clear and complete in structure, neatly and densely arranged, with the nucleus located in the center, the cytoplasm of the cells uniformly colored, and a small number of light red COL fibers uniformly distributed around the extracellular matrix. Compared with the Sham group, myocardial fibers of rats in the model group were broken and disorganized, cardiomyocytes were hypertrophied, square, poorly demarcated, and died in large numbers, and there were a large number of reddish COL fibers in the extracellular matrix, which indicated that the degree of myocardial fibrosis was severe. Compared with the model group, the degree of myocardial injury and the number of cell deaths reduced in the rats of each treatment group, the arrangement of cardiomyocytes was more regular and

orderly, the nuclei of cardiomyocytes were more clearly defined, and the deposition of COL fibers in the extracellular matrix reduced. Among them, the therapeutic effect of Traditional Chinese Medicine was dose-related, and the therapeutic effect of the ONSMP-H group was close to that of the ENP group (Figure 2A). In masson-stained sections, normal myocardial tissue is red and COL fibers are blue. Sham group rat myocardial tissue transverse muscle was visible, with a regular arrangement of myocardial fibers and only a few blue COL fibers in the intercellular matrix. Compared with the sham group, rats in the model group showed loss of rhabdomyosin in the myocardial tissue, large areas of myocardial fibers that were necrotic and irregularly arranged, and excessive blue COL fibers in the extracellular matrix. Quantitative results showed that CVF was significantly higher in the model group than in the sham group ($P < 0.01$). Compared with the model group, all treatment groups showed a significant decrease in CVF ($P < 0.01$). Compared with the ONSMP-L group, CVF was significantly lower in the ONSMP-M, ONSMP-H, and ENP groups ($P < 0.01$). Significant reductions in CVF in both the ONSMP-H and ENP groups compared to the ONSMP-M group ($P < 0.01$). There was no difference in CVF between ONSMP-H and ENP groups (Figure 2B, 2C).

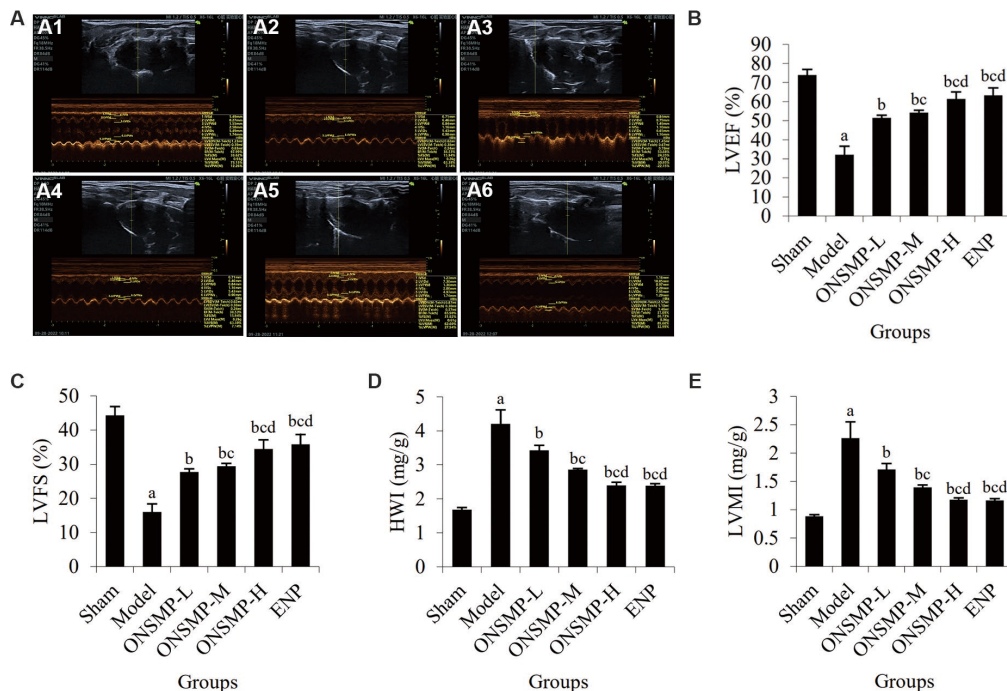


Figure 1 Comparison of cardiac function and organ coefficient in rats of each group

A: typical echocardiographic image. A1-A6: the echocardiographic profile of sham (A1), model (A2), ONSMP-L (A3), ONSMP-M (A4), ONSMP-H (A5), and ENP (A6); B: statistic of LVEF of rats in each group; C: Statistic of LVFS of rats in each group; D: Statistic of HMI of rats in each group; E: statistic of LVMI of rats in each group. Sham group: sham operated; model group: ligation of the left anterior descending coronary artery without treatment; ONSMP-L: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 4.05 g crude drug/kg for 4 weeks; ONSMP-M: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 8.1 g crude drug/kg for 4 weeks; ONSMP-H: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 16.2 g crude drug/kg for 4 weeks; ENP: ligation of the left anterior descending coronary artery rats treated with ENP intragastrically at a daily dose of 0.9 g/kg for 4 weeks. ONSMP: Optimized New Shengmai powder; ENP: enalapril; LVEF: left ventricular ejection fractions; LVFS: left ventricular fractional shortening; HWI: heart weight index; LVMI: left ventricular mass index. Data represent the mean $\pm$ standard deviation using one-way analysis of variance ($n = 6$). Compared with the sham group, ^a $P < 0.01$; compared with the model group, ^b $P < 0.01$; compared with the ONSMP-L group, ^c $P < 0.01$; compared with the ONSMP-M group, ^d $P < 0.01$.

3.3. Differences in the levels of COL I and COL III in rat myocardial tissue between groups

Compared with the sham group, the COL I and COL III levels in the model group were significantly increased ($P < 0.01$). Compared with the model group, the COL I level in ONSMP-L, ONSMP-M, and ONSMP-H groups decreased significantly ($P < 0.01$), the COL III level in the ONSMP-L group decreased ($P < 0.05$), and the COL III level in the ONSMP-M and ONSMP-H groups decreased significantly ($P < 0.01$). Compared with the ONSMP-L group, the COL I level was significantly decreased in the ONSMP-H group ($P <$

0.01), while the COL III level was decreased ($P < 0.05$). Compared with the ONSMP-M group, the COL I level in the ONSMP-H group decreased ($P < 0.05$) (Table 2).

3.4. Differences in the mRNA expression of COL I, COL III, α -SMA, and c-Fos in rat myocardial tissue between groups

Compared with the sham group, the mRNA expressions of COL I, COL III, α -SMA, and c-Fos in the model group were significantly increased ($P < 0.01$). Compared with the model group, the mRNA expressions of COL I, COL III, α -SMA, and c-Fos in ONSMP-L, ONSMP-M,

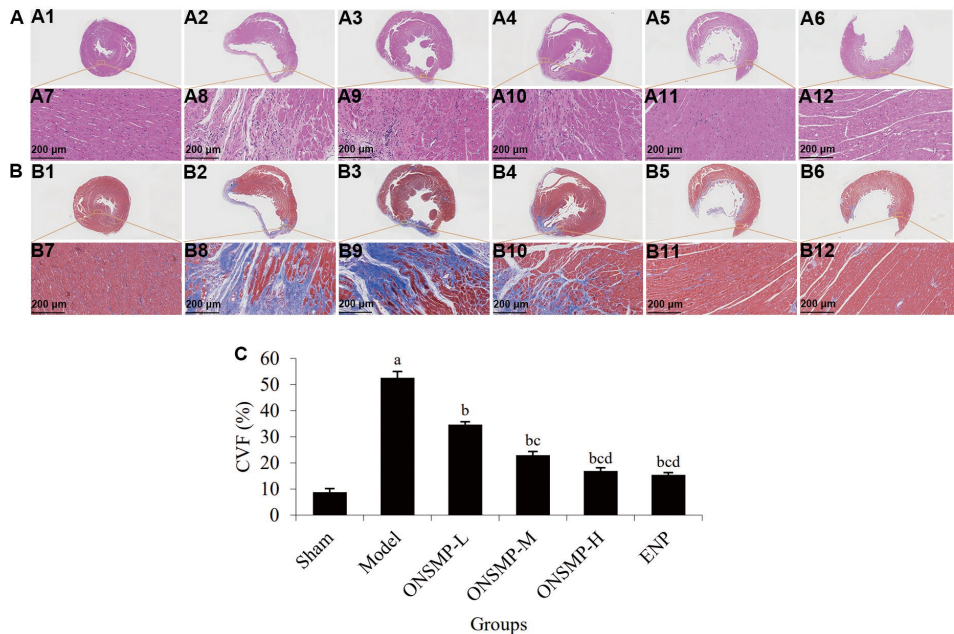


Figure 2 Pathological section results of myocardial tissue of rats in each group

A: typical myocardial HE-stained section image. A1-A6: HE-stained section images of sham (A1), model (A2), ONSMP-L (A3), ONSMP-M (A4), ONSMP-H (A5), and ENP (A6) ($\times 1$); A7-A12: HE-stained section images of sham (A7), model (A8), ONSMP-L (A9), ONSMP-M (A10), ONSMP-H (A11), and ENP (A12) ($\times 200$). B: Typical myocardial masson-stained section image. B1-B6: masson-stained section images of sham (B1), model (B2), ONSMP-L (B3), ONSMP-M (B4), ONSMP-H (B5), and ENP (B6) ($\times 1$); B7-B12: masson-stained section images of sham (B7), model (B8), ONSMP-L (B9), ONSMP-M (B10), ONSMP-H (B11), and ENP (B12) ($\times 200$). C: statistics of CVF of rats in each group. Sham group: sham operated; model group: ligation of the left anterior descending coronary artery without treatment; ONSMP-L: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 4.05 g crude drug/kg for 4 weeks; ONSMP-M: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 8.1 g crude drug/kg for 4 weeks; ONSMP-H: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 16.2 g crude drug/kg for 4 weeks; ENP: ligation of the left anterior descending coronary artery rats treated with ENP intragastrically at a daily dose of 0.9 g/kg for 4 weeks. ONSMP: Optimized New Shengmai powder; ENP: enalapril; CVF: collagen volume fraction. Data represent the mean $\pm$ standard deviation using one-way analysis of variance ($n = 3$). Compared with the sham group, ^a $P < 0.01$; compared with the model group, ^b $P < 0.01$; compared with the ONSMP-L group, ^c $P < 0.01$; compared with the ONSMP-M group, ^d $P < 0.01$.

Table 2 Content of COL I and COL III in myocardial tissue of rats in each group ($\mu\text{g/L}$, $\bar{x} \pm s$)

Group	<i>n</i>	COL I	COL III
Sham	6	34.5 $\pm$ 1.4	20.4 $\pm$ 1.6
Model	6	44.3 $\pm$ 1.3 ^a	27.1 $\pm$ 1.3 ^a
ONSMP-L	6	40.5 $\pm$ 1.7 ^c	24.7 $\pm$ 1.5 ^b
ONSMP-M	6	39.5 $\pm$ 1.8 ^c	23.1 $\pm$ 1.1 ^c
ONSMP-H	6	36.1 $\pm$ 1.8 ^{cef}	22.4 $\pm$ 0.8 ^{cd}

Notes: sham group: sham operated; model group: ligation of the left anterior descending coronary artery without treatment; ONSMP-L: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 4.05 g crude drug/kg for 4 weeks; ONSMP-M: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 8.1 g crude drug/kg for 4 weeks; ONSMP-H: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 16.2 g crude drug/kg for 4 weeks; ENP: ligation of the left anterior descending coronary artery rats treated with ENP intragastrically at a daily dose of 0.9 g/kg for 4 weeks. COL I: collagen I; COL III: collagen III; ONSMP: optimized new Shengmai powder. The data were expressed as the mean $\pm$ standard deviation ($n = 6$). Compared with the sham group, ^a $P < 0.01$; compared with the model group, ^b $P < 0.05$, ^c $P < 0.01$; compared with the ONSMP-L group, ^d $P < 0.05$, ^e $P < 0.01$; compared with the ONSMP-M group, ^f $P < 0.05$.

and ONSMP-H groups were significantly decreased ($P < 0.01$). Compared with the ONSMP-L group, the mRNA expressions of COL I, COL III, α -SMA, and c-Fos in the ONSMP-M and ONSMP-H groups were significantly decreased ($P < 0.01$). Compared with the ONSMP-M group, the mRNA expressions of COL I, COL III, α -SMA, and c-Fos in the ONSMP-H group were significantly decreased ($P < 0.01$) (Table 3).

3.5. Differences in the protein expressions of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, p-c-Fos, COL I, COL III, and α -SMA in rat myocardial tissue between groups

Compared with the sham group, the protein expressions of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, p-c-Fos, COL I, COL III, and α -SMA were significantly increased in model group ($P < 0.01$). Compared with the model group, the protein expressions of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, p-c-Fos, COL I, COL III, and α -SMA in ONSMP-L, ONSMP-M, and ONSMP-H groups were significantly decreased ($P < 0.01$). Compared with the ONSMP-L group, the protein expressions of p-MEK1/2 in the ONSMP-M group and p-RAS, p-MEK1/2, p-ERK1/2, p-ELK1, COL I, COL III in the ONSMP-H group were decreased ($P < 0.05$), the protein expressions of p-RAF, p-c-Fos, and α -SMA in the ONSMP-H group were significantly decreased ($P < 0.01$). Compared with the ONSMP-M group, the expressions of p-ELK1, p-c-Fos, and COL III in the ONSMP-H group were decreased ($P < 0.05$) (Figure 3).

4. DISCUSSION

HF is considered the leading cardiovascular epidemic of the 21st century, and ventricular remodeling due to myocardial infarction is a common cause of HF. Continuous stimulation of pathological factors such as ischemia, hypoxia, and inflammation induced by myocardial infarction can cause myocardial injury. Due to the complete differentiation of myocardial cells, they do not have the ability of mitosis, so they can only rely on myocardial fibrosis to repair the damage.¹¹ Compensatory hyperplasia of COL fibers in the injured

area and formation of scar tissue can ensure the integrity of tissue structure and function of the heart during the healing period, improve its ability to withstand pressure and maintain the electromechanical function of the heart. With the development of myocardial fibrosis, cardiac function gradually decreases. This pathological change severely limits the systolic and diastolic capacity of the myocardium and the conduction of neural electrical signals. The risk of arrhythmias and sudden death increases, and heart failure progresses from compensatory to decompensated.¹² The causes of cardiac discomfort in patients with myocardial fibrosis are broadly defined as necrosis of myocardial tissue and stiffness of the ventricular wall induced by insufficient local myocardial blood supply and low oxygen saturation, leading to a decrease in myocardial compliance, contractility, and synchronization, and disturbances in myocardial electrophysiological activity (including reentrant arrhythmias and unidirectional conduction block, etc.), which ultimately hurt cardiac electrophysiological characteristics and organization, resulting in significant changes in cardiac pathophysiological function.¹³ Due to its severe complications and high mortality, it has become a hotspot and difficulty in the field of cardiovascular research.¹⁴ Reducing myocardial fibrosis is a significant part of HF treatment, and multiple pathways and greater inhibition of myocardial fibrosis have become a key research direction for regulating cardiac function and inhibiting the development of HF.¹⁵ Given the importance of the RAS/RAF/MEK/ERK signaling pathway in the life activities of cardiac fibroblasts, we prepared a rat model of postmyocardial infarction HF by ligating the left anterior descending branch of the coronary artery and intervened with ONSMP, and analyzed the mechanisms of inhibition of MF and slowing down of HF with ONSMP at the molecular level of this signaling pathway. It provides ideas and approaches for developing new drugs to prevent and treat HF.

The RAS/RAF/MEK/ERK signaling pathway is one of the core members of the mitogen-activated protein kinase signaling pathway family, which plays a significant role in controlling the survival and proliferation of cardiac fibroblasts. Excessive secretion of growth factors and

Table 3 mRNA of COL I, COL III, α -SMA, and c-Fos in myocardial tissue of rats in each group ($\bar{x} \pm s$)

Group	n	COL I/ β -actin	COL III/ β -actin	α -SMA/ β -actin	c-Fos/ β -actin
Sham	3	1.04 $\pm$ 0.29	1.04 $\pm$ 0.28	1.01 $\pm$ 0.15	1.08 $\pm$ 0.40
Model	3	7.69 $\pm$ 0.50 ^a	7.03 $\pm$ 0.74 ^a	7.36 $\pm$ 1.62 ^a	6.45 $\pm$ 0.70 ^a
ONSMP-L	3	4.85 $\pm$ 0.42 ^b	4.12 $\pm$ 0.22 ^b	5.02 $\pm$ 0.70 ^b	2.92 $\pm$ 0.16 ^b
ONSMP-M	3	2.73 $\pm$ 0.33 ^{bc}	2.19 $\pm$ 0.30 ^{bc}	3.26 $\pm$ 0.68 ^{bc}	2.05 $\pm$ 0.33 ^{bc}
ONSMP-H	3	1.32 $\pm$ 0.11 ^{bcd}	1.30 $\pm$ 0.05 ^{bcd}	1.79 $\pm$ 0.32 ^{bcd}	1.35 $\pm$ 0.05 ^{bcd}

Notes: sham group: sham operated; model group: ligation of the left anterior descending coronary artery without treatment; ONSMP-L: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 4.05 g crude drug/kg for 4 weeks; ONSMP-M: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 8.1 g crude drug/kg for 4 weeks; ONSMP-H: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 16.2 g crude drug/kg for 4 weeks; ENP: ligation of the left anterior descending coronary artery rats treated with ENP intragastrically at a daily dose of 0.9 g/kg for 4 weeks. COL I: collagen I; COL III: collagen III; α -SMA: α -smooth muscle actin; c-Fos: c-Fos proto-oncogene; ONSMP: optimized New Shengmai powder. The data were expressed as the mean $\pm$ standard deviation ($n = 3$). Compared with the sham group, ^a $P < 0.01$; compared with the model group, ^b $P < 0.01$; compared with the ONSMP-L group, ^c $P < 0.01$; compared with the ONSMP-M group, ^d $P < 0.01$.

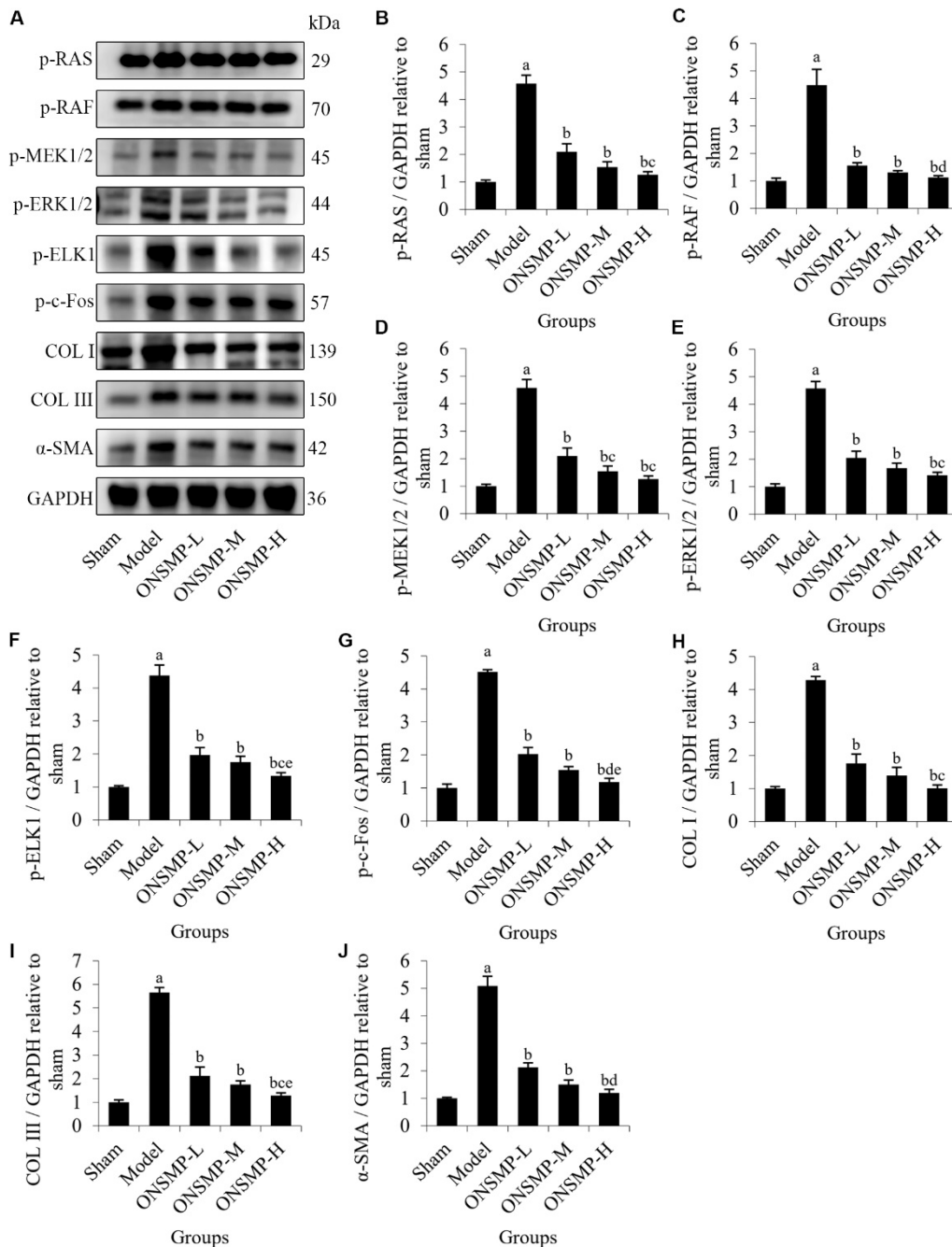


Figure 3 ONSMP inhibited the RAS/RAF/MEK/ERK pathway and downstream protein expression

A: Western blotting representative images of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, p-c-Fos, COL I, COL III, and α-SMA. B: protein expression levels of p-RAS; C: protein expression levels of p-RAF; D: protein expression levels of p-MEK1/2; E: protein expression levels of p-ERK1/2; F: protein expression levels of p-ELK1; G: protein expression levels of p-c-Fos; H: protein expression levels of COL I; I: protein expression levels of COL III; J: protein expression levels of α-SMA. Sham group: sham operated; model group: ligation of the left anterior descending coronary artery without treatment; ONSMP-L: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 4.05 g crude drug/kg for 4 weeks; ONSMP-M: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 8.1 g crude drug/kg for 4 weeks; ONSMP-H: ligation of the left anterior descending coronary artery rats treated with ONSMP intragastrically at a daily dose of 16.2 g crude drug/kg for 4 weeks; ENP: ligation of the left anterior descending coronary artery rats treated with ENP intragastrically at a daily dose of 0.9 g/kg for 4 weeks. p-RAS: phosphor-rat sarcoma; p-RAF: phosphor-rapidly accelerated fibrosarcoma; p-MEK1/2: phosphor-mitogen-activated protein kinase kinase 1/2; p-ERK1/2: phosphor-extracellular regulated protein kinases; p-ELK1: phosphor-ETS-like-1 transcription factor; p-c-Fos: phosphor-c-Fos proto-oncogene; COL I: collagen I; COL III: collagen III; α-SMA: α-smooth muscle actin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ONSMP: optimized New Shengmai powder; ENP: enalapril. Data represent the mean ± standard deviation using one-way analysis of variance ($n = 3$). Compared with the sham group, ^a $P < 0.01$; compared with the model group, ^b $P < 0.01$; compared with the ONSMP-L group, ^c $P < 0.05$, ^d $P < 0.01$; compared with the ONSMP-M group, ^e $P < 0.05$.

angiotensin II (Ang II) during the occurrence and development of HF will continuously stimulate the receptor tyrosine kinase (RTK) or G protein-coupled

receptor family member Ang II type 1 receptor (AT1R) on the cell membrane surface of cardiac fibroblasts, and initiate signal transmission at different positions in the

resting state.^{16,17} After RTK and AT1R are activated, they first phosphorylate src tyrosine kinase (Src) in a positive feedback manner, and p-Src then activates the proliferation of cardiac fibroblasts by activating downstream effector molecules. The process is as follows: (1) activated RTK directly phosphorylates Src;¹⁸ (2) Activated AT1R binds to and phosphorylates G protein-coupled receptor kinase (GRK), which then binds to β -arrestin to form a trimer that recruits and activates Src;¹⁹ (3) activated AT1R conformation occurs Changed, exposing the G protein binding site to stimulate G protein activation, G protein α Subunits ($G\alpha$) dissociates from G protein $\beta\gamma$ subunits ($G\beta\gamma$) to form a $G\alpha$: β -arrestin dimer with β -arrestin, recruiting and activating Src.²⁰ Activated Src phosphorylates and activates the adaptor protein p66 Shc (Shc), followed by the recognition and binding of phosphotyrosine residues of Shc by growth factor receptor-bound protein 2 (Grb2), which in turn binds to the son of sevenless protein (SOS), resulting in the formation of the Shc/Grb2/SOS complex.^{20,21} Both Shc/Grb2/SOS and $G\beta\gamma$ induced RAS activation.²²⁻²⁴ The p-RAS can bind to the amino terminus of RAF and induce its phosphorylation. On this basis, p-RAF catalyzes two regulatory serines on MEK1/2 to form p-MEK1/2, and p-MEK1/2 further selectively activates ERK1/2 and maintains the strong phosphorylation effect of ERK1/2.^{25,26} ERK1/2 translocates into the nucleus after receiving mitogen stimulation of upstream cascade response signals and activates ELK1, a transcriptional activator containing the ETS transcription factor domain, which is a transcriptional activator that responds extracellularly by inducing the expression of its target gene c-Fos Stimulate. The c-Fos gene can form a mature mRNA (c-Fos mRNA) of about 2.2 kb by transcription, and then the c-Fos mRNA is translated in the cytoplasm to produce a 55 ku c-Fos protein.²⁷⁻²⁹ Phosphatases that regulate the activity of c-Fos protein are not fully understood. Potential phosphatases are for regulation kinase (FRK) and ribosomal protein S6 kinase (S6K).^{30,31} When c-Fos protein is phosphorylated, it interacts with transcription factor Jun (c-Jun) through a basic “leucine zipper” to form c-Fos:c-Jun with transcriptional activation activity Heterodimer, c-Fos:c-Jun is a member of the AP-1 transcription factor family.³² Activator protein-1 (AP-1) then crosses the nuclear membrane into the nucleus, binds to the AP-1 site in the promoter regions of many genes, and increases the transcriptional activity of specific genes. Studies have confirmed that AP-1 can initiate myocardial fibroblast proliferation after being activated by Ang II.³³ Both the c-Jun subunit and the c-Fos subunit of AP-1 are critical for the progression of the cardiac fibroblast proliferation cycle.³⁴ The c-Jun recognizes and activates specific DNA sequences, such as cyclin D1,³⁵ which accelerates cell proliferation in the G1 phase, and Lysyl oxidase (LOX),³⁶ which is responsible for post-translational modification of COL I/III; AP-1 formed by binding of c-Fos to c-Jun is the most structurally stable of the many AP-1 dimers and

possesses enhanced DNA-binding and transcription-stimulating activities compared to the c-Jun:c-Jun complex.^{37,38} In any case, the half-life of the c-Jun:c-Fos/DNA complex is longer than that of the c-Jun:c-Jun/DNA complex.³⁹ Increased c-Fos protein shifts the c-Jun:c-Jun equilibrium to a c-Jun:c-Fos equilibrium, which alters the expression pattern of AP-1-dependent genes, leading to overexpression of cyclin D1 and LOX.⁴⁰ The up-regulation of cyclin D1 and LOX protein expression can accelerate the proliferation, migration, and differentiation of cardiac fibroblasts into myofibroblasts, and α -SMA is a marker of myofibroblasts.⁴¹ Myofibroblasts tend to promote the accumulation of extracellular matrix more rapidly than cardiac fibroblasts, which synergistically secrete large amounts of COL I and COL III, which cross-link with elastin residues, ultimately leading to the deposition of COL in the matrix, an increase in the content of mature COL fibers, and the generation of myocardial fibrosis and scarring that maintains the structural integrity of the myocardium under pathologic conditions.

The results of the ultrasound, organ coefficient, and cardiac histopathology in rats showed that ONSMP could improve LVEF and LVFS, reduce heart mass index and left ventricular mass index, and reduce myocardial fibrosis area in HF rats to varying degrees compared with the model group. A dose-effect relationship existed to some extent, with the high-dose group showing similar results to enalapril in improving cardiac function. The results of ELISA, RT-PCR, and Western Blot showed that ONSMP could dose-dependently inhibit the excessive accumulation of COL I/III fibers in myocardial tissue of HF rats, down-regulate the mRNA expression of COL I, COL III, α -SMA, c-Fos, and reduce the protein expression of p-RAS, p-RAF, p-MEK1/2, p-ERK1/2, p-ELK1, p-c-Fos, COL I, COL III, and α -SMA. This study suggests that ONSMP can effectively reduce the degree of myocardial fibrosis in HF rats, improve myocardial hypertrophy and remodeling, increase left ventricular contractility, and play a role in the treatment of HF, which may be related to the inhibition of the RAS/RAF/MEK/ERK signaling pathway activation to inhibit the proliferation and differentiation of cardiac fibroblasts. In conclusion, ONSMP may inhibit the proliferation and differentiation of cardiac fibroblasts by inhibiting the activation of the RAS/RAF/MEK/ERK signaling pathway, thereby reducing the excessive accumulation of COL I/III fibers, effectively reducing the degree of myocardial fibrosis in HF rats, improving myocardial hypertrophy and remodeling, improving left ventricular contractility, and ultimately playing a role in the treatment of HF.

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