Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Saponins from *Rhizoma Panacis Majoris* Attenuate Myocardial Ischemia/Reperfusion Injury via the Activation of the Sirt1/FoxO1/Pgc-1α and Nrf2/Antioxidant Defense Pathways in Rats

Hai-Bo He[1,2], Xiao-Mei Li[1], Dao-Jun Li[3], Yong-Hong Ding[1], Yong-Feng Zhang[1], Hai-Yan Xu[1], Min-Lu Feng[1], Chang-Qing Xiang[4], Ji-Gang Zhou[5], Ji-Hong Zhang[5], Hong-Jun Liu[5], Mao-Hua Chen[5]

[1]Department of Pharmacology, Hubei Key Laboratory of Natural Products Research and Development, College of Biological and Pharmaceutical Sciences, China Three Gorges University, [2]Institute of Cardiovascular Diseases, Key Laboratory of Ischemic Cardiovascular and Cerebrovascular Disease Translational Medicine, Three Gorges University, [3]Institute of Cancer Research, The First Clinical Medical College and Yichang Central Hospital, China Three Gorges University, [4]Institute of Integration of Traditional Chinese and Western Medicine, The Second People's Hospital, China Three Gorges University, [5]Institute of Cardiovascular Disease, Traditional Chinese Medicine Hospital of China Three Gorges University & Yichang Hospital of Traditional Chinese Medicine, Yichang, Hubei, China

Submitted: 10-10-2017 Revised: 05-02-2018 Published: 14-08-2018

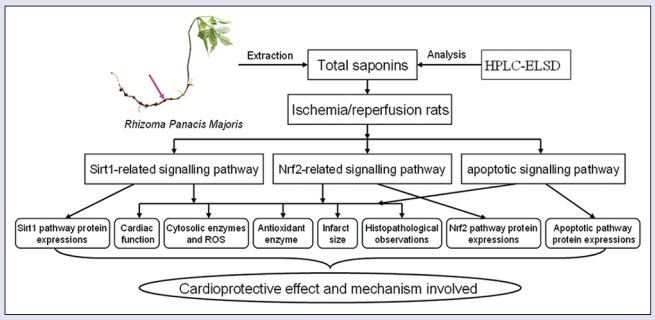
ABSTRACT

Background: Saponins from *Rhizoma Panacis Majoris* (SRPM) are confirmed to have cardioprotective effect against myocardial ischemia injury by reducing oxidative stress, while its underlying mechanism has not been elucidated until recently. **Objective:** The objective of this study was to investigate the SRPM's cardioprotection and elucidate its underlying mechanisms. **Materials and Methods:** Adult male rats were received SRPM treatment in the presence or the absence of the silent information regulator 1 (Sirt1) inhibitor Ex-527 or nuclear factor erythroid 2-related factor 2 (Nrf2) inhibitor ATRA for 14 days and subjected to myocardial ischemia for 0.5 h and then 2 h reperfusion. Cardiac function, infarct size, antioxidant enzyme activities, ROS level and the related mRNA and protein expressions of antiapoptosis protein Bcl-2, proapoptosis protein Bax, caspase-3, caspase-9, Sirt1 and Nrf2-relatived signaling pathways were assessed. **Results:** SRPM was confirmed to have cardioprotective effects by ameliorating cardiac function, decreasing infarct size, reducing serum creatine kinase (CK), creatine kinase isoenzyme, lactate dehydrogenase and ROS releases and malondialdehyde level, raising total antioxidant capacity, superoxide dismutase, glutathione peroxidase, catalase activities, upregulating myocardial Sirt1, Nrf2, Bcl-2 protein and manganese superoxide dismutase, heme oxygenase-1, NAD(P)H-quinone oxidoreductase 1 and γ-glutamylcysteine synthetase mRNA expressions and downregulating acetylated forkhead box O 1, acetylated peroxisome proliferator-activated receptor γ coactivator 1α, Bax, cleaved caspase-3 and cleaved caspase-9 protein expressions; histopathological observations provided supportive evidence for the aforementioned results. Interestedly, its protective effects were significantly blocked for its combination with Ex-527 or ATRA. **Conclusion:** The studies demonstrated that SRPM exerted beneficially protective effects on myocardial ischemia/reperfusion injury. It was possibly related to reducing oxidative stress damage by activations of Sirt1 and Nrf2-related antioxidant signaling pathways.

Key words: Cardioprotection, ischemia reperfusion, Nrf2 signaling pathway, oxidative stress, saponins from *Rhizoma Panacis Majoris,* Sirt1 signaling pathway

SUMMARY

- Extraction and analysis of saponins from *Rhizoma Panacis Majoris* (SRPM)
- Cardioprotective effect of SRPM was evaluated by cardiac function, infarct size, histopathological observations, biochemical assays, western blot analysis, and quantitative real-time polymerase chain reaction analysis as well as reactive oxygen species measurement in myocardial ischemia/reperfusion (I/R) rats
- SRPM exerted beneficially protective effects on myocardial I/R injury by activating silent information regulator 1 and Nrf2-related antioxidant signaling pathways and then reducing oxidative stress damage.



Abbreviations used: Ac-FoxO1: Acetylated FoxO1, Ac-Pgc-1α: Acetylated Pgc-1α, ARE: Antioxidant-response element, CK: Creatine kinase, CK-MB: Creatine kinase isoenzyme, DEPPD: N, N-diethyl-paraphenylendiamine, ELSD: Evaporative light scattering detection, FoxO: Forkhead box O, GCLC: Glutamate-cysteine ligase catalytic subunit, GSH-Px: Glutathione peroxidase, HO-1: Heme oxygenase-1, H_2O_2: Hydrogen peroxide, Keap-1: Kelch-like ECH-associated protein 1, HPLC: High-performance liquid chromatography, LDH: Lactate dehydrogenase, MDA: Malondialdehyde, NAD+: Nicotinamide adenine dinucleotide, MnSOD: Manganese superoxide dismutase, NF-κB: Nuclear factor-kappa B, NQO1: Nicotinamide quinone oxidoreductase 1, NAD(P) H: NAD(P)H-quinone oxidoreductase 1, Nrf2: Nuclear factor erythroid 2-related factor 2, Pgc-1α: Peroxisome proliferator-activated receptor γ coactivator 1α, Sirt1: Silent information regulator 1, SOD: Superoxide dismutase, SRPM: Saponins from *Rhizoma Panacis Majoris*, I/R: Ischemia/reperfusion, T-AOC: Total antioxidant capacity, TTC: 2,3,5-triphenyltetrazolium chloride.

Correspondence:

Dr. Mao-Hua Chen,
College of Clinical Traditional Chinese Medical Science, China Three Gorges University, 2 Shengli Three Road, Yichang 443001, Hubei, China.
E-mail: 79198685@qq.com
Prof. Dao-Jun Li,
The First Clinical Medical College and Yichang Central Hospital, China Three Gorges University, Yichang 443001, Hubei, China.
E-mail: doyleli@sina.com
DOI: 10.4103/pm.pm_467_17

Access this article online
Website: www.phcog.com
Quick Response Code:

Cite this article as: He HB, Li XM, Li DJ, Ding YH, Zhang YF, Xu HY, *et al*. Saponins from *Rhizoma Panacis Majoris* attenuate myocardial ischemia/reperfusion injury via the activation of the Sirt1/FoxO1/Pgc-1α and Nrf2/antioxidant defense pathways in rats. Phcog Mag 2018;14:297-307.

INTRODUCTION

Myocardial ischemia injury is a major cause of mortality in today's world. Although early reperfusion is necessary for myocardial ischemia injury salvage, reperfusion itself also exacerbates myocardial injury.[1] By analyzing the many theories regarding the development of myocardial ischemia/reperfusion (I/R) injury, we found that the enhanced oxidative stress during the acute reperfusion phase is the most likely one. In this process, reactive oxygen species (ROS) overproduction and accumulation, such as superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical, can overwhelm the intrinsic antioxidants and bring about a damage to cardiomyocytes, result in peroxidation of lipids, proteins, carbohydrates, and DNA, at last appear massive cell loss, cardiac dysfunction, and inevitably lead to heart failure.[2,3] Although many current interventions alleviating the extent of myocardial injury in myocardial I/R animals and patients have been tested, thus far none of them have exhibited definitive advantages over the control, which suggests that a novel mechanism of intervention is needed.[4]

Silent information regulator 1 (Sirt1), a key member of the mammalian sirtuin family, is a nicotinamide adenine dinucleotide (NAD+)-dependent histone deacetylase. It is confirmed to have a wide variety of functions in different biologic systems including gene transcription silencing, cell growth cycle, energy metabolism, insulin secretion, angiogenesis, and cellular senescence.[5] Using many histones and nonhistone proteins as substrates, Sirt1 may deacetylate and activate many transcription factors, such as forkhead box O (FoxO), p53, nuclear factor-kappa B (NF-κB) and so on, and nuclear coactivators, for example peroxisome proliferator-activated receptor γ coactivator 1α (Pgc-1α), c-AMP-responsive element-binding protein regulate transcription coactivator 2 and so forth, and then synthesize antioxidants, for instance manganese superoxide dismutase (MnSOD) and catalase (CAT), thereby promoting cellular resistance against oxidative stress.[6,7] Previous studies have shown that the deacetylased Sirt1 is specifically involved in cardioprotective effect against myocardial I/R injury, which affects apoptotic pathway via regulating the acetylation levels of FoxO1, Pgc-1α in the heart.[1,8] For instance, Hsu and his team confirmed that Sirt1 conferred a cardioprotective effect against myocardial I/R injury by upregulating antioxidants and downregulating proapoptotic molecules through the activation of FoxO1 as well as decreasing oxidative stress.[9]

Nuclear factor erythroid 2-related factor 2 (Nrf2), which plays a key role in cellular antioxidant defense, is a redox sensitive transcription factor. Usually the case, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap-1); when oxidative stress occurs, Nrf2 is rapidly degraded by the proteasome system, enters the nucleus, binds to antioxidant-response element (ARE), and activates ARE-dependent transcription of phase II and antioxidant defense enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H-quinone oxidoreductase 1 (NQO1), and γ-glutamylcysteine synthetase (GCLC, the rate-limiting enzyme for glutathione synthesis).[10] Accumulating evidences have demonstrated that Nrf2 is activated in various oxidative stress-mediated diseases, such as cardiovascular disease, neurodegenerative disease, and so forth.[11,12] Thus, Nrf2-mediated antioxidant signaling pathway plays an important role in strengthening cellular defence against oxidative stress.

Rhizoma Panacis Majoris, a common traditional herbal medicine in China Tujia and the Hmong, belongs to Araliaceae *Panax* family in the plant kingdom, which grows wild throughout the southwest region of China and Vietnam, Japan, Korea of Asian countries. In minority areas, it is used as substitutes for *Panax ginseng* and *Panax notoginseng*.[13] Saponins from *Rhizoma Panacis Majoris* (SRPM) are the most abundant and bioactive members in *Rhizoma Panacis Majoris*[14-16] and proved to have a wide range of biological activity, such as antioxidant, anti-inflammatory, antiapoptosis, antifatigue, antitumor, antiulcer, analgesia, immunoregulation, and hematogenesis.[14,17] Our previous study also confirmed that SRPM attenuated ischemia injury by reducing ischemia-induced mitochondrial oxidative damage through the removal of excessive ROS.[18,19] Although its role as a protective agent against myocardial oxidative damage mediated by I/R has also been investigated, the potential mechanisms for the antiapoptosis and antioxidant effects of SRPM have not been elucidated until recently.[20,21] In this study, we introduced Sirt1 inhibitor (Ex-527, a selective inhibitor of Sirt1) and Nrf2 inhibitor (ATRA, a selective inhibitor of Nrf2) to investigate whether Sirt1 and Nrf2-related signaling pathways play an important role in the SRPM-mediated cardioprotection, based on these results, explore its possible mechanisms.

MATERIALS AND METHODS

Materials

Authentic standards of Ginsenosides Rg1, Ginsenosides Re, Ginsenosides Ro, Pjs-2, Chikusetsusaponin IV, Chikusetsusaponin IVa, Notoginsenoside R2, Ginsenosides Rb1, Ginsenosides Rc, Ginsenosides Rd, and so on were purchased from National Institutes for Food and Drug Control (Beijing, China). Ex-527, ATRA, Evans blue dye, 2,3,5-triphenyltetrazolium chloride (TTC), N, N-diethyl-paraphenylendiamine (DEPPD) and ferrous sulfate were purchased from Sigma-Aldrich Co., LLC (St. Louis, USA). H_2O_2 was obtained from Sinopharm Chenmical Reagent Co., Ltd (Shanghai, China). Commercial kits used for determination of lactate dehydrogenase (LDH), creatine kinase (CK), CK-isoenzyme (CK-MB), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Polymerase chain reaction (PCR) primers of MnSOD, HO-1, NQO-1, GCLC and β-actin were synthesized by Shanghai Sangon Biotech Co., Ltd (Shanghai, China). Trizol, Prime Script RT reagent kit with gDNA Eraser, and SYBR Premix Ex TaqTM were purchased from Danlian TaKaRa Biotech Co., Ltd (Dalian, China). Antibodies for Sirt1, acetylated FoxO1 (Ac-FoxO1), acetylated Pgc-1α (Ac-Pgc-1α), Nrf2, Bcl-2, Bax, cleaved caspase-3, cleaved caspase-9, TBP and β-actin were obtained from Cell Signaling Technology, Inc (Danvers, USA). All other chemicals were of analytical grade.

Extraction and analysis of Saponins from *Rhizoma Panacis Majoris*

SRPM was extracted and analyzed according to the method of our previous study.[18] Briefly, the root of dried *Panacis Majoris* (1000 g), collected from Shennongjia Forest Region, was crushed into coarse powder and efluxed for extraction with 60% ethanol for three times per 2 h. The above three times of filtration was combined and then decompressed to concentrate. The obtained ethanol extract (about 375.3 g) was dissolved in distilled water and further extracted with petroleum ether, ethyl acetate, and n-butanol in turn, then the obtained extracts were concentrated, at last got 219.5 g n-butanol extract. It redissolved in water and poured into chromatographic column containing macroporous adsorption resin D101 (Tianjin, China). At first, it was rinsed with water, and then eluted with 30%, 60% and 90% ethanol to afford three fractions; finally, we got 91.3 g in the range of 90% as refined n-butanol extract. High performance liquid chromatography (HPLC) was performed with the above refined n-butanol extract to quantify and identify the species of SRPM in the sample. At first, it was redissolved in water at a concentration of 10 mg/mL, and then diluted with methanol: water (50:50) to 1 mg/mL. HPLC analysis was performed on an YMC-Pack ODS-AQ column (4.6 mm × 250 mm, 5 μm) eluted with mobile phases of acetonitrile (A) and 0.5% phosphate solution (B) at a flow rate of 1.0 mL/min. The

elution program was as follow: 0〜5 min, 5% A; 5〜20 min, 5%〜20% A; 20〜30 min, 20% A; 30〜50 min, 20%〜80% A; 50〜60 min, 80% A. Sample injection volume was 10 μL and the temperature of column was 30°C. Detection was evaporative light scattering detection (ELSD), the drift tube temperate was 40°C, and the nitrogen pressure was 33 Psi.

Surgical preparation

Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China Three Gorges University, and approved by the ethics committee, and the whole laboratory procedure was carried out under the permission and surveillance of the ethics committee. Male Wistar rats initially weighing 200 ± 20 g were purchased from the Laboratory Animal Research Center of China Three Gorges University, which were housed in constant conditions at a temperature of 20°C〜25°C, humidity of 50%〜60%, and on a 12 h light-dark cycle with free access to water and food. After adaptation of 1 week, the rat mycardial I/R operation was performed according to our previous study.[19] Briefly, the rats were anesthetized by 3% pentobarbital sodium and then ventilated in a volume-regulated respirator. Rat myocardial ischemia was produced by exteriorizing the heart through a left thoracic incision and placing a 6-0 silk suture and making a slipknot around the proximal left anterior descending coronary artery. Ischemia was confirmed by an ST elevation on the electrocardiography and the ischemic area change in color of the myocardial tissue. After 0.5 h of ischemia, the slipknot was released, and the myocardium was reperfused for 2 h. Sham-operated rats underwent the same surgical procedures except that the suture passed under the proximal left anterior descending coronary artery was left untied. Before the surgery, animals received different treatments. After 2 h reperfusion, the corresponding experiment were performed in rats. All surgical procedures were carried out under sterile conditions.

Experimental protocol

Male Wistar rats were randomly assigned to following experimental groups: (1) Sham: normal rats receiving no treatment but sham operation; (2) I/R + vehicle (I/R): normal rats receiving vehicle (0.5% carboxymethyl cellulose sodium, 0.5% CMC-Na) treatment and myocardial I/R operation; (3) I/R + SRPM (SRPM): normal rats receiving SRPM intragastric administration (200 mg/kg, 14 days before myocardial I/R operation; 200 mg/kg, 30 min before reperfusion) and myocardial I/R operation; (4) I/R + SRPM + Ex-527 (a selective Sirt1 inhibitor): normal rats receiving SRPM (following the same routine) and Ex-527 (5 mg/kg, 14 days before myocardial I/R operation; 5 mg/kg, 20 min before reperfusion) intraperitoneally and myocardial I/R operation; (5) I/R + SRPM + ATRA (a selective Nrf2 inhibitor): normal rats receiving SRPM (following the same routine) and ATRA (10 mg/kg, 14 days before myocardial I/R operation; 10 mg/kg, 20 min before reperfusion) intraperitoneally and myocardial I/R operation. SRPM was dissolved in 0.5% CMC-Na. Ex-527 and ATRA were first dissolved in dimethyl sulfoxide (DMSO), and then diluted to the final concentration with sterile saline (the final DMSO concentration <2%).

Measurement of hemodynamic parameters

Two hours reperfusion later, the rats were separated the right carotid artery and inserted the catheter into it. The catheter which connected to a multichannel recorder to measure pressure was advanced into the left ventricle and connected to the pressure transducer. Hemodynamic parameters were recorded using the MP150 Biopac system multichannel recorder (California, USA). After hemodynamic measurement, the blood samples were taken from abdominal aorta, and the hearts were quickly removed and then washed several times with normal saline.

Measurement of infarct zone and risk area

The myocardial infarct size was evaluated by Evans blue/TTC dual staining as our previous study.[22] Briefly, 2 h reperfusion later, the ligature around the coronary artery was retied before 1 mL of 2% Evans blue dye was injected into carotid artery to delineate the area at risk (AAR). After 5 min, the heart was excised and sliced horizontally to yield five slices of equal thickness. The slices were incubated for 15 min in 1% TTC at 37°C and then fixed in 4% paraformaldehyde. After stained by Evans blue/TTC, the viable myocardium appeared blue, AAR was red, and necrotic myocardium appeared pale white. The slice that had the longest circumference was imaged, and the area of infarction on both sides of the slice was determined by the Image J Morphology Analysis System (exploited by National Institutes of Health).

Histomorphological observation

Four rats' hearts in each group were randomly selected, and cut from apex to base into five equal transverse sections. The middle slice of heart tissue was fixed in 4.0% paraformaldehyde for 24 h, dehydrated in the different concentrations of alcohol, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin. At last, the sections were observed under microscope.

Ultrastructural observation

The random selected left ventricular tissues (about 1 mm × 1 mm × 1 mm from peri-infarct area in each rat) of four animals from each group were fixed in the 2% glutaraldehyde for 24 h, washed with 0.2M phosphate buffer for three times, fixed in the 1% osmium tetroxide, and dehydrated by ethanol at different concentrations. The samples were immersed in epoxy resins for 0.5 h, embedded for convergence for 12 h. The tissue was cut into slices of 50 nm thickness by ultramicrotome and then observed the ultrastructure of cardiac muscle cells by transmission electron microscopy (Hitachi, Japan).

Measurement of serum reactive oxygen species

The collected blood samples were centrifuged at 3500 rpm at 4°C for 10 min. Supernatant serums were transferred to clean EP tubes and stored at −80°C. Serum ROS level was detected according to Hayashi and his colleague previous study.[23] Briefly, in 0.1M pH = 4.8 sodium acetate buffer, DEPPD was dissolved to get 100 μg/mL final concentration, and ferrous sulfate was dissolved in sodium acetate buffer to achieve 4.37 μM final concentration. DEPPD and ferrous sulfate were mixed in a ratio of 1:25 to make analysis solution. This solution was then added as starter in a 3 mL cuvette followed by the addition of sodium acetate buffer and H_2O_2 in order to be used as positive control, while the absorbance of serum samples was measured at 505 nm using ultraviolet-visible spectrophotometer (Hitachi, Japan).

Biochemical analysis

LDH, CK, CK-MB, T-AOC, SOD, GSH-Px activities, and MDA level in serum were measured by chemichromatometry according to the directions of the reagent kits.

Western blotting analysis

Nuclear and total proteins were extracted from the peri-infarct area tissue with a nuclear and total protein extract kits. Protein concentration was determined using a bicinchoninic acid protein assay kit. Samples (80 μg protein each) were separated by 10%〜15% SDS-PAGE gels, and then transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk with TBST buffer (20 mm Tris-HCl, 150 mm NaCl, 0.05% [V/V] Tween

20 for 1 h). After blocking, the membranes were incubated with the following primary antibodies: anti-Sirt1 (1:500), anti-Ac-FoxO1 (1:400), anti-Ac-Pgc-1α (1:400), anti-Nrf2 (1:400), anti-Bcl-2 (1:400), anti-Bax (1:400), cleaved caspase-3 (1:400), cleaved caspase-9 (1:400), β-actin (1:1000) and TBP (1:1000) overnight at 4°C, and subsequently with horseradish peroxidase-conjugated secondary antibody (1:3000) at room temperature for 1 h. Protein bands were visualized on X-ray film using ECL, and molecular expressions were normalized to β-actin or TBP. Quantitative analysis was performed by Image J software.

Quantitative real-time polymerase chain reaction analysis

Total RNA of the peri-infarct area tissue was extracted using Trizol reagent according to the manufacturer's protocol. Quantity and purity of RNA were evaluated by the ratio of absorbance at 260 and 280 nm by using a spectrophotometer. cDNA was prepared from the total RNA (1 μg) with a reverse transcriptase Primer Mix using the PrimeScript RT reagent Kit with gDNA Eraser according to the instruction. PCR primers of MnSOD, HO-1, NQO-1, GCLC and β-actin were synthesized by Shanghai Shenggong Bilocolor BioScience and Technology (Shanghai, China). Primers for quantitative real-time PCR analysis in this study were shown in Table 1. Subsequent PCR amplification was carried out on a Light Cycler system (software v 4.0; Roche Applied Science) using 40 cycles of 94°C for 5 s, 58°C for 30 s, and 72°C for 45 s. β-actin was used as an internal control. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method using cycle time values and data for normalization.

Statistical analysis

All data were shown as the mean ± standard deviation. Statistical analyses were performed using SPSS 19.0 software package (Armonk, NY, USA). Differences among groups were analyzed by one-way analysis of variance. *Post hoc* testing was performed for intergroup comparisons using the least significant difference test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Composition and content of the saponins

In the chromatographic fingerprint of SRPM analyzed by HPLC-ELSD detector, 12 chromatogram peaks were marked. According to compound molecular weight data and comparing their retention times with those of authentic standards, peaks numbered 1–10 were successively identified as Ginsenosides Rg1, Ginsenosides Re, Ginsenosides Ro, Pjs-2, Chikusetsusaponin IV, Chikusetsusaponin IVa, Notoginsenoside R2, Ginsenosides Rb1, Ginsenosides Rc, Ginsenosides Rd. The other two peaks numbered 11 and 12 were unidentified. By HPLC-ELSD analysis, SRPM contents were 92.65% [Figure 1].

Improvement of Saponins from *Rhizoma Panacis Majoris* on cardiac function

In the I/R group, there were markedly compromised systolic and diastolic functions compared with the sham group ($P < 0.01$, respectively). SRPM treatment significantly improved myocardial I/R injury heart systolic and diastolic functions compared with the I/R group ($P < 0.01$, respectively). However, in the I/R + SRPM + Ex-527 and I/R + SRPM + ATRA groups, there is little amelioration of the systolic and diastolic functions compared with the I/R group. On the contrary, the I/R+SRPM+Ex-527 and I/R+SRPM+ATRA groups significantly decreased in improving cardiac function compared with the I/R+SRPM group ($P<0.05$ or $P<0.01$, respectively), HR did not significantly differ among all groups. The above results demonstrated that Ex-527 and ATRA attenuated the protective action of SRPM against myocardial I/R injury in the rats [Table 2].

Attenuation of Saponins from *Rhizoma Panacis Majoris* on myocardial damage

CK, CK-MB, and LDH activities were used to monitor the myocardial damage. In the I/R group, serum CK, CK-MB, and LDH activities were remarkably raised compared the sham group ($P < 0.01$, respectively). SRPM treatment effectively reversed the aforementioned abnormal changes compared with the I/R group ($P < 0.01$, respectively). However, its combination with Ex-527 or ATRA directly resulted in appearing the significant inhibitory effects compared with the I/R + SRPM group ($P < 0.05$ or $P < 0.01$, respectively) [Table 3].

Regulatory role of Saponins from *Rhizoma Panacis Majoris* on serum reactive oxygen species level

To determine whether SPRM treatment mitigated myocardial I/R-induced oxidative stress, serum ROS level was detected by the derivatives of reactive oxygen metabolites test. As shown in Figure 2, serum ROS level of the I/R group significantly increased compared with sham group ($P < 0.01$). SRPM treatment effectively decreased the ROS level compared with the I/R group ($P < 0.01$), and the effect was also significantly inhibited by its combination with Ex-527 or ATRA compared with the I/R + SRPM group ($P < 0.05$ or $P < 0.01$, respectively). These results suggested that SRPM was capable of reducing intracellular ROS overproduction following myocardial I/R injury stimulation, which indicated that SPRM's cardioprotective effect owed to scavenging oxidative stress-triggered overgeneration and accumulation of ROS during myocardial I/R injury.

Enhancement of Saponins from *Rhizoma Panacis Majoris* on antioxidant enzyme activities

In the I/R group, the serum antioxidant activity was found in the descended activities of T-AOC (64.7%), SOD (32.1%), GSH-Px (31.0%) and CAT (47.5%) and increased MDA level (104.9%) compared with the sham group ($P < 0.01$, respectively). SRPM treatment significantly increased T-AOC, SOD, GSH-Px, CAT activities and decreased MDA level compared with the I/R group, but these improved effects were remarkably inhibited for its combination with Ex-527 or ATRA compared with the I/R + SRPM group ($P < 0.05$ or $P < 0.01$, respectively) [Table 4].

Table 1: Primer sequences quantitative real-time polymerase chain reaction used in the study

Genes	Forward primer	Reverse primer	Lengths of amplicons (bp)
MnSOD	GAGCAAGGTCGCTTACAGA	CTCCCCAGTTGATTACATTC	195
HO-1	TCCTGCGATGGGTCCTCACACTC	CAGCCGCCTCTACCGACCACAG	340
NQO1	GCGTCTGGAGACTGTCTGGG	CGGCTGGAATGGACTTGC	170
GCLC	TTGCGGGGGTGCTTGTTTATGG	GTTACTGAATGGCGGCGATGTT	357
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC	150

MnSOD: Manganese superoxide dismutase; HO-1: Heme oxygenase-1; NQO1: NAD(P)H-quinone oxidoreductase 1; GCLC: γ-glutamylcysteine synthetase

Table 2: Effect of Saponins from Rhizoma Panacis Majoris on hemodynamic parameters in the ischemia/reperfusion rats

Group	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	LV+dp/dtmax (mmHg/s)	LV-dp/dtmin (mmHg/s)
Sham	381.4±22.0	143.2±3.5	5.33±0.66	3920.8±135.1	−3358.6±146.5
I/R	386.6±18.3	119.8±3.8##	15.21±1.76##	2940.2±288.8##	−2786.9±157.0##
I/R + SRPM	382.9±15.6	134.1±4.3**	9.69±1.10**	3568.9±161.2**	−3122.2±105.3**
I/R + SRPM + Ex-527	383.6±13.1	125.4±5.2†	13.10±1.23††	3231.2±185.9†	−2902.4±123.8†
I/R + SRPM + ATRA	384.2±18.2	126.3±5.3†	12.53±1.50*,††	3292.0±197.6†	−2963.4±105.3†

##*P*<0.01 compared with the sham group; **P*<0.05, ***P*<0.01 compared with the I/R group; †P<0.05, ††P<0.01 compared with the I/R + SRPM group. Data are shown as the mean±SD (*n*=10). I/R: Ischemia/reperfusion; SRPM: Saponins from Rhizoma Panacis Majoris; HR: Heart rate; LV: Left ventricular; LVSP: Left ventricular systolic pressure; LVEDP: Left ventricular end-diastolic pressure; LV+d*p*/d*t*max: Maximal rate of LV systolic pressure; LV-d*p*/d*t*min: Minimum rate of LV systolic pressure; SD: Standard deviation

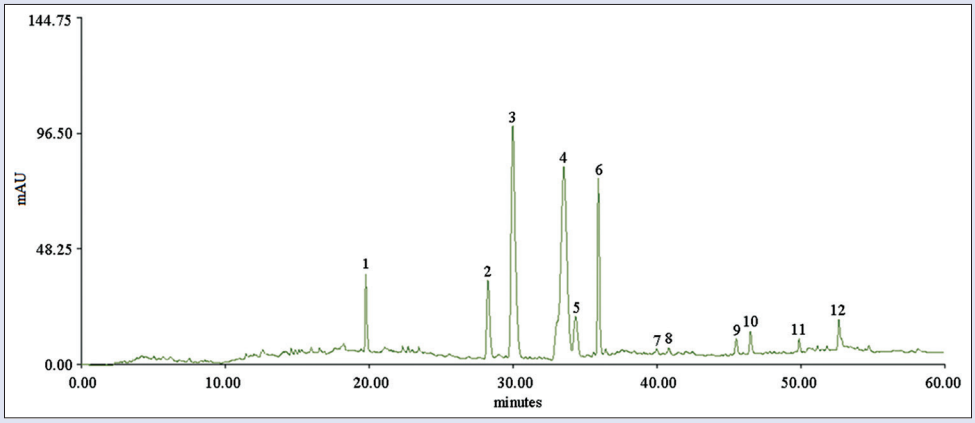


Figure 1: The typical chromatographic fingerprint of SRPM decoction analyzed by high performance liquid chromatography evaporative light scattering detection. 1: Ginsenosides Rg1, 2: Ginsenosides Re, 3: Ginsenosides Ro, 4: Pjs-2, 5: Chikusetsusaponin IV, 6: Chikusetsusaponin IVa, 7: Notoginsenoside R2, 8: Ginsenosides Rb1, 9: Ginsenosides Rc, 10: Ginsenosides Rd. SRPM: Saponins from *Rhizoma Panacis Majoris*

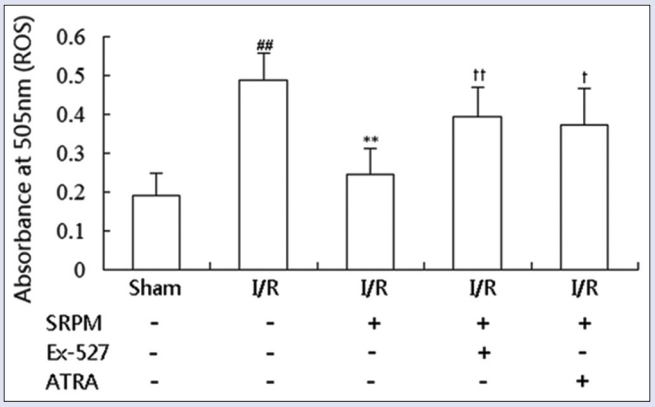


Figure 2: Effect of SRPM on ROS in the serum of the I/R injury rats. Data are shown as the mean ± standard deviation (*n* = 10). ##*P* < 0.01 compared with the sham group; **P* < 0.01 compared with the I/R group; †*P* < 0.05, ††*P* < 0.01 compared with the I/R + SRPM group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*; ROS: reactive oxygen species

Reducement of Saponins from *Rhizoma Panacis Majoris* on the infarct size

To evaluate the direct effect of SRPM treatment on rat myocardial I/R injury, we detected the infarct size by using Evans-TTC method. Representative images of infarct size were shown in Figure 3a. In the I/R group, the IS/ARR and IS/LV of the heart were up to 70.8% (70.8 ± 7.1) and 31.6% (31.6 ± 5.5) compared with the sham group (*P* < 0.01, respectively). After treated with SRPM, there was conspicuously decrease in IS/ARR and IS/LV by 55.1% (55.1 ± 3.0) and 20.9% (20.9 ± 1.8) compared with the I/R group (*P* < 0.01, respectively). However, there is little amelioration of the infarct size in the I/R + SRPM + Ex-527 and I/R + SRPM + ATRA groups compared with the I/R group and significantly decreased compared with the I/R + SRPM group (*P* < 0.05 or *P* < 0.01, respectively). There was no remarkable alteration in AAR/LV among the I/R and SRPM treated groups [Figure 3b-d].

Improvement of Saponins from *Rhizoma Panacis Majoris* on myocardial histopathological architecture

In the sham group, the myocardium appeared normal tissue structure and shape, chear nuclei, no inflammatory cell infiltration. Whereas heart tissues from I/R rats presented extensive myocardial structure disorder and rupture, the necrosis and "wavy" forms of myocardial fibers were observed, myocardial cell nuclei disappeared. SRPM treatment might significantly rescued these changes compared with the I/R group. However, its improvement was markedly inhibited for its combination with Ex-527 or ATRA compared with the I/R + SRPM group [Figure 4].

Improvement of Saponins from *Rhizoma Panacis Majoris* on myocardial ultrastructure

Ultrastructure changes of cardiac myocytes were observed by transmission electron microscopy. As shown in Figure 5, clear sarcomere, intact cell membrane, rich mitochondria in normal size and regular shape with high density of cristae were seen in cardiac myocytes. Abnormal sarcomere and structural changes in mitochondria, for example, widespread vacuolization, vague, and partly dissolved cristales were observed. After treated with SRPM, impaired sarcomeres were

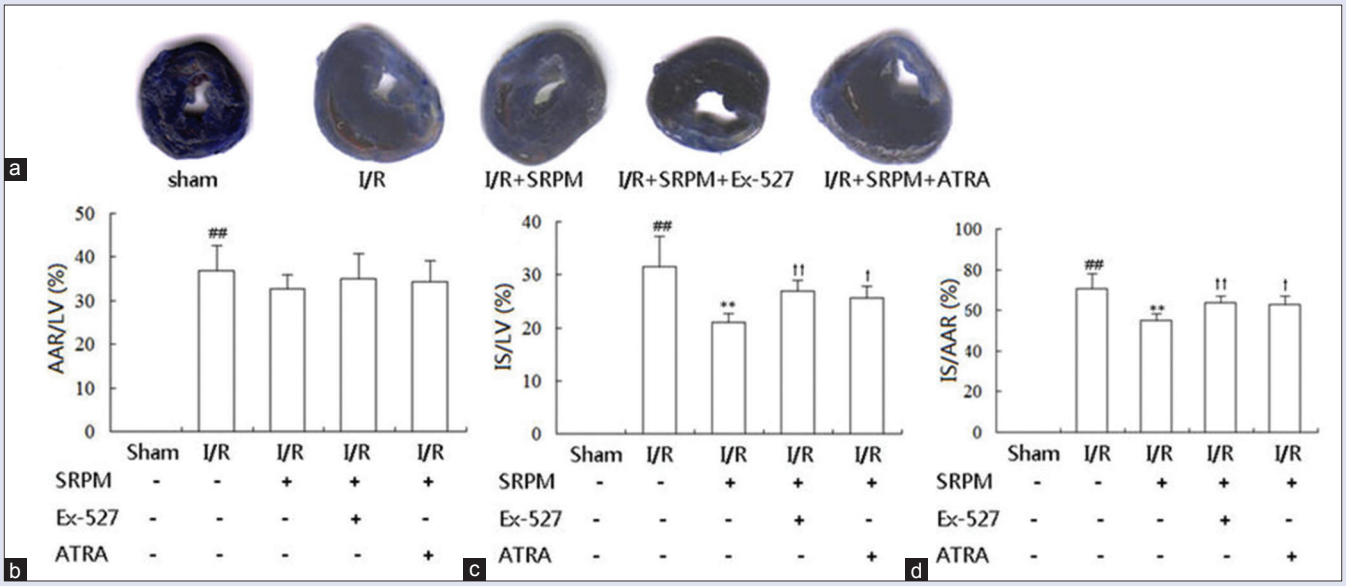


Figure 3: Effect of SRPM on infarct size in the I/R rats. (a) Representative images of infarct size as stained by Evans blue and TTC; (b) percentage of area at risk to left ventricular; (c) percentage of infarcted size to left ventricular area; (d) percentage of infarcted size to area at risk. Data are shown as the mean ± standard deviation (n = 8). $^{\#\#}P < 0.01$ compared with the sham group; $^{**}P < 0.01$ compared with the I/R group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the I/R + SRPM group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*; TTC: 2,3,5-Triphenyltetrazolium chloride

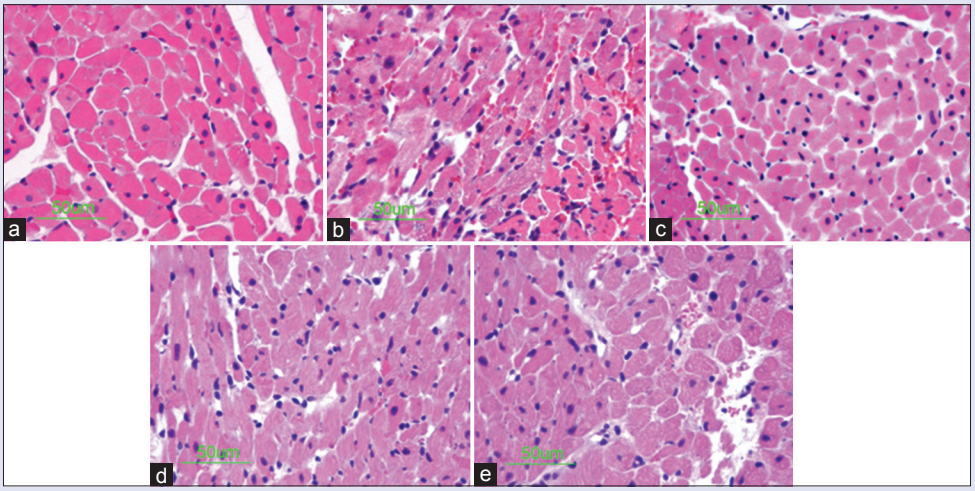


Figure 4: Effect of SRPM on I/R induced myocardial damage in rats by hematoxylin and eosin (H and E) staining. (a): Sham group; (b): I/R group; (c): I/R + SRPM group; (d): I/R + SRPM + Ex-527 group; (e): I/R + SRPM + ATRA group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*

Table 3: Effect of Saponins from Rhizoma Panacis Majoris on serum creatine kinase, creatine kinase isoenzyme and lactate dehydrogenase activities in the ischemia/reperfusion rats

Group	CK (U/mL)	CK-MB (U/mL)	LDH (U/mL)
Sham	0.52±0.08	0.33±0.06	0.86±0.14
I/R	1.51±0.19$^{\#\#}$	1.13±0.27$^{\#\#}$	3.81±0.47$^{\#\#}$
I/R + SRPM	0.96±0.12**	0.57±0.12**	1.72±0.22**
I/R + SRPM + Ex-527	1.38±0.16††	0.89±0.15††	2.94±0.31*,††
I/R + SRPM + ATRA	1.29±0.22†	0.82±0.19†	2.87±0.42*,††

$^{\#\#}P<0.01$ compared with the sham group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the I/R group; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared with the I/R + SRPM group. Data are shown as the mean±SD (n=10). CK: Creatine kinase; CK-MB: Creatine kinase-MB; LDH: Lactate dehydrogenase; I/R: Ischemia/reperfusion; SRPM: Saponins from Rhizoma Panacis Majoris; SD: Standard deviation

regularly rearranged, welling and vacuolization were significantly reduced, even a part of mitochondria structure had returned to normal. However, when it was combined with Ex-527 or ATRA, the aforementioned improvements on myocardial ultrastructure were significantly inhibited.

Effect of Saponins from *Rhizoma Panacis Majoris* on silent information regulator 1-related signaling pathway

To determine whether SRPM administration affected Sirt1/FoxO1/Pgc-1α-mediated antioxidant signaling, we examined Sirt1, Ac-FoxO1, and Ac-Pgc-1α protein expressions. As shown in Figure 6, Sirt1 expression was markedly downregulated, and Ac-FoxO1

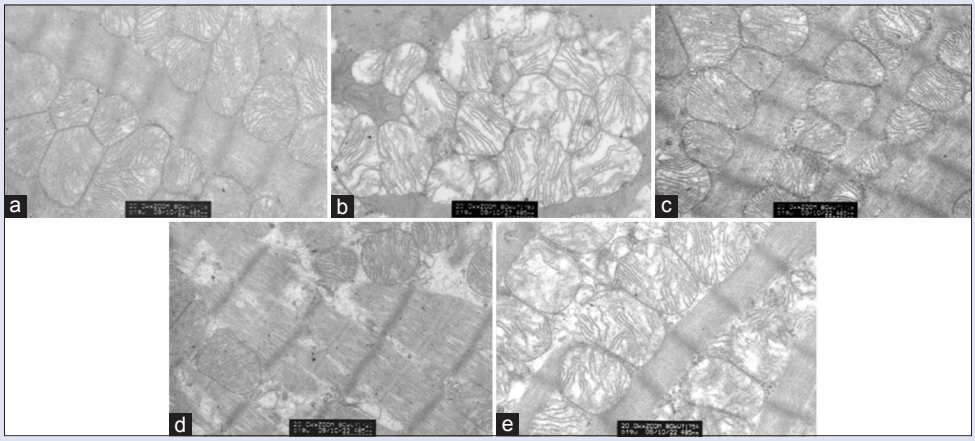


Figure 5: Effect of SRPM on ultrastructural changes in the I/R injury rats. (a): Sham group (×20,000); (b): I/R group (×20,000); (c): I/R + SRPM group (×20,000); (d): I/R + SRPM + Ex-527 group (×20,000); (e): I/R + SRPM + ATRA group (×20,000). I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*

Table 4: Effect of Saponins from Rhizoma Panacis Majoris on antioxidant assay in the serum of the ischemia/reperfusion rats

Group	T-AOC (U/mL)	SOD (U/mL)	GSH-Px (U/L)	CAT (U/mL)	MDA (nmol/mL)
Sham	32.74±4.15	171.9±13.4	840.2±35.6	21.79±1.55	2.47±0.35
I/R	11.57±2.02$^{\#\#}$	116.8±14.1$^{\#\#}$	579.5±50.8$^{\#\#}$	11.43±1.43$^{\#\#}$	5.06±0.39$^{\#\#}$
I/R + SRPM	25.94±3.90**	153.0±12.2**	697.1±40.3**	15.83±1.39**	3.65±0.37**
I/R + SRPM + Ex-527	15.09±2.90††	128.3±11.0††	603.7±34.7††	12.78±1.50††	4.59±0.41††
I/R + SRPM + ATRA	16.76±3.62*,††	133.4±12.9†	621.9±39.2†	13.35±1.43†	4.49±0.43††

$^{\#\#}P$<0.01 compared with the sham group; *P<0.05, **P<0.01 compared with the I/R group; $^{\dagger}P$<0.05, $^{\dagger\dagger}P$<0.01 compared with the I/R+SRPM group. Data are shown as the mean±SD (n=10). I/R: Ischemia/reperfusion; SRPM: Saponins from Rhizoma Panacis Majoris; SD: Standard deviation; T-AOC: Total antioxidant capacity; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde; CAT: Catalase

and Ac-Pgc-1α expressions were significantly upregulated in the I/R group compared with the sham group (P < 0.01, respectively). SRPM treatment remarkably upregulated Sirt1 expression and downregulated Ac-FoxO1 and Ac-Pgc-1α expressions compared with the I/R group (P < 0.01, respectively). However, when it was combined with Ex-527, SRPM-induced reductions in Ac-FoxO1 and Ac-Pgc-1α expressions, and augmentation in Sirt1 were significantly abolished compared with the I/R + SRPM group (P < 0.01, respectively). These results indicated that SRPM's cardioprotective effect was associated with Sirt1-related signaling pathway activation.

Effect of Saponins from *Rhizoma Panacis Majoris* on nuclear factor erythroid 2-related factor 2-related signaling pathway

In order to corroborate whether SRPM effected myocardial antioxidant signaling pathway, activated Nrf2 (translocation into nuclei) and its downstream regulatory enzymes in heart were investigated. As shown in Figure 7a and b, Nrf2 protein level in nuclei was significantly downregulated in the I/R group compared with the sham group (P < 0.01), whereas SRPM treatment effectively promoted Nrf2 translocation into nucleus compared with the I/R group (P < 0.01). Furthermore, mRNA levels of MnSOD, a key antioxidative enzyme downstream of Sirt1, and HO-1, NQO-1 and GCLC, the important phase II enzymes downstream of Nrf2, were examined using quantitative real-time PCR. As shown in Figure 7c and d, mRNA levels of MnSOD, HO-1, NQO-1 and GCLC in the I/R group significantly decreased compared with the sham group. SRPM treatment significantly reversed those to similar levels as in the sham group. However, when it was combined with Ex-527, SRPM-induced augmentation in MnSOD expression was significantly attenuated compared with the I/R + SRPM group (P < 0.01); When it was combined with ATRA, SRPM-induced augmentations in HO-1, NQO-1 and GCLC expressions were remarkably abolished compared with the I/R + SRPM group (P < 0.01, respectively). The aforementioned results indicated that SRPM treatment activated the Sirt1 and Nrf2 expression, thus increasing MnOD, HO-1, NQO-1 and GCLC levels. Our results demonstrated that SRPM's cardioprotective effect was related to Sirt1 and Nrf2-related signaling pathway activation.

Effect of Saponins from *Rhizoma Panacis Majoris* on apoptotic signaling pathway

To further confirm whether the potential signaling pathways also involved in SRPM's protective effects, we next evaluated myocardial apoptotic signaling pathway. As showed in Figure 8, Bcl-2 expression was markedly downregulated, and Bax expressions were significantly upregulated in the I/R group compared with the sham group (P < 0.01, respectively). SRPM treatment significantly upregulated Bcl-2 expression, and downregulated Bax expression (P < 0.01, respectively). In addition, I/R-induced cleaved caspase-3 and cleaved caspase-9 activations was markedly inhibited after SRPM treatment compared with the I/R group (P < 0.01, respectively). These results indicated that SRPM treatment may inhibit the apoptotic signaling pathway. To establish a more distinct relationship between SRPM treatment and its antiapoptotic property in I/R injury, we analyzed whether the combination of Ex-527 or ATRA abolished SRPM's antiapoptotic effect. As shown in Figure 8, its combination with Ex-527 or ATRA attenuated antiapoptotic signaling by decreasing Bcl-2 expression level, while increasing Bax, cleaved caspase-3 and cleaved caspase-9 expression levels compared with the I/R + SRPM group (P < 0.05, P < 0.01 respectively). These results indicated that SRPM's protective effect was associated with apoptotic signaling pathway inhibition.

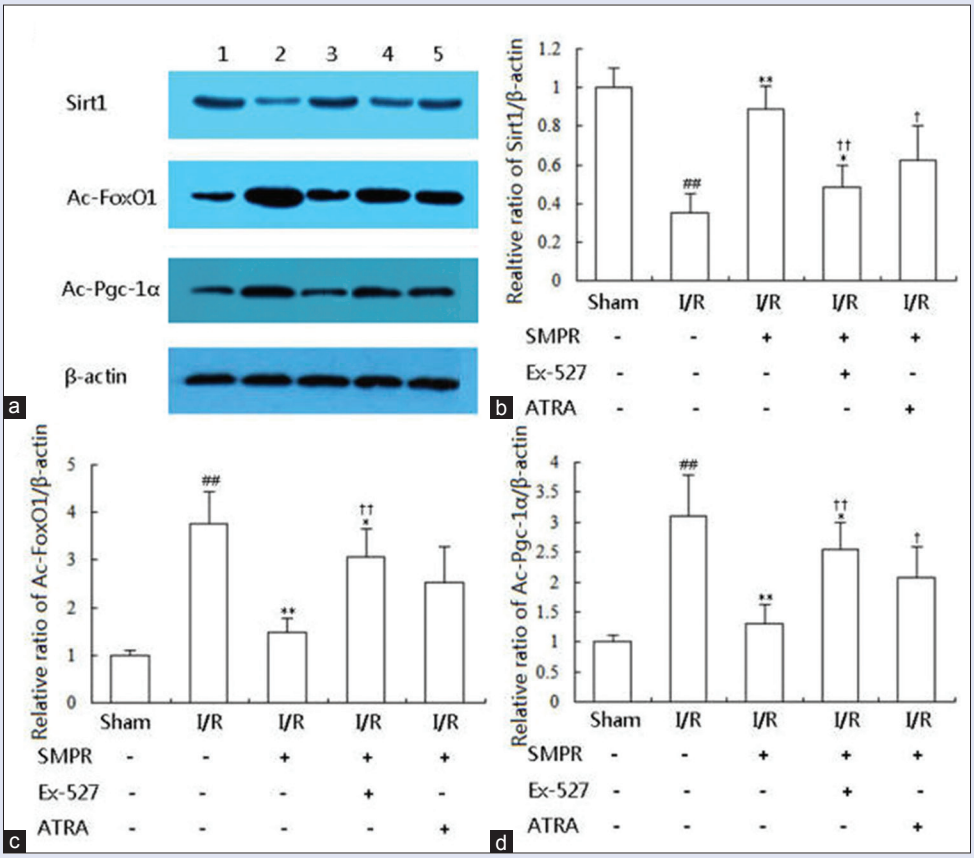


Figure 6: Effect of SRPM treatment on Sirt1-related signaling pathway in the I/R injury rats. (a) Representative western blotting bands of Sirt1, Ac-FoxO1, and Ac-Pgc-1α (1: Sham group, 2: I/R group, 3: I/R + SRPM group, 4: I/R + SRPM + Ex-527 group, 5: I/R + SRPM + ATRA group); (b) Sirt1 expression; (c) Ac-FoxO1 expression; (d) Ac-Pgc-1α expression. Data are shown as the mean ± standard deviation ($n = 5$). $^{\#\#}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the I/R group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the I/R + SRPM group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*

DISCUSSION

In this study, our results demonstrated that SRPM had beneficial effect on cardiac function in myocardial I/R rats, which was in line with the improvements in decreasing infarct size, ameliorating myocardial histopathological observations, reducing serum CK, CK-MB, LDH and ROS releases and MDA level, raising T-AOC, SOD, GSH-Px, CAT activities, upregulating myocardial Sirt1, Nrf2, Bcl-2 protein and MnSOD, HO-1, NQO1 and GCLC mRNA expressions, and downregulating Ac-FoxO1, Ac-Pgc-1α, Bax, cleaved caspase-3 and cleaved caspase-9 protein expressions. Interestedly, its protective effects were significantly attenuated for its combination with Ex-527 or ATRA. Our present study indicated that SRPM's cardioprotective effect was possibly related to reducing oxidative stress damage by activating Sirt1 and Nrf2-related antioxidant signaling pathways.

Generally, LDH, CK, CK-MB, and so on in the cytosolic enzymes served as the myocardial I/R injury diagnostic markers leak out from the damaged myocardial tissues to the blood stream. Clinically, these releases are usually the main indicators of the extent of myocardial ischemia injury, and also an important indicator to determine the cardiac cell from reversible damages to irreversible ones.[3,24] In the present study, we found that SPRM might remarkably reduce the elevated serum LDH, CK and CK-MB levels in the I/R rats. Interestingly, these aforementioned effects were markedly inhibited for its combination with Ex-527 or ATRA, which were also in line with Yin and other author previous reports.[25] These results indicated that SPRM decreased cell membrane damage, permeability and protein release in myocardial I/R heart, and then delayed the myocardial I/R injury process.

It is well known that oxidative stress causes the overproduction and accumulation of ROS, such as H_2O_2, superoxide anion, and hydroxyl radicals and so forth, which are thought to be the key players in myocardial I/R injury.[26] Usually, the generation of ROS is a part of normal cell metabolism and defense, cellular systems also produce antioxidant defenses against ROS. Myocardial I/R causes alterations in the defense mechanism against oxygen free radicals, overproduction ROS exceeds the capacity of antioxidant defenses, and at last brings about oxidative damage of membrane lipids, proteins, carbohydrates, and DNA.[27] Clinical evidence shows that oxidative stress is associated with cardiac dysfunction after surgical reperfusion or after thrombolysis of the entire heart.[28] In addition, ROS significantly contributes to the acceleration of necrosis in myocardial injury caused by I/R, resulting in the extension of infarction, apoptosis, arrhythmogenesis, and endothelial dysfunction.[28] Based on these conditions, the prevention of oxidative stress is considered as a reasonable therapeutic strategy for alleviating myocardial I/R injury.[26] In the study, we found that serum ROS level in I/R rats significantly increased. However, treated with SPRM, the increased ROS level in the I/R + SRPM group was markedly inhibited. Interestedly, the effect was significantly inhibited for its combination with Ex-527 or ATRA. The result indicated that SPRM exerted protective effect on I/R-induced myocardial injury by scavenging oxidative stress-triggered ROS overproduction, which was similar to the result of our previous result.[29] To defend against possible

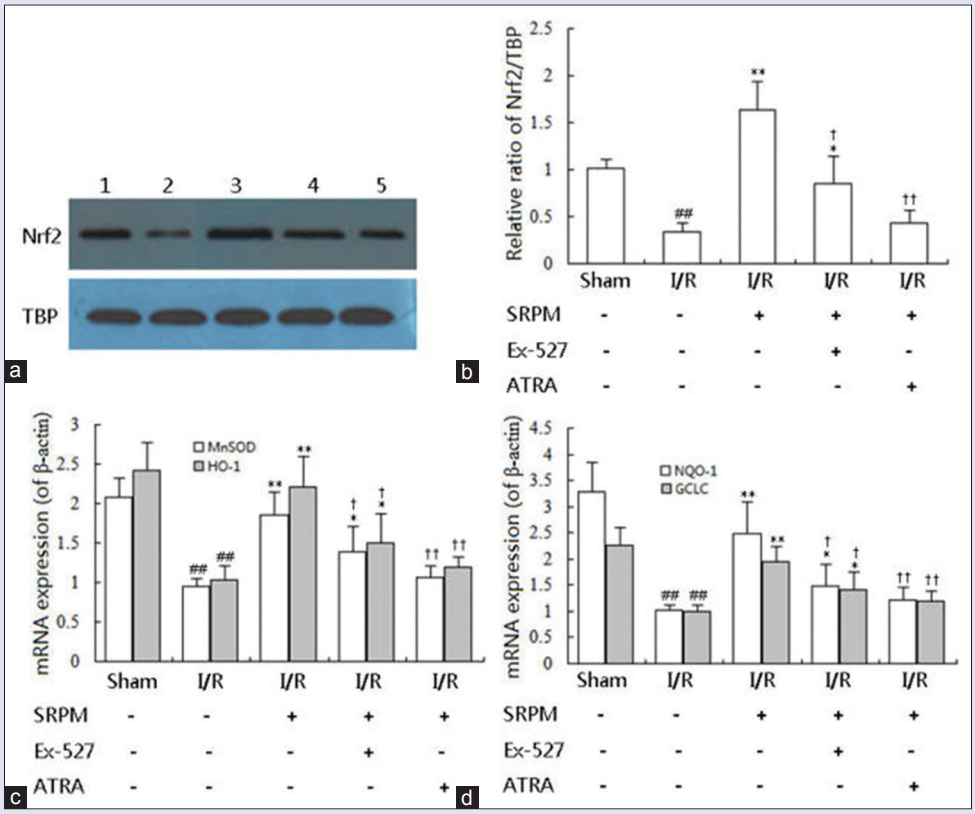


Figure 7: Effect of SRPM treatment on Nrf2-related signaling pathway in the I/R injury rats. (a) Representative western blotting bands of Nrf2 (1: Sham group; 2: I/R group; 3: I/R + SRPM group; 4: I/R + SRPM + Ex-527 group; 5: I/R + SRPM + ATRA group); (b) Nrf2 protein expression; (c) MnSOD and HO-1 mRNA expressions; (d) NQO-1 and GCLC mRNA expressions. Data are shown as the mean ± standard deviation (n = 5). $^{\#\#}P < 0.01$ compared with the sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the I/R group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the I/R + SRPM group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris;* Nrf2: Nuclear factor erythroid 2-related factor 2; MnSOD: manganese superoxide dismutase; NQO-1: NAD(P)H-quinone oxidoreductase 1; GCLC: glutamate-cysteine ligase catalytic subunit

deleterious effects of ROS, cells maintain an endogenous antioxidative capacity consisting of SOD, CAT, and GSH-Px enzyme systems that remove ROS by metabolic conversion.[30]

While, T-AOC activity is often considered the cumulative action of all the antioxidants present *in vivo*, thus providing an integrated parameter rather than the simple sum of measurable antioxidants. Therefore, the capacity of antioxidants and their synergistic interaction are assessed, thus giving an insight into the delicate balance between oxidants and antioxidants.[3,31] MDA is the degradation product of the oxygen-derived free radicals and lipid oxidation, its content contributes to increased generation of free radicals and/or decreased activities of antioxidant system.[32] In the present study, the serum level of T-AOC in the I/R group was a significant decrease compared with the sham group. Meanwhile, the decreases of SOD, GSH-Px, CAT activities and increase of MDA content were associated with decreased T-AOC. The aforementioned results demonstrated that myocardial I/R injury resulted in oxidative stress occurrence. SPRM might significantly increase the serum antioxidant enzymes SOD, GSH-Px and CAT activities, decrease MDA content, and then increase T-AOC level. Interestedly, when its combination with Ex-527 or ATRA, these above advantageous effects were remarkably inhibited. Taken together, our results demonstrated that SRPM scavenged ROS mainly via increasing antioxidant enzymes activity, inhibited ROS overproduction and accumulation, and consequently protected myocardial I/R injury.

Sirt1, a nicotinamide adenosine dinucleotide (NAD+)-dependent protein deacetylase known to be a member of the mammalian sirtuin family of proteins, regulates genomic stability, gene expression, and targets protein activity, therefore, participates in a series of physiological functions, including metabolism, aging and so on.[5] Meanwhile, Sirt1 is also sensitive, and responds to oxidative stress challenge. It exerts significant resistance against oxidative stress damage by increasing the levels of enzymes that scavenge ROS, including CAT and MnSOD through modulating FoxO1, Pgc-1α, peroxisome proliferator-activated receptor γ, NK-κB and so forth.[33] Recent studies indicate that Sirt1 plays a key role in myocardial ischemia, and confirms that the deacetylase activity of Sirt1 regulates the acetylation levels of FoxO1 and Pgc-1α to affect apoptotic pathways in the heart, and then enduces its protective effect on myocardial I/R injury.[1,8] For example, Hsu and his team indicated that Sirt1 protected against myocardial I/R injury by upregulating antioxidants and downregulating proapoptotic molecules through the deacetylation and activation of Pgc-1α and FoxO1, as well as decreasing oxidative stress.[9,34] In our study, we found that Sirt1 expression was significantly downregulated 2 h after myocardial I/R, accompanied by increased Ac-Foxo1, Ac-Pgc-1α expressions and enhanced oxidative stress. SRPM significantly upregulated Sirt1 and downregulated Ac-Foxo1 and Ac-Pgc-1α expressions. However, the effects of SRPM treatment on the expressions of Sirt1, Ac-Foxo1 and Ac-Pgc-1α were markedly abolished for its combination with Ex-527. These results suggested that SRPM's cardioprotection involved the activation of Sirt1, and then SRPM-induced Sirt1 activation might further decrease the acetylations of Foxo1 and Ac-Pgc-1α.

Accumulating evidences have indicated that Nrf2 is an important protective survival factor for cardiovascular system and a major

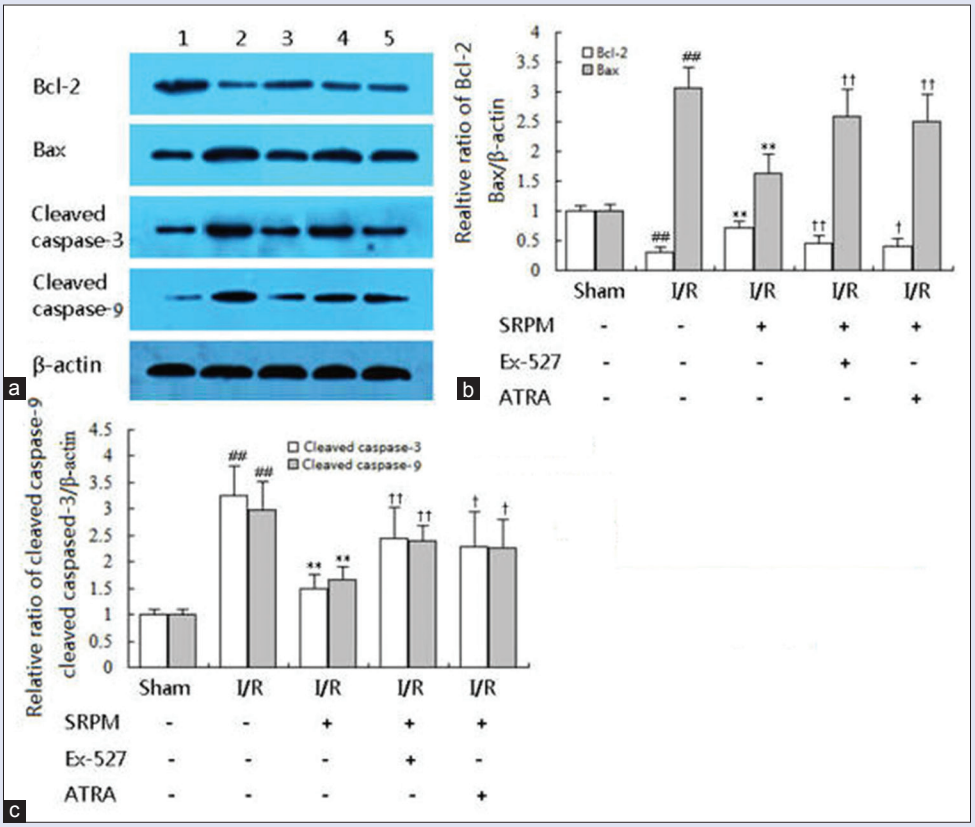


Figure 8: Effect of SRPM treatment on apoptotic signaling pathway in the I/R injury rats. (a) Representative western blotting bands of Bcl-2, Bax, cleaved caspase-3 and cleaved caspase-9 (1: Sham group; 2: I/R group; 3: I/R + SRPM group; 4: I/R + SRPM + Ex-527 group; 5: I/R + SRPM + ATRA group); (b) Bcl-2 and Bax expressions; (c) cleaved caspase-3 and cleaved caspase-9 expressions. Data are shown as the mean ± standard deviation ($n = 5$). $^{\#\#}P < 0.01$ compared with the sham group; $^{**}P < 0.01$ compared with the I/R group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with the I/R + SRPM group. I/R: ischemia/reperfusion; SRPM: Saponins from *Rhizoma Panacis Majoris*

regulator of antioxidative responses,[35,36] which is a basic leucine zipper transcription factor that binds and activates the ARE in the promoters of many antioxidant and detoxification genes such as HO-1, NQO1, and GCLC and thus maintains redox homeostasis.[37] Under condition of oxidative stress, Nrf2 interacts with Keap-1, a cytosolic repressor protein that limits Nrf2-mediated gene expression. On stimulation, Nrf2 is released from Keap-1 and translocates to the nucleus. It binds ARE and activates ARE-dependent transcription of phase II and antioxidant defense enzymes, such as HO-1, NQO1, and GCLC.[10,38] In our present study, we found that Nrf2 activation and expressions of downstream genes including HO-1, NQO-1 and GCLC in the I/R group were lower than those in sham group. SRPM enhanced Nrf2 nuclear translocation and ARE binding, which further increased mRNA expressions of HO-1, NQO-1, and GCLC. However, the effects of SRPM on the Nrf2 activation and mRNA expressions of HO-1, NQO1, and GCLC were significantly abolished for its combination with ATRA. These results suggested that SRPM's cardioprotection involved the activation of Nrf2, and then SRPM-induced Nrf2 nuclear translocation might further promote the expressions of HO-1, NQO-1, and GCLC.

Previous studies have demonstrated that Sirt1 and Nrf2 activations might reduce oxidative stress and maintain mitochondrial function.[1,39] To establish a more distinct relationship between SRPM treatment and its antiapoptotic property in mycardial I/R injury, we needed to further confirm whether the administration of Ex-527 or ATRA could abolish SRPM's antiapoptotic effect. In the present study, we found that the SRPM-induced Sirt1 and Nrf2 activations were also associated with an increase in the antiapoptotic factor Bcl-2 and decreases in the proapoptotic factor Bax, cleaved caspase-3, and cleaved caspase-9. As expected, Ex-527 and ATRA indubitably abolished SRPM's antiapoptotic effect. These results all suggested that the cardioprotection of SRPM involved to the activations of Sirt1 and Nrf2, and SRPM-induced Sirt1 and Nrf2 activations might decrease the acetylation of Foxo1 and Pgc-1α, promote the expressions of HO-1, NQO-1 and GCLC, and then promote antiapoptotic signaling pathway activation in the heart.

CONCLUSION

Our study demonstrated that SRPM exerted a profound cardioprotective effect against myocardial I/R injury. This protection appeared to be largely due to the activations of Sirt1 and Nrf2-related signaling pathways and the attenuation of oxidative stress. These results revealed that SRPM might be a promising candidate for the treatment of myocardial I/R injury in cardiac surgery and ischemic heart diseases.

Financial support and sponsorship

This study was supported by the grants from the National Nature and Science Foundation of China (No. 81341141), the common project from Health and Family Planning Commission of Hubei Province (No. 2012Z-Y07, 2011JX5C21, 201759), the common project from Science and Technology Bureau of Yichang city (No. A15-301-33, A16-301-03), and the Open Fund from Key Laboratory of Ischemic Cardiovascular and Cerebrovascular Disease Translational Medicine (2016KXN03).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Shin B, Cowan DB, Emani SM, Del Nido PJ, McCully JD. Mitochondrial transplantation in myocardial ischemia and reperfusion injury. Adv Exp Med Biol 2017;982:595-619.
2. Zweier JL, Talukder MA. The role of oxidants and free radicals in reperfusion injury. Cardiovasc Res 2006;70:181-90.
3. He HB, Xu J, Xu YY, Zhang CC, Wang HW, He YM, *et al.* Cardioprotective effects of saponins from *Panax japonicus* on acute myocardial ischemia against oxidative stress-triggered damage and cardiac cell death in rats. J Ethnopharmacol 2012;140:73-82.
4. Hu MZ, Zhou B, Mao HY, Sheng Q, Du B, Chen JL, *et al.* Exogenous hydrogen sulfide postconditioning protects isolated rat hearts from ischemia/reperfusion injury through Sirt1/PGC-1α signaling pathway. Int Heart J 2016;57:477-82.
5. Singh CK, Chhabra G, Ndiaye MA, Garcia-Peterson LM, Mack NJ, Ahmad N, *et al.* The role of sirtuins in antioxidant and redox signaling. Antioxid Redox Signal 2018;28:643-61.
6. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, *et al.* Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004;303:2011-5.
7. Webster BR, Lu Z, Sack MN, Scott I. The role of sirtuins in modulating redox stressors. Free Radic Biol Med 2012;52:281-90.
8. Yang Y, Duan WX, Li Y, Jin ZX, Yan JJ, Yu SQ, *et al.* Novel role of silent information regulator 1 in myocardial ischemia. Circulation 2013;128:2232-40.
9. Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, *et al.* Silent information regulator 1 protects the heart from ischemia/reperfusion. Circulation 2010;122:2170-82.
10. Smith RE, Tran K, Smith CC, McDonald M, Shejwalkar P, Hara K, *et al.* The role of the Nrf2/ARE antioxidant system in preventing cardiovascular diseases. Diseases 2016;4. pii: E34.
11. Zrelli H, Kusunoki M, Miyazaki H. Role of hydroxytyrosol-dependent regulation of HO-1 expression in promoting wound healing of vascular endothelial cells via Nrf2 *de novo* synthesis and stabilization. Phytother Res 2015;29:1011-8.
12. Wang T, Di GJ, Yang L, Dun YY, Sun ZW, Wan JZ, *et al.* Saponins from *Panax japonicus* attenuate D-galactose-induced cognitive impairment through its anti-oxidative and anti-apoptotic effects in rats. J Pharm Pharmacol 2015;67:1284-96.
13. State Administration of Traditional Chinese Medicine. Editorial Board of Chinese Materia Medica. Chinese Materia Medica. Shanghai: Shanghai Science and Technology Press; 1999. p. 835-8.
14. Zhao R, Zhao Y, Li DM, Wang JY, Zeng Y. Research progress of Rhizoma Panacis majoris. Mod Chin Med 2008;10:3-6.
15. Song XM, Liu Y, Cai BC. Chemical constituents of rhizome of Panacis Majoris. J Shenyang Pharm Univ 2010;27:626-30.
16. Shi XL, Wang JF, Yao H, Zhang PX, Zhang HY, Jin YR. Chemical constituents of roots of *Panax japonicus* C. A. Mey. var. major (Burk.) C.Y. Wu et K.M. Feng. Chem J Chin Univ 2015;34:381-5.
17. Chen T, Hu YQ, Deng LR, Gong ZP, Yu XQ. Effects of polysaccharides extracted from zhu zi shen (Rhizoma Panacis majoris) on oxidative stress and hemodynamics in rats with adriamycin-induced chronic heart failure. J Tradit Chin Med 2011;31:235-40.
18. Jin JH, He HB, Shi MQ, Lu XC, Chen LJ. Protective effects of total saponin Rhizoma Panacis majoris on acute cerebral ischemia injury in mice. J Third Mil Med Univ 2011;33:2631-3.
19. Liu AH, Shi MQ, Yang WY, Yan WH, Gong XQ, Xiong YQ, *et al.* Cardioprotective effect and its mechanism of total saponin from Rhizoma Panacis majoris on myocardium ischemia/reperfusion injury in rats. Chin J Clin Phamacol Ther 2013;18:1224-32.
20. Bai CH, He HB, Cheng F, Wang JZ, Chen X, Zou K, *et al.* Cardioprotective effects of Saponins from Rhizoma Panacis Majoris on myocardial ischemia/reperfusion injury via scavenging excessive reactive oxygen species. Adv Mater Res 2014;934:165-72.
21. Bao ZW, Qin HL, Shi MQ, Chen ZF, Deng W, Yang XJ, *et al.* Cardioprotective effect and its mechanism of total saponins of panacis majoris rhizoma in myocardial infarction rats. Zhong Yao Cai 2015;38:1230-6.
22. Wei N, Zhang CC, He HB, Wang T, Liu ZQ, Liu GY, *et al.* Protective effect of saponins extract from *Panax japonicus* on myocardial infarction: Involvement of NF-κB, Sirt1 and mitogen-activated protein kinase signalling pathways and inhibition of inflammation. J Pharm Pharmacol 2014;66:1641-51.
23. Hayashi I, Morishita Y, Imai K, Nakamura M, Nakachi K, Hayashi T, *et al.* High-throughput spectrophotometric assay of reactive oxygen species in serum. Mutat Res 2007;631:55-61.
24. Yan Y, OW LJ, Zhao XJ, Ye XC, Zhang C, Hao JJ, *et al.* Effect of essential oil of *Syringa pinnatifolia* hemsl. var. alashanensis on ischemia of myocardium, hypoxia and platelet aggregation. J Ethnopharmacol 2010;131:248-55.
25. Yin Y, Guan Y, Duan JL, Wei G, Zhu YR, Quan W, *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;699:219-26.
26. Liou SF, Hsu JH, Liang JC, Ke HJ, Chen IJ, Wu JR, *et al.* San-huang-xie-xin-tang protects cardiomyocytes against hypoxia/reoxygenation injury via inhibition of oxidative stress-induced apoptosis. J Nat Med 2012;66:311-20.
27. Leonard SS, Harris GK, Shi X. Metal-induced oxidative stress and signal transduction. Free Radic Biol Med 2004;37:1921-42.
28. Moris D, Spartalis M, Tzatzaki E, Spartalis E, Karachaliou GS, Triantafyllis AS, *et al.* The role of reactive oxygen species in myocardial redox signaling and regulation. Ann Transl Med 2017;5:324.
29. He HB, Shi MQ, Luo T, Chen LJ, Zhou JG, Zhang JH, *et al.* Total saponin from Rhizoma Panacis majoris protects neonatal rat cardiomyocytes against oxidative stress-induced injuries by improving Nrf2 translocation. Acta Acade Med Milit Terti 2012;34:1527-32.
30. Sun X, Sun GB, Wang M, Xiao J, Sun XB. Protective effects of cynaroside against H_2O_2-induced apoptosis in H9c2 cardiomyoblasts. J Cell Biochem 2011;112:2019-29.
31. Qin F, Liu YX, Zhao HW, Huang X, Ren P, Zhu ZY, *et al.* Chinese medicinal formula Guan-Xin-Er-Hao protects the heart against oxidative stress induced by acute ischemic myocardial injury in rats. Phytomedicine 2009;16:215-21.
32. Zhou R, Xu QB, Zheng P, Yan L, Zheng J, Dai GD, *et al.* Cardioprotective effect of fluvastatin on isoproterenol-induced myocardial infarction in rat. Eur J Pharmacol 2008;586:244-50.
33. Huang KP, Huang J, Xie X, Wang SG, Chen C, Shen XY, *et al.* Sirt1 resists advanced glycation end products-induced expressions of fibronectin and TGF-β1 by activating the Nrf2/ARE pathway in glomerular mesangial cells. Free Radic Biol Med 2013;65:528-40.
34. Kulkarni SR, Donepudi AC, Xu J, Wei W, Cheng QC, Driscoll MV, *et al.* Fasting induces nuclear factor E2-related factor 2 and ATP-binding cassette transporters via protein kinase A and sirtuin-1 in mouse and human. Antioxid Redox Signal 2014;20:15-30.
35. Ping Z, Zhang LF, Cui YJ, Chang YM, Jiang CW, Meng ZZ, *et al.* The protective effects of salidroside from exhaustive exercise-induced heart injury by enhancing the PGC-1 α -NRF1/NRF2 pathway and mitochondrial respiratory function in rats. Oxid Med Cell Longev 2015;2015:876825.
36. Zhao MY, Guo HM, Chen JM, Fujino M, Ito H, Takahashi K, *et al.* 5-aminolevulinic acid combined with sodium ferrous citrate ameliorates H2O2-induced cardiomyocyte hypertrophy via activation of the MAPK/Nrf2/HO-1 pathway. Am J Physiol Cell Physiol 2015;308:C665-72.
37. Shelton P, Jaiswal AK. The transcription factor NF-E2-related factor 2 (Nrf2): A protooncogene? FASEB J 2013;27:414-23.
38. Bayram B, Ozcelik B, Grimm S, Roeder T, Schrader C, Ernst IM, *et al.* A diet rich in olive oil phenolics reduces oxidative stress in the heart of SAMP8 mice by induction of Nrf2-dependent gene expression. Rejuvenation Res 2012;15:71-81.
39. Yamamoto T, Byun J, Zhai P, Ikeda Y, Oka S, Sadoshima J, *et al.* Nicotinamide mononucleotide, an intermediate of NAD+synthesis, protects the heart from ischemia and reperfusion. PLoS One 2014;9:e98972.