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Beneficial effects of a polysaccharide from *Salvia miltiorrhiza* on myocardial ischemia–reperfusion injury in rats

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ABSTRACT

In the present study, one water-soluble polysaccharide (SMP1) was isolated from the roots of *Salvia miltiorrhiza*. The cardio-protective potential of SMP1 was studied in the ischemia–reperfusion (I/R) model of rats in vivo. Results showed that 30 min of left anterior descending coronary artery occlusion (LAD) followed by 4 h of reperfusion markedly decreased myocardial superoxide dismutase (SOD), Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities and increased myocardial malondialdehyde (MDA) level and serum activities of creatine kinase (CK) and lactate dehydrogenase (LDH) in I/R rats. An increase in infarct size and high apoptosis index of cardiac cell were also observed in IR rats. Administration of SMP1 400 and 800 mg/kg significantly reversed these biochemical parameters in the I/R rats to the normal levels in sham control rats. The infarct sizes and the percent of TUNEL-positive cells were found significantly decreased in SMP1-treated groups compared to I/R rats. Taken together, the present study clearly suggests SMP1 has a protective effect against myocardial I/R injury in rats by ameliorating oxidative stress and inhibiting myocardial apoptosis.

1. Introduction

Cardiovascular disease, especially ischemic heart disease (IHD), remain the leading cause of death in industrialized countries and its incidence is also rising at an alarming rate in the developing world (Chan et al., 2011; Zhu, Zhu, Gohlke, & Unger, 1998). Several life style associated factors, e.g. sedentary habits, overeating, smoking, excessive alcohol consumption and psychological stress are known to contribute to the higher incidence of IHD (Wasir, 1990). Ischemic reperfusion (I/R) injury is a major pathological factor in the natural course of IHD (Hansen, 1995). Reduction of mortality rate and prevention of I/R injury are of utmost importance. Western drugs such as angiotensin-converting enzyme (ACE) inhibitors, calcium channel blockers, angiotensin II receptor antagonists, etc. have been proven to have cardioprotective effects in both preclinical and clinical studies. However, there is a growing interest in the use of alternative medicine for maintaining heart health and curing heart attack in high risk patients.

Danshen, the dried roots of *Salvia miltiorrhiza*, is a traditional Chinese herbal medicine in the treatment of many diseases, especially ischemic cardiovascular diseases (Sun et al., 2005). According to Chinese medicine theory, Danshen promotes blood flow and resolves blood stasis (Song et al., 2008). Experiments using modern techniques showed that Danshen dilates coronary arteries, increases coronary blood flow and scavenges free radicals in patients with ischemic diseases, thus reducing cellular damage from ischemia (Anversa, Olivetti, Meggs, Sonnenblick, & Capasso, 1993). Clinical trials have also indicated that Danshen is an effective medicine for angina pectoris, myocardial infarction and stroke (Sun et al., 2005). There are many traditional Chinese medicine preparations containing *S. miltiorrhiza*, including Fufang Danshen tablets, Compound Danshen dripping pills, Danshen injections, and Xiangdan injections (Zhang, Li, & Wang, 2010). Up to now, studies regarding protective effects of *S. miltiorrhiza* on IHD mainly focus on low molecular-weight substances. More frequently, polysaccharides from Danshen were reported to possess a wide range of biological properties, such as antioxidant (Wu, Zhu, Zhang, Yang, & Zhou, 2012), hepatoprotective (Song et al., 2008), antidiabetic (Zhang, Zheng, Zhang, & Hai, 2012), immunoregulatory and anti-tumor activities (Liu et al., 2013). Despite all this, information regarding the cardioprotective effect of the polysaccharide from *S. miltiorrhiza* is still

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limited. Therefore, in this present study, we intend to evaluate whether *S. miltiorrhiza* polysaccharide could ameliorate myocardial IR injury in rats and to investigate the possible underlying mechanism.

2. Materials and methods

2.1. Materials and chemicals

The roots of *S. miltiorrhiza* were collected from a local herb shop, Chongqing City, China in July 2012. The samples were washed, cut into small pieces and further ground into a fine powder in a high speed disintegrator and dried at 65 °C. The ground material, which passed through 80-mesh sieve, was collected for subsequent experiment. Sepharose CL-6B and DEAE-cellulose were purchased from Amersham (Sweden). T-series dextran, trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO) and standard sugars were obtained from Sigma (St. Louis, MO, USA). All other chemical reagents were analytical grade.

2.2. Isolation and purification of polysaccharide SMP1

The ground roots of *S. miltiorrhiza* were subjected to successive extraction with 95% ethanol to remove lipids and colored materials. Subsequently the residues were extracted with distilled water at 80 °C for 3 times and 3 h for each time. The aqueous extract was combined, concentrated under reduced pressure at 50 °C and precipitated with ethanol (final concentration 75% (v/v)) at 4 °C overnight. After that, the associated proteins in the precipitate were removed, using the Sevag method (Staub, 1965). The resulting precipitate was dissolved in water and dialyzed against distilled water (MW cut-off 3000 Da). After removal of the Sevag reagent, the condensed supernatant was lyophilized and a primrose yellow crude polysaccharide (CSMP) was obtained.

The CSMP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of anion exchange chromatography on DEAE-cellulose column (30 cm × 2.6 cm), eluting with 0–2 M gradient NaCl solution. The fractions eluted with 0–2 M gradient NaCl were collected, concentrated, dialyzed and lyophilized to get the purified polysaccharides. The collected fractions were further purified on a Sepharose CL-6B gel filtration column (100 cm × 2.6 cm) and eluted with deionized water at a flow rate of 1 mL/min. Fractions (8 mL) were collected by a fraction collector. All of these fractions were analyzed for the carbohydrate content by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Major fractions obtained were lyophilized to afford four white purified polysaccharides (SMP1, SMP2, SMP3 and SMP4), of which the SMP1 was used in the subsequent studies.

2.3. Analytical methods

The carbohydrate content of the solution was determined by phenol–sulfuric acid method (Dubois et al., 1956), using glucose as standard. Protein content was determined by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as the standard. Total uronic acid content was determined by photometry with m-hydroxybiphenyl at 523 nm (Blumenkrantz & Asboe-Hansen, 1973), using GalA as standard. The monosaccharide composition analysis was performed by gas chromatography (GC-14A, Shimadzu, Japan). The polysaccharides (10 mg) were first hydrolyzed using 2 M TFA (100 °C, 2 h) into monosaccharides (Parikh & Madamwar, 2006) before derivatization as their alditol acetates by the previous method (Jones & Albersheim, 1972; Oades, 1976), and were analyzed by GC instrument as previously mentioned. The molecular weight of SMP1 was determined by high-performance gel permeation chromatography (HPGPC), which was performed on a Waters HPLC instrument (Waters 515, USA), equipped with a TSK gel 4000 PWXL column and eluted with 0.05 M Na_2SO_4 solution at a flow rate of 0.8 mL/min. Elution was monitored by an a Waters 2410 refractive index detector (RID). A standard calibration curve was obtained by plotting the elution volume versus the logarithm of the corresponding molecular weight of Dextran T (10–270 kDa).

2.4. Experimental animals and treatment

Sprague-Dawley male rats, 250–300 g, were supplied by Chongqing Tengxin Animal Center (Chongqing, China). Rats were maintained at 25 ± 2 °C and allowed free access to a standard laboratory diet and tap water ad libitum during the experimental period. The rats were acclimated for one week prior to the following experiments. The project was approved by the Third Military Medical University Animal Experimentation Ethics Committee, and all experiments were based on internationally accepted guidelines for the care and use of laboratory animals.

Animals were randomly divided into the following 4 groups with 10 mice in each group: I/R control group, fed with vehicle (distilled water) at dose of 10 mL/kg body weight; Sham control group, fed with vehicle (distilled water) at dose of 10 mL/kg body weight (b.w.); IR+SMP1L-treated group, fed with the SMP1 (400 mg/kg b.w.); IR+SMP1H-treated group, fed with the SMP1 (800 mg/kg b.w.). All administrations were given by gavage once daily for one week, along with standard rat chow or water ad libitum before surgical induction of myocardial I/R injury. Rats in the model and SMP1-treated groups were induced with I/R injury by ligation of the left anterior descending (LAD) coronary artery, after one week of oral administration. Rats in the sham group were subject to the same procedure except ligation.

2.5. Ischemia–reperfusion procedure

After rats were anesthetized with sodium pentobarbital (40 mg/kg) through intraperitoneal injection, the chest was opened to exposure the heart. Then the left anterior descending coronary artery was identified and occluded by a knot for 30 min to create ischemia. After 30 min of ischemia, the ligature was removed for 4 h of reperfusion. At the end of reperfusion, blood samples were taken from the abdominal aortic artery and the serum was separated. After collection of serum samples, the heart was excised immediately and washed with cold saline. The serum samples were kept at −20 °C and the tissue samples were kept in liquid nitrogen at −80 °C until analysis.

2.6. Evaluation of myocardial infarct size

Measurement of heart infarct size was performed by TTC staining method as described previously (Miki et al., 2000; Oron et al., 2001). After the completion of the infarct protocol and infusion, the heart was quickly removed, frozen and cut into 2-mm transverse slices. The sections were incubated for 10 min at 37 °C with 1% TTC in PBS (pH 7.4) followed by fixing in a 10% formalin solution (pH 7.0) for 24 h. TTC stains living tissue deep red (TTC positive) while necrotic tissue appears tan in color (TTC negative). The TTC-positive and TTC-negative areas were digitally captured and measured by Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA). The infarct area was defined as the percentage of the total TTC-negative area over the total ventricle areas measured. Myocardial tissues from the area at risk were collected to measure the biochemical indexes.

2.7. Measurement of LDH and CK activities in serum

Myocardial damage was evaluated by measuring the myocardial specific enzyme, including the activity of creatine phosphokinase (CK) and lactate dehydrogenase (LDH). At the end of reperfusion, blood samples were drawn from the abdominal aortic artery and sera were prepared by centrifuging at 2000 × *g* for 10 min at room temperature. Serum activities of LDH and CK were determined by using commercial assay kits from Sigma according to manufacturer's instructions. Values were expressed in international units (IU) per liter serum.

2.8. Measurement of MDA level and SOD activity in myocardial tissue

The individual tissue samples from the area at risk were added to 10 volumes of cold saline and were homogenized with a tissue homogenizer and centrifugated at 2000 × *g* for 15 min. Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were used as indices of oxygen free radical and lipid superoxide level. The content of MDA and activity of SOD were measured using commercial kits (JianCheng Bioengineering Institute, Nanjing, China) with a spectrophotometer (Beckman, U.S.A.).

2.9. Measurement of Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in myocardial tissue

Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities were assayed by spectrophotometrically measuring the release of inorganic phosphate from reaction of the tissue extract with disodium ATP (Sigma, England) as reported elsewhere (Grosman, 2001; Vér, Csermely, Bányász, Kovács, & és Somogyi, 1995).

2.10. Measurement of cardiomyocyte apoptosis

Terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay was carried out with the recommended protocol. TUNEL positive (brown) cardiac myocytes were regarded as apoptotic cells and the apoptosis index was calculated from the number of TUNEL-positive cells divided by the total number of cells by a reader who was blinded to the animals' treatment status. The experiment was repeated on three different sections for each specimen. Ten random fields (400×) per section were analyzed.

2.11. Statistical analysis

Data are expressed as the mean ± S.D. of results obtained from the number (9–10) of animals used. Differences between data sets were determined by one-way analysis of AVONA followed by Tukey's test. $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Isolation and characterization of SMP1

The water-soluble crude polysaccharide (CSMP) was obtained as a primrose yellow powder from the roots of *S. miltiorrhiza* by removing lipids and colored materials with 95% ethanol reflux extraction, hot-water extraction, ethanol precipitation, deproteinization, dialysis and lyophilization. CSMP was further purified by DEAE-cellulose column and Sepharose CL-6B gel filtration column chromatography to afford four white purified polysaccharides (SMP1, SMP2, SMP3 and SMP4), of which the SMP1 collected and lyophilized for further study. This polysaccharide showed a single and symmetrical peak on HPGPC, indicating its homogeneity (Fig. 1). Its molecular weight was determined as 5.5×10^5 Da according to the calibration standards. SMP1 appeared as a heterogeneous mixture of polysaccharides which consisted of neutral sugars (91.3%), uronic acid (2.81%) and protein (4.34%). According to GC analysis, SMP1 was composed of galactose, glucose, fucose, rhamnose, arabinose and mannose with molar ratios of 1.0:1.2:0.3:1.5:1.3:1.9.

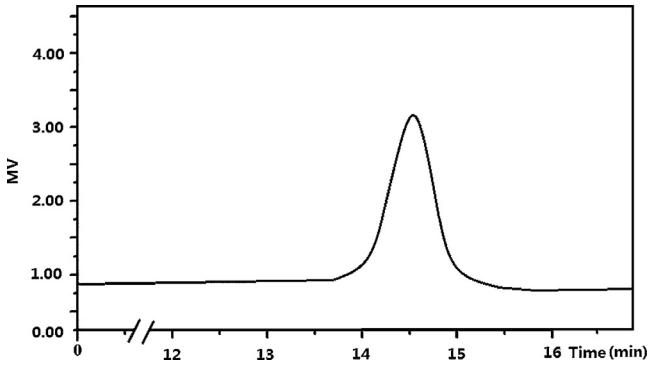

Fig. 1. HPGPC profile of the polysaccharide SMP1 isolated from the roots of *S. miltiorrhiza*.

3.2. Effect of SMP1 on myocardial infarct size

Myocardial infarct size is the gold standard for assessing the cardioprotective capability of a drug; we therefore employed it in the current study to examine the cardioprotective effect of SMP1. Fig. 2 shows TTC-stained infarct areas expressed as a percentage of the left ventricular area at risk in control, IR, and IR+SMP1 groups. The untreated IR group showed a substantial increase in infarct area (39.05%) compared with noninfarct controls. However, SMP1 treatment at dose of 400 and 800 mg/kg, when administered orally one week before acute ischemia, could significantly reduce the infarct sizes in rats, with infarct areas percentage of 25.84% and 21.23%, respectively, when compared with the IR group ($P < 0.001$).

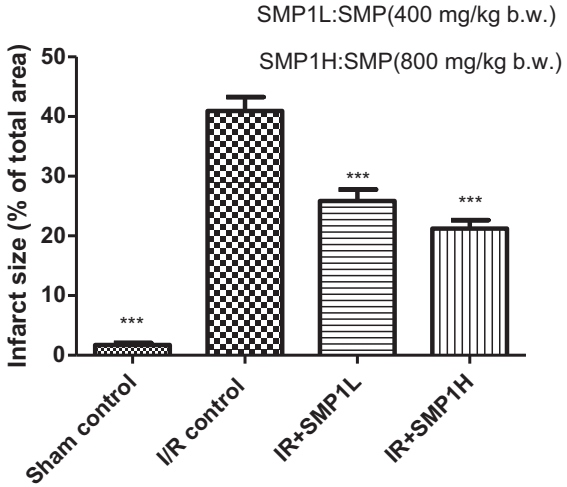

Fig. 2. Effect of SMP1-treatment on I/R-induced myocardial infarct size determined by TTC staining. Sham control: rats received vehicle (distilled water, 10 mL/kg b.w.) treatment without I/R injury; I/R control: rats received vehicle treatment with I/R injury; IR+SMP1L: I/R rats received SMP1 (400 mg/kg b.w.) treatment; IR+SMP1H: I/R rats received SMP1 (800 mg/kg b.w.) treatment. Values are mean ± S.D. ($n = 9$–10 in each group). $^{***}P < 0.001$ compared to IR group.

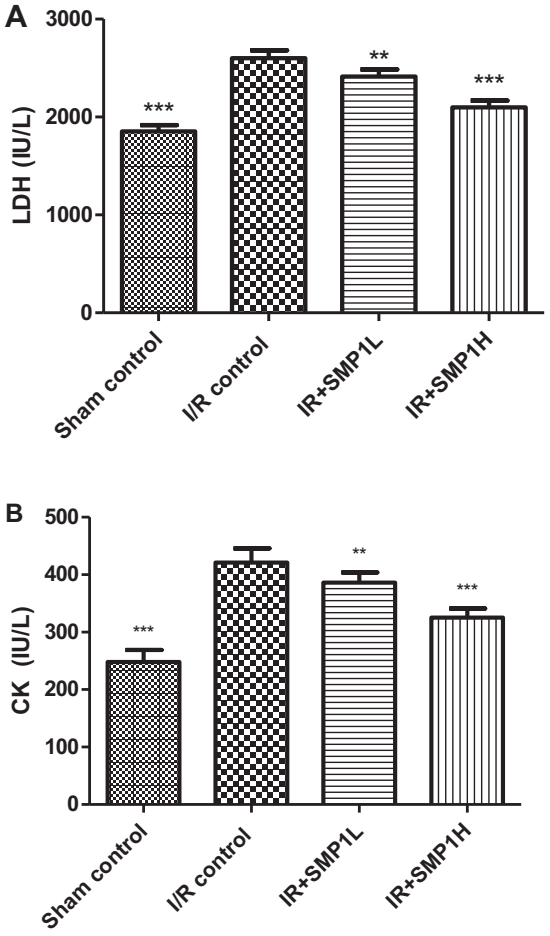

Fig. 3. Effect of SMP1-treatment on LDH and CK activities in sera of rats after myocardial I/R injury. Sham control: rats received vehicle (distilled water, 10 mL/kg b.w.) treatment without I/R injury; I/R control: rats received vehicle treatment with I/R injury; IR+SMP1L: I/R rats received SMP1 (400 mg/kg b.w.) treatment; IR+SMP1H: I/R rats received SMP1 (800 mg/kg b.w.) treatment. Values are mean ± S.D. (n = 9–10 in each group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to IR group.

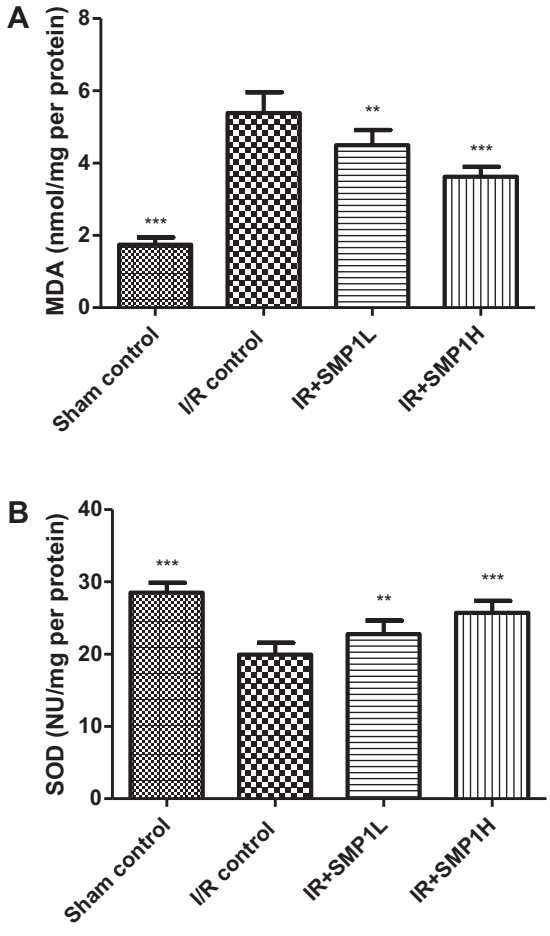

Fig. 4. Effects of SMP1-treatment on the content of MDA and SOD activity in myocardial tissue of rats after myocardial I/R injury. Sham control: rats received vehicle (distilled water, 10 mL/kg b.w.) treatment without I/R injury; I/R control: rats received vehicle treatment with I/R injury; IR+SMP1L: I/R rats received SMP1 (400 mg/kg b.w.) treatment; IR+SMP1H: I/R rats received SMP1 (800 mg/kg b.w.) treatment. Values are mean ± S.D. (n = 9–10 in each group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to IR group.

3.3. Effect of SMP1 on LDH and CK activities in serum

The myocardial specific enzymes activities of serum LDH and CK was used as a marker of myocardial injury (Bassenge, Sommer, Schwemmer, & Bunger, 2000). As a consequence of I/R injury, the serum activities of CK and LDH were significantly increased in the IR group when compared with the sham group, but pretreatment with 400 and 800 mg/kg of SMP1 significantly inhibited the elevation of LDH (by 7.21% and 19.36%, $P < 0.01$ and $P < 0.001$, respectively) or CK (by 8.34% and 22.75%, $P < 0.01$ and $P < 0.001$, respectively) activities induced by I/R injury compared with the vehicle-treated I/R animals (Fig. 3).

3.4. Effect of SMP1 on MDA level and SOD activity in myocardial tissue

Oxidative stress is one of the major concerns in the treatment of ischemic heart diseases. There is ample evidence for a detrimental role of ROS in cardiovascular disease (Dhalla, Temsah, & Netticadan, 2000; Sawyer et al., 2002). This usually results from deficient natural antioxidant defense (Ferrari et al., 1991). SOD is a very endogenous antioxidants enzyme; an augmentation in its activity been identified as a promising therapeutic approach in myocardial diseases associated with increased oxidative stress (Jin, Qin, & Wu, 2003). MDA formed by the breakdown of lipid peroxides is an indicative of an enhanced oxidative stress, which is documented both in clinical and experimental studies in conditions of myocardial I/R injury (Dhalla, Elmoselhi, Hata, & Makino, 2000), so MDA level reflects the damage caused by ROS. To elucidate the cardioprotective mechanism of SMP1, its effect on the production of MDA and the SOD activity in myocardial tissues in response to I/R injury were subsequently investigated (see Fig. 4). I/R caused a significant increase in the content of MDA in the myocardial tissue, while this parameter decreased significantly ($P < 0.01$ and $P < 0.001$) in those groups treated with SMP1 (400 and 800 mg/kg) in comparison with untreated I/R group. SOD activity was declined significantly in the I/R control group compared with the sham-operation group, but SOD levels increased significantly ($P < 0.01$ and $P < 0.001$) in groups treated by SMP1 (400 and 800 mg/kg) compared with untreated I/R group.

3.5. Effect of SMP1 on Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in myocardial tissue

Decreased myocardial Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities may induce myocardial damage such as ischemic cardiac dysfunction and arrhythmias (Ke, Wang, Wang, & Yang, 2004). Fig. 5 shows the effects of SMP1 on myocardial Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in experimental rats.

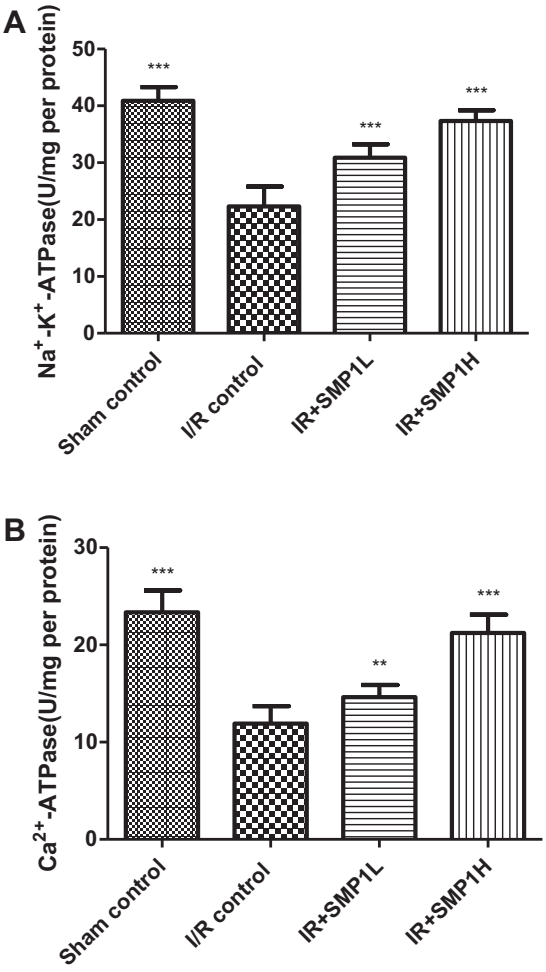

Fig. 5. Effects of SMP1-treatment on the Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in myocardial tissue of rats after myocardial I/R injury. Sham control: rats received vehicle (distilled water, 10 mL/kg b.w.) treatment without I/R injury; I/R control: rats received vehicle treatment with I/R injury; IR+SMP1L: I/R rats received SMP1 (400 mg/kg b.w.) treatment; IR+SMP1H: I/R rats received SMP1 (800 mg/kg b.w.) treatment. Values are mean ± S.D. (n = 9–10 in each group). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to IR group.

Myocardial Na^+–K^+-ATPase and Ca^{2+}-ATPase activities were significantly lower in the in I/R model group than in the sham group. Oral administration of SMP1 at either 400 or 800 mg/kg b.w. markedly increased their activities in rats when compared with the I/R model group ($P < 0.01$ and $P < 0.001$). These results suggest that one of the mechanisms whereby SMP1 prevents development of I/R-induced cardiac dysfunction may be attributed to enhancement of myocardial Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities.

3.6. Effect of SMP1 on myocardial cell apoptosis

Accumulating evidence indicates that cell loss through apoptosis is the predominant form of post-ischemic cardiomyocyte death in the heart and contributes significantly to the impairment of cardiac performance, suggesting that reducing cardiocyte loss through suppression of cell death is a logical strategy to protect cardiomyocytes (Gottlieb & Engler, 1999; Haunstetter & Izumo, 1998; Wang, Wang, Zhang, & Zheng, 2009). To examine the effect of SMP1 on myocardial cell apoptosis, TUNEL staining was performed. TUNEL-positive cells indicate typical apoptosis. The changes in the apoptosis index are summarized in Fig. 6. The apoptosis index in I/R group was significantly higher than that in sham group ($P < 0.001$). However pretreatment with SMP1 significantly reduced the

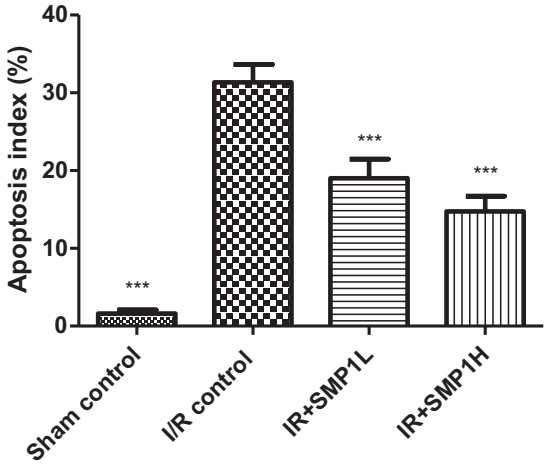

Fig. 6. Effects of SMP1-treatment on the apoptosis index of rats after myocardial I/R injury. Sham control: rats received vehicle (distilled water, 10 mL/kg b.w.) treatment without I/R injury; I/R control: rats received vehicle treatment with I/R injury; IR+SMP1L: I/R rats received SMP1 (400 mg/kg b.w.) treatment; IR+SMP1H: I/R rats received SMP1 (800 mg/kg b.w.) treatment. Values are mean ± S.D. (n = 9–10 in each group). $^{***}P < 0.001$ compared to IR group.

percent of TUNEL-positive cells by 12.34% for 400 mg/kg and 16.60% for 800 mg/kg, respectively, versus the I/R control group ($P < 0.001$). The results suggest that SMP1 could inhibit the cardiac cell apoptosis of rats after myocardial I/R injury.

4. Conclusions

In the present study, we successfully purified and partially characterized a homogenous polysaccharide SMP1 fraction from the roots of *S. miltiorrhiza*. The average molecular weight of SMP1 was 5.5×10^5 Da, which was detected by the HPSEC. The monosaccharide composition study demonstrated that SMP1 is a heteropolysaccharide consisting of galactose, glucose, fucose, rhamnose, arabinose and mannose in a relative molar ratio of 1.0:1.2:0.3:1.5:1.3:1.9. SMP1 contained 91.3% of total carbohydrate, 2.81% of uronic acid and 4.34% of protein. The protective effect of SMP1 on myocardial I/R injury was studied in rats which was induced by the occlusion of LAD coronary artery for 30 min followed by reperfusion for 4 h. Pretreatment with SMP1 (400 and 800 mg/kg) one week before the ligation of the LAD coronary artery significantly caused a significant reduction in infarct size in I/R rats. Moreover, the increases in the levels of serum LDH, serum CK and myocardial MDA and the decrease in the myocardial SOD, Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities in I/R rats were reversed by oral administration of SMP1 at dose of 400 and 800 mg/kg. In addition, a TUNEL assay indicated that SMP1 could suppress cardiocyte apoptosis. Taken together, the results of the present study demonstrate cardioprotective properties of SMP1 and suggest that such cardioprotective properties appears to be achieved by alleviating oxidative stress and inhibiting myocardial apoptosis in rats subjected to myocardial I/R injury. The results further strengthen the concept of using natural products in degeneration diseases like IHD. This issue needs to be investigated with new experimental and clinical studies to evaluate the efficacy of SMP1 as a protective agent against myocardial I/R injury in near future.

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