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Effects of *Salviae Mitiorrhizae* and *Cortex Moutan* extract on the rat heart after myocardial infarction: A proteomic study

Yi Wang, Li Liu, Chuchu Hu, Yiyu Cheng *

Department of Chinese Medicine Science & Engineering, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, 310027, P.R. China

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ABSTRACT

In this study, we characterized the therapeutical effects of *Salviae Mitiorrhizae* (Danshen) and *Cortex Moutan* (Danpi) extract (SDD) on Sprague-Dawley rats subjected to coronary artery ligation, and applied proteomic approach to investigate its potential mechanism of action. The chemical composition of SDD was investigated by HPLC/MS^n analysis. Measurement for serum levels of creatine kinase (CK), creatine kinase-MB (CK-MB), nitrite and histological study for infarct area of heart were performed. Moreover, protein abundance profiles of myocardium were compared by two-dimensional gel electrophoresis and altered proteins were identified by MALDI-TOF–MS. The results showed SDD significantly decreased CK, CK-MB concentration in serum and infarct area of heart, while increased the release of nitrite in rats with coronary occlusion. Increased concentration of ATP and total adenine nucleotide indicated the energy metabolism has been improved in ischemic heart induced by SDD. Proteomic data revealed that 23 proteins associated with energy metabolism, oxidative stress and cytoskeleton were modulated in SDD treated rats.

1. Introduction

Coronary heart disease (CHD) is the leading cause of death in developed countries. Although Asian population has traditionally represented a low-risk group in term of CHD, approximately 1,300,000 people in China are affected. In spite of numerous improvements in diagnosis and therapy in recent years, 450,000 persons die per year [1,2]. Many single-entity drugs such as beta-blockers and angiotensin converting enzyme inhibitor (ACEI) had significantly improved survival rate of CHD patients. However, people living in Asian nations still prefer to use herbal medicine due to their cultural beliefs and local health care system [3]. ShuangDan Decoction (SDD), made from *Radix Salviae Mitiorrhizae* and *Cortex Moutan* (traditionally named as Danshen and Danpi), is one of the most widely used herbal medicines for treating myocardial infarction, angina and coronary heart disease in China [4,5]. Pharmacological studies demonstrated that some active compounds of SDD such as Salviae Mitiorrhizaesu, paeonol and salvianolic acid B can dilate coronary arteries [6], block Ca^{2+} channels [7] and possess superoxide radical scavenger properties [8]. However, there is lack of scientific proof of pharmacological activity of SDD on a molecular basis.

The proteomic approach has great potential to provide global, holistic profiling and characterization of protein expression in disease state or in response to drug treatment. Recently, two-dimensional electrophoresis (2DE) with matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS) has been applied to study differential proteomic profiles of various cardiovascular diseases [9,10]. There are

* *Corresponding author*. Tel.: +86 571 87951138; fax: +86 571 87951138.
E-mail address: chengyy@zju.edu.cn (Y. Cheng).

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CK, creatine kinase; CK-MB, creatine kinase-MB; NO, nitric oxide; SDD, *Salviae Mitiorrhizae* and *Cortex Moutan* extract

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also some investigations on differential proteomics after drug treatment, to reveal toxicological and pharmacological effect on molecular level [11,12]. However, few proteomic reports are available about cardioprotective effects of herbal medicine [13]. As far as we know, there is no report about proteomic approach used to explore the cardioprotective effect of SDD.

The objective of the current study was to investigate the action mechanism of SDD for the treatment of coronary heart disease by proteomic approach. Myocardial infarct size, adenine nucleotides concentrations in left ventricle, and serum levels of creatine kinase (CK), CK-MB, and nitrite were measured to evaluate the cardioprotective effect of SDD. By comparing the protein profile of myocardium in rats treated with SDD to those untreated, we identified differentially expressed proteins and discussed the functions of these proteins to obtain molecular level insights into the action of mechanism of SDD.

2. Materials and methods

2.1. Reagents

Bisacrylamide(bis), Tris(hydroxymethyl) aminomethane (Tris), sodium dodecyl sulfate (SDS), glycine, *N*,*N*,NU,NU-tetramethylethyldiamide (TEMED), ammonium persulfate (APS), glycerol, ultra pure urea, bromophenol blue, protease inhibitor cocktail, 2D cleanup kit, 2D Quant Kit were purchased from GE Healthcare (Amersham, Freiburg, Germany). Acrylamide, dithiothreitol (DTT), 3-3-1-propane-sulfonate (CHAPS), Coomassie G-250 (ultra pure grade) and agarose were obtained from Shanghai Biotech Co. Ltd (Shanghai, China). Iodoacetamide (IAA) was purchased from Fluka BioChemika (Buchs, Switzerland). HPLC-grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). HPLC-grade trifluoroacetic acid (TFA) was purchased from Tedia (Fairfield, USA). Formic acid (FA) was purchased from Acros Organics (Fairlawn, NJ). All other solvents were of analytical grade.

2.2. Animals

Male Sprague-Dawley rats (~200 g) ($n = 28$) were purchased from Zhejiang Experimental Animal Center (Grade II, Certificated SCXK2003-0001). All rats were provided with water and standard chow *ad libitum* for 1 week before experiment. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The Animal Ethic Review Committees of Zhejiang University approved all procedures.

2.3. Preparation of SDD and HPLC/MS^n analysis

The SDD was prepared according to the protocol of the manufacturer. Briefly, dried powder of *Salviae Mitiorrhizae* and *Cortex Moutan* was soaked with water (10 v/w) for 2 h, and then extracted for 1 h at 100 °C with refluxing. Extracts were evaporated on a rotary evaporator, and the residue was dried in a vacuum oven at 50 °C for 12 h. The yield of SDD was 33%.

To illustrate chemical composition of SDD, an HPLC–DAD–MS analysis was performed to determine its major compounds using a modified method from our previous description [14]. The result indicated phenolic acids, diterpenoid quinines in *Salviae Mitiorrhizae* and monoterpenoids in *Cortex Moutan* are major components of SDD. Representative chromatogram of SDD and structures of constituents were shown in Fig. 1.

2.4. Myocardial infarction and treatment

Myocardial infarction was produced by occlusion of the left anterior descending coronary artery according to the method of Yamaguchi [15,16]. Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and ventilated by a respirator (DH-150, Zhejiang, China) with room air (tidal volume, 3 mL/100 g; respiratory rate, 60 cycles/min). A thoracotomy was performed in the fourth intercostal space and a 5/0 Proline suture was tied around the left anterior descending

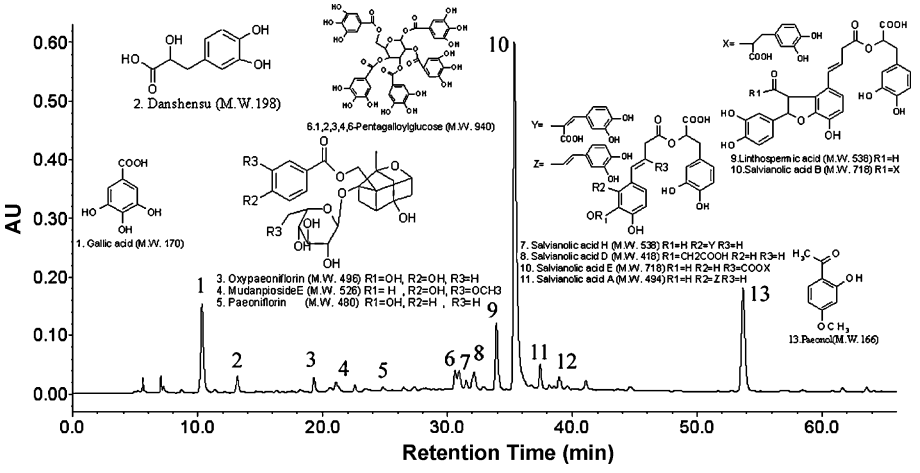

Fig. 1 – LC-UV chromatogram of SDD. Mobile phase of (A) 0.5% acetic acid solution and (B) 0.5% acetic acid–acetonitrile. The initial condition was set at 5% of (B), gradient up to 25% in 25 min, up to 35% at 50 min and up to 90% at 70 min. Detection wavelength was 254 nm. Oven temperature was 30 °C and flow rate was 1.0 mL/min. The chemical structures of marked peaks are illustrated.

coronary artery a few millimeters from its origin ($n = 20$). Among 20 operated animals, 4 animals died during operation. The surviving 16 animals were randomly divided into two groups, myocardial infarction (MI) group ($n = 8$) and SDD treatment (MI + SDD) group ($n = 8$). In sham-operated rats ($n = 8$), used throughout this study as controls, the ligation suture was not placed in the heart.

Because herbal medicine is commonly used by oral administration, intraduodenal administration was performed on anesthetized rats with/without LAD ligation to allow possible reaction available during adoption. The dosage of SDD used in current study was calculated according to the daily consumption of human and was confirmed by our preliminary study. The amount of SDD used for per kg rat weight was extracted from 4 g raw material of *Salviae Mitiorrhizae* and *Cortex Moutan*. Intraduodenal injection of aqueous solution of SDD was performed at 15 min after LAD ligation in rats of MI + SDD group. Rats in control and MI group were administered with saline in the same manner. All animals tolerated the treatment.

2.5. Sample preparation and histological study

Rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg) at 8 h after the treatment and then bled from inferior vena cava. Blood samples were centrifuged at $1000 \times g$ for 10 min and serum was stored at $-20\,^{\circ}C$ until used. Serum concentrations of creatine kinase, CK-MB were determined by the use of diagnostic kit (Jiancheng, Nanjing, China) according to the supplier's instructions. Serum nitrite was determined by the Griess reaction [17].

After removal of blood, hearts were harvested either for histological assessments ($n = 4$) or for protein extraction ($n = 4$). To measure infarct size, the heart was rapidly excised and serially sectioned into five slices (1 mm thick) from the base to the atrioventricular groove in a plain parallel. The slices were then incubated in 1% triphenyltetrazolium chloride (Sigma, St. Louis, MO, USA) for 5 min at 37 °C to distinguish viable tissue and the necrotic tissue. Each slice was traced along the borders of the infarction area and the non-infarction area. The corresponding areas were weighed out. The infarction area as a percent ratio of the left ventricular mass was calculated as:

$$\frac{\text{IA}}{\text{LV}} = \frac{\text{weight of infarcted area}}{\text{weight of left ventricle}} \times 100\% \quad (1)$$

For protein extraction, the fresh hearts were quickly removed and perfused for 1–2 min with cold modified Krebs–Henseleit solution (200 mM sucrose, 200 mM Tris–HCL, 0.4 mM $CaCl_2$, pH 7.0). After removing fat and connective tissues, the left ventricle was snap frozen in liquid nitrogen and stored at $-80\,^{\circ}C$. To obtain protein extracts, 100 mg tissue was ground in liquid nitrogen and homogenized for 15 s twice in lysis buffer (9 M urea, 2% CHAPS, 0.5% DTT, protease inhibitor cocktail consisted of AEBSF, EDTA, Bestatin, pepstatin A and E-64 (Sigma, MI, USA)) at the maximal speed. The resulting homogenate was centrifuged for 10 min at $10{,}000 \times g$, supernatant was collected and treated with 2D Cleanup Kit (Amersham Biosciences). Protein concentration of sample was measured using 2D Quant Kit (Amersham Biosciences).

2.6. Proteomic analysis

Proteins (100 μg) were applied to a 24 cm non-linear gradient IPG-strips, pH 3–10 (Amersham Biosciences). Strips were rehydrated for 12 h at 30 V, followed by focusing for 1 h at 100 V, 1 h at 200 V, 1 h at 500 V, 1 h at 1000 V, 30 min for voltage increasing to 8000 V, and remaining 8000 V for 73 kVh on an IPGPhor (Amersham Biosciences). The rehydration solution contained: 8 M urea, 2% CHAPS, 0.5% IPG Buffer, 0.02% bromophenol blue and DTT (2.8 mg/mL) was added just prior to use. Focused IPG strips were equilibrated twice for 15 min with gentle shaking in equilibration buffer (50 mM Tris–HCL buffer pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% Bromophenol blue and 10 mg/mL DTT). In the second equilibration buffer, DTT was replaced with 25 mg/mL iodoacetamide as suggested by Görg et al. [18]. The equilibrated IPG strips were gently rinsed with SDS electrophoresis buffer, blotted to remove excessive buffer and then applied onto a 12.5% polyacrylamide gradient gel (26 cm × 20 cm). The second dimension separation was performed sequentially with a constant voltage of 5 W/gel for 1 h, followed by 20 W/gel for 6 h using the Ettan DALT system (Amersham Biosciences). After SDS-PAGE, the separated gels were visualized by silver staining [19] or Coomassie Brilliant Blue (CBB) [20].

Silver-stained gels were scanned using Powerlook 2100XL (Umax, Hanchu, Taiwan) scanner and gel images were analyzed using commercially available software (Image Master 2D Platinum software; Amersham Biosciences; USA). In order to minimize the contribution of experimental variations, three separate gels of same sample were analyzed. Those spots displaying the same pattern in triplicate gels were selected for further analysis. The intensity of each spot was expressed as a proportion of the total intensity detected for the entire gel. The list of candidate differential proteins was automated selected by software and then visually checked to eliminate artificial influences. A subset of the most promising candidate spot was then selected for excision from a preparative gel for MS.

CBB-stained gels were used for harvesting proteins. The in-gel digestion of CBB-stained spots was performed according to the method by Jensen et al. [21]. The peptide extracts were combined and concentrated to about 5 μL under the protection of N_2.

2.7. MALDI-TOF–MS and database search for protein identification

Each peptide sample was re-suspended with 0.7 μL matrix solution (CHCA in ACN/water, 1:1, acidified with 0.1% TFA). The mixture was immediately spotted on the MALDI target and allowed to dry and crystallize. The analyses were performed on a 4700 Proteomics Analyzer (TOF/TOF™) (Applied Biosystems, USA) equipped with a 355-nm ND:YAG laser. The instrument operated in the positive ion reflection mode at 20 kV accelerating voltage and batch mode acquisition control. Reflector spectra were obtained over a mass of 700–3500 Da. The first five precursor ions with highest intensity were selected for fragmentation. The spectra were internally calibrated using two trypsin autolysis peaks at m/z 842.510 and 2211.105. The proteins were identified by peptide mass fingerprinting (PMF) or MS/MS using the program MASCOT (Matrix Science, London,

UK) against an NCBInr database with GPS explorer software (Applied Biosystems, USA). The searching was carried out in *Rattus norvegicus* species. A maximum of one missed cleavage peptide was allowed, a mass tolerance of 0.3 Da, and MS/MS tolerance of 0.4 Da were used, and variable modifications, such as carbamidomethylation for cysteine and oxidation for methionine, were taken into account. Tryptic autolytic fragments and contamination were removed from the data set used for database search.

2.8. Measurement of myocardial energy metabolism

To monitor myocardial energy status after ischemia and SDD treatment, collected cardiac tissues were prepared according to the modified method of Volonté et al. [22]. Briefly, left ventricle sample (100 mg) was mechanically homogenized with 0.5 mL perchloric acid (0.4 M). Centrifuged at 4 °C (10,000 × *g*, 5 min), 400 μL of the supernatant was transferred to a test tube, neutralized with 50 μL potassium phosphate (0.2 M) and centrifuged again. The supernatant was used for quantitative analysis for ATP, ADP and AMP. High performance liquid chromatography (HPLC) was applied to measure adenine nucleotides under the described experimental conditions. Results were expressed as median in nmol/mg protein. Total adenine nucleotide concentration (TAN), a measure of pool of all nucleotides containing adenine moiety (ATP + ADP + AMP), and adenylate energy charge (AEC), calculated by (ATP + ADP/2)/(ATP + ADP + AMP), were used to evaluate the status of myocardial high-energy phosphate contents [23].

2.9. Western blot analysis

Twenty micrograms of cardiac protein was separated by 10% SDS-PAGE using a Bio-Rad mini-protein II electrophoresis, and transferred into polyvinylidene difluoride membrane (PVDF, Amersham Pharmacia Biotech, Piscataway, NJ). Blots were blocked (2 h) with 5% non-fat dry milk in Tris-buffered saline (TBS) at room temperature and then incubated overnight at 4 °C with antibody against Hsp27 (1:2000) (Sigma, St. Louis, MO, USA), and ATP Synthase-β (1:10000) (BD Biosciences, USA) diluted in blocking buffer. Blots were incubated with HRP-conjugated secondary anti-rabbit antiserum (Santa Cruz, CA, USA) diluted 1:5000 in TBS. After several washes with 0.1% TBS-Tween 20, immunoreactive proteins were visualized with an enhanced chemiluminescence (ECL) and captured on an X-ray film. Protein levels were quantitated using Biosense 300 software (Oberhaching, Germany). Each membrane was routinely stained with Ponceau S to ensure equivalent loading. Common sample were included on each blot to allow quantitative between-blot comparisons. Initial quantities were adjusted by subtracting the background, then normalized for any loading inequities based on the Ponceau S stained image of the gel, and finally adjusted for signal intensity differences among groups.

2.10. Data analysis and bioinformatics

All data were expressed as mean ± S.D. Difference in means between groups was tested by one-way ANOVA. The *P*-values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

Principal component analysis (PCA), the most commonly used algorithm in proteomic studies [24], was employed to process protein abundance data using the program contained in Matlab 6.5 (Mathwork Inc.). The simultaneous comparison of a large number of complex objects was facilitated by reducing the dimensionality of the data set via two-dimensional mapping procedures. The resulting data were displayed as ''score plots'', which represent the distribution of samples in multivariate space. The score plots of the first two principal components were applied to visualize the drug-induced difference in proteomic expression profile.

3. Results

3.1. Effect of SDD on infarct size and serum CK, CK-MB level

To evaluate cardioprotective effects of SDD, the infarct size of heart, serum CK and CK-MB levels in rats with LAD ligation were measured. Infarct size was expressed as a ratio of the mass of ischemic zone over the left ventricular mass (IA/LV). In MI group, IA/LV was 35.66% ± 7.31. Treatment with SDD resulted in smaller infarction with 24.18% ± 4.09 for IA/LV (Fig. 2A).

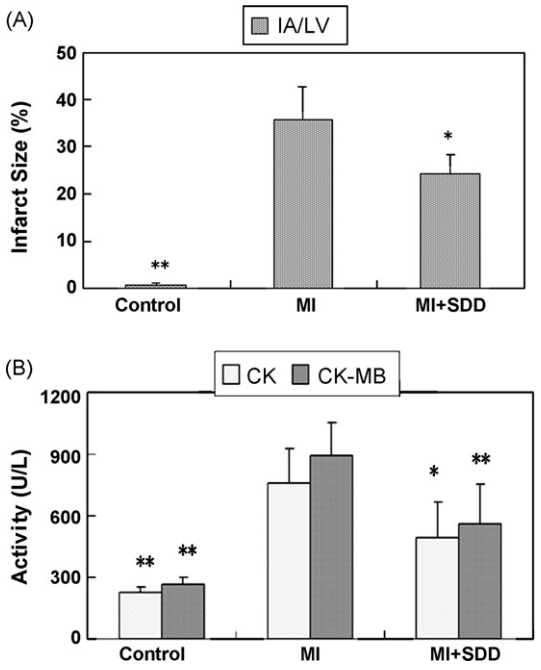

Fig. 2 – Effect of SDD on coronary ligated rats. (A) Comparing with MI group, significant difference in the infarct size occurred in treated and control group. The IA/LV and IA/H were 0.62% ± 0.30 and 0.38% ± 0.17 for sham-operated rats, 24.18% ± 4.09 and 16.52% ± 2.34 for SDD treated rats, 35.66% ± 7.31 and 23.58% ± 4.72 for MI group, respectively. (B) Effect of SDD on the serum CK and CK-MB levels. Each column represents mean ± S.D. (*) $P < 0.05$, (**) $P < 0.01$ vs. MI group.

Table 1 – Concentration of adenine nucleotides and TAN, AEC in rat heart tissue

	ATP (nmol/mg protein)	ADP (nmol/mg protein)	AMP (nmol/mg protein)	TAN (nmol/mg protein)	AEC (%)
Control	14.22 ± 5.64**	10.68 ± 2.71*	22.36 ± 8.38*	47.26 ± 15.46**	0.41 ± 0.03**
MI	0.79 ± 0.25	5.74 ± 1.09	9.04 ± 3.00	15.56 ± 4.09	0.24 ± 0.03
MI + SDD	3.88 ± 1.41*	7.17 ± 1.28	12.32 ± 3.09*	23.38 ± 4.54**	0.32 ± 0.02*

* $P < 0.05$.
** $P < 0.01$, vs. MI group.

As important indicators of myocardial damage, CK and CK-MB levels were monitored in control, MI and MI + SDD groups. Treatment with SDD resulted in a marked reduction in serum CK and CK-MB activity, compared with the MI rats (492 ± 175 and 561 ± 192 U/L versus 762 ± 166 and 896 ± 157 U/L, $P < 0.05$ and $P < 0.01$, respectively), while CK and CK-MB in control group were 224 ± 30 and 266 ± 36 U/L (Fig. 2B). These results indicate that SDD can effectively diminish ischemic injury in the heart of rats with LAD ligation.

3.2. *Contents of adenine nucleotides in heart tissue*

We next addressed to measure the concentrations of ATP, ADP and AMP in heart tissue for evaluating the energy status of cells in each group. Significant decrease of ATP, ADP and AMP levels were observed after LAD ligation, which coincides with previous report that adenine nucleotides was depleted under ischemia condition [25]. The loss of ATP in MI rats may be attributed to the large infarct size in left ventricle (~36%) and almost 50% of myocardial perfusion blocked due to LAD ligation. Compared to the MI group, level of ATP and ADP in SDD treated rats was elevated, approximately five-folds and 25% respectively. Moreover, the up-regulation of TAN and AEC in treated group indicated that energetic process of myocardial tissue was improved by SDD significantly (Table 1).

3.3. *Measurement of Nitric Oxide Metabolites*

In the cardiovascular system, NO appears to play a significant role in the regulation of heart contractile function, oxygen consumption and energy metabolism by a variety of mechanisms [26,27]. We used Griess assay to measure the amount of nitrite in serum. As shown in Fig. 3, there was no significant difference between serum nitrite content in control and MI group, while SDD treatment significantly increased the amount of synthesized NO.

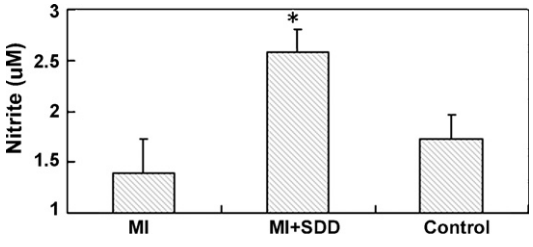

Fig. 3 – Effect of SDD on serum nitrite level. The nitrite concentration in MI, SDD-treated and sham-operated rats was 1.40 ± 0.34, 2.58 ± 0.23, 1.73 ± 0.24, respectively. Each column represented mean ± S.D. (*) $P < 0.05$ vs. MI group.

3.4. *Differential expression of rat heart proteins*

Protein samples from the control, MI and MI + SDD group were prepared as described above. The procedure of sample preparation and 2DE separation ensured the reproducibility of the proteomic analysis. Proteins were resolved on 2DE gels and visualized by silver staining. Using Image Master 2D Platinum, means of 760, 750 and 730 protein spots were detected from the control, MI and SDD treated group, respectively. The expression patterns of heart proteins are shown in Fig. 4 (A) MI + SDD, (B) MI, (C) Control. Most of the protein spots were distributed in the region of isoelectric point (pI) 4–9 and had molecular weight between 15 and 70 kDa. Compared with MI group, 23 protein spots were found to be significantly altered in SDD treated group. Out of these proteins, 14 proteins were upregulated and 9 proteins were downregulated. The significance of the changes in protein abundance was calculated by student *t*-test. For all the selected plots, the *p*-value between MI and treated group or between control and treated group was less than 0.05. The relative spot intensity of the proteins in MI and MI + SDD group was calculated, taking control group as standard. To illustrate the change in intensity of the protein spots, enlarged 2D gel images are shown in Fig. 5.

The PCA plot map of those proteins was shown in Fig. 6. In the PCA map, each spot represented a rat. It is clear that the spots representing the sham-operated, MI and SDD treated rats are shifted away, suggesting that the different proteomic patterns have been established due to the drug effect.

3.5. *Identification of altered proteins in rat myocardium*

The differentially expressed protein spots were isolated from the 2D gel and subjected to trypsin digestion and identified using MALDI-TOF–MS. The peptides mass peaks were compared with those in the NCBI database. As a demonstration, the mass spectrum of Cu/Zn superoxide dismutase protein was shown in Fig. 7. The complete list of protein data identified by MS was listed in Table 2. The altered proteins induced by SDD treatment were categorized into several groups according to their functions. The first group of altered proteins was related to energy metabolism: including the increase in the mitochondrial precursors of NADH ubiquinone oxidoreductase, cytochrome *c* oxidase subunit Va, malate dehydrogenase 1, long-chain acyl-CoA dehydrogenase, glyceraldehydes 3-phosphate-dehydrogenase (GAPDH), aldolase A, lactate dehydrogenase B, creatine kinase, and the significant down-regulation of NADH dehydrogenase 1 alpha 10, ATP synthase H(+)-transporting and triose phosphate isomerase. The second group of proteins was associated with oxidative stress, including the up-regulation of Cu/Zn superoxide dismutase

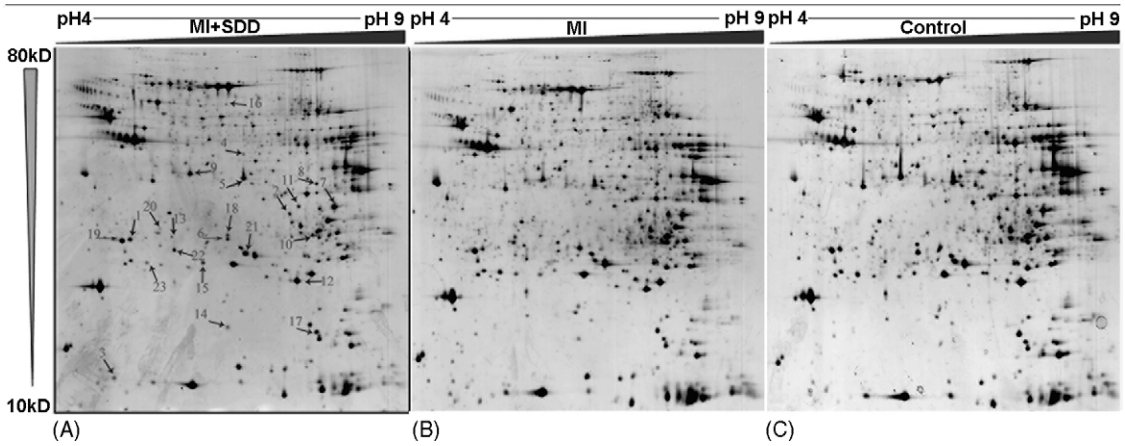

Fig. 4 – Representative 2D gel images obtained from heart protein extracts from SDD treated (A), MI (B) and sham-operated (C) rats. Proteins on 2DE gels were visualized by silver staining. Differential proteins were marked by arrow and number.

(SOD), precursor of peroxiredoxin 5, mitochondrial GrpE-like 1, and the downregulation of 58 kDa glucose-regulated protein (Grp58), αB-crystallin and heat shock protein 27 (Hsp27). In the third group, skeletal proteins, α-Actin, heavy chain myosin polypeptide 6 and myosin light polypeptide 3, were down-regulated. Other proteins, DJ-1 protein, phosphatidylethanolamine binding protein (PEBP) and apolipoprotein A-I, were increased.

3.6. *Western blot analysis*

The 27 kDa heat shock protein (Hsp27) and 55 kDa ATP Synthase-β were selected and subjected to Western blot analysis. The immunoblotting data (Fig. 8) confirmed the 2DE gel data. Compared with the control group, the levels of Hsp27 and ATP Synthase in the LAD ligation hearts increased, which is in consistent with previous proteomic findings in ischemic rabbit myocardium [28]. SDD treatment restored the Hsp27 and ATP Synthase levels to the control level.

4. Discussion

In China, SDD is commonly used as an agent for acute ischemia and ischemia-induced angina. Traditional Chinese Medicine (TCM) doctors usually suggest persons with stable

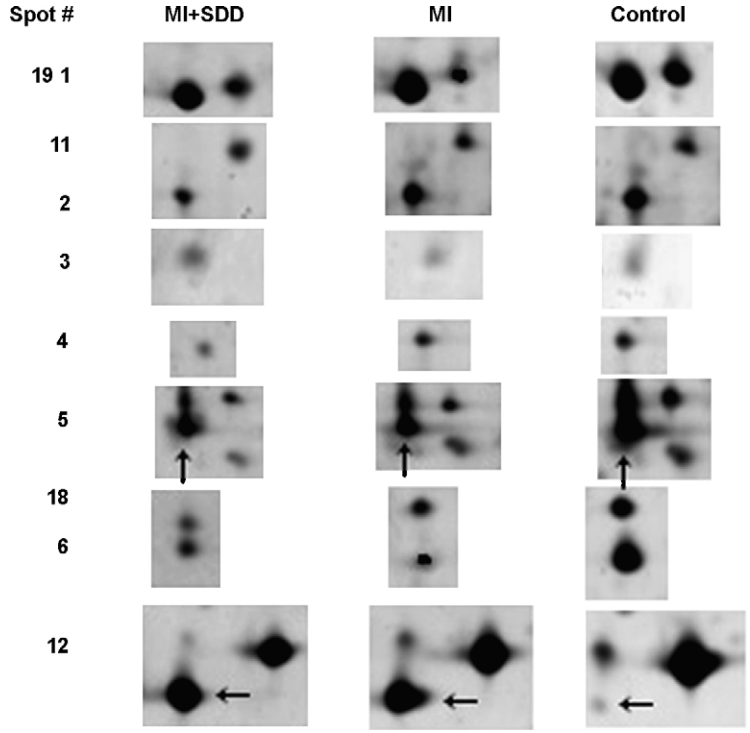

Fig. 5 – Selected regions of 2D image showing examples of protein spots with significantly altered expression in control, MI and MI + SDD group. Information on each numbered spot is listed in Table 2.

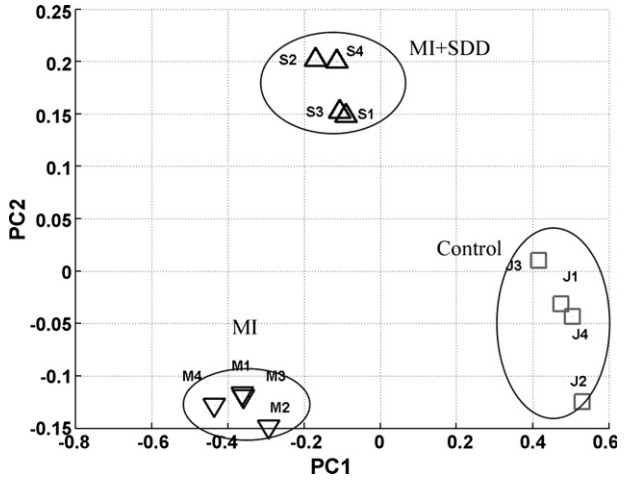

Fig. 6 – PCA map of rats from MI group (▽), control group (□) and MI + SDD group (△).

and unstable angina pectoris use SDD for long time because side effect of SDD has rarely been reported. SDD preparations have also been used to help patients against acute ischemic stroke [29]. The common dosage of SDD for human is in a range of 0.3–0.9 g/kg raw material per day. In present study, the dosage for rat was calculated according to body surface area (BSA) based on daily consumption of humans, which is approximate six-fold. Acute ischemic rats induced by LAD ligation were used to investigate therapeutical effect of SDD, and 2D gel electrophoresis was applied to study change of protein profile in myocardium. 2D gel electrophoresis is the most classical and popular approach for cardiac proteome separation [30], though it also contains several drawbacks, such as lacking of ability for detecting low-abundance proteins and very small or very large proteins [31]. In current study, the detectable molecular weight for proteins was between 10 and 100 kDa, which covers primary cardiovascular biomarkers reported by other proteomic studies [10].

Acute ischemia may occur as a result of increased myocardial metabolic demand and decreased supply of

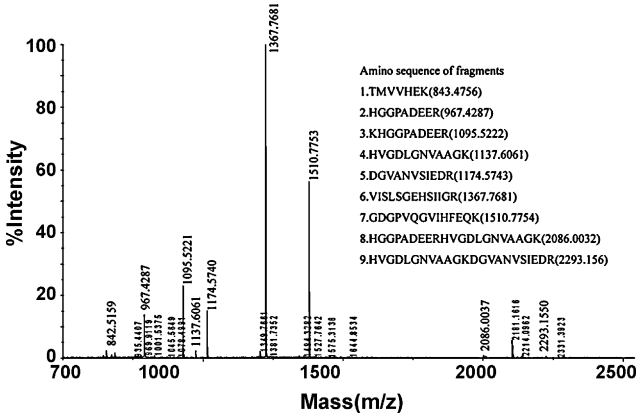

Fig. 7 – MALDI-TOF spectra of Cu/Zn superoxide dismutase protein (SOD) isolated from 2D gel. Out of 11 representative spectra, 9 peptides were matched and listed.

oxygen and nutrients to the myocardium via the coronary circulation. Oxidative stress is also regarded as an important risk factor [32]. The cardioprotective effects of SDD may be caused at several levels of the cardiovascular system, such as heart rate, aortic pressure and hemorheology [33]. Although precise mechanism of beta-blocker-induced improvement in heart failure is not fully understood, it has been reported that some beta-blockers such as propranolol can improve contractile force and reduce catecholamine-induced cardiac oxidative stress in the myocardial infarction rat model [34]. In present study, we showed SDD reduced infarct size of heart, decreased serum CK and CK-MB activity, improved energy status and prompted serum nitrite content. Differential proteomic profiles induced by SDD treatment in rat myocardium were resolved by 2D and MALDI-TOF–MS. Most of the identified spots are proteins involved in the energy/metabolism, stress response and cytoskeleton.

Myocardial ischemia can be regarded as a metabolic problem involving disruption of mitochondrial oxygen consumption and subnormal aerobic ATP resynthesis, resulting in ATP depletion, decrease of mitochondrial NADH oxidation, activation of glycolysis and accumulation of lactate and H^+. In this study, 11 proteins associated with energy metabolism were identified, related to several pathways of energy metabolism including respiratory chain (Rsp-chain), TCA-cycle, β-oxidation, glycolysis and phosphocreatine.

NADH ubiquinone oxidoreductase (Complex I) is the largest of five mitochondrial electron transport chain complexes comprising of at least 46 subunits. In this study, several components of Complex I, the NADH ubiquinone oxidoreductase (NADH-Uq) complex and NADH dehydrogenase subunit alpha 10 (NDUFA10), were altered. A decrease in abundance of NADH-Uq 24 kDa subunit was observed in MI group, which may be induced by the generation of reactive oxygen species during ischemia. SDD treatment can recuperate the expression of NADH-Uq to the level of control group. Cytochrome *c* oxidase (CcO), located in the inner mitochondrial membrane, is an important component in electron transport chain. We also observed the significant decrease of cytochrome *c* oxidase subunit VA (CcO5a) in MI group, which is consistent with the previous report of reduced CcO content during myocardial ischemia [35]. Compared with MI group, SDD can prevent this decrease. This effect may be attributed to the observed increased release of nitric oxide, which can upregulate CcO mRNA expression in post-ischemic myocardium [36].

Moreover, according to our data, two β-oxidation and TCA-cycle related proteins, long-chain acyl-CoA dehydrogenase and malate dehydrogenase (MDH), are markedly downregulated in MI group, which suggested β-oxidation and TCA-cycle are almost ceased after prolonged ischemia due to the deficient oxygen and other substrates supply. After SDD treatment, the significant upregulation of long-chain acyl-CoA dehydrogenase and MDH was observed. It is worth noticing that in SDD-treated rats, coronary microcirculation may be improved due to the increased release of NO. It is believed that endothelial-derived NO plays an important role in regulating ischemia-induced angiogenesis [37], which is beneficial to improve coronary microcirculation and correct the imbalance between the perfusion capacity of coronary vessels and the

Table 2 – Identified altered proteins and relative spot intensity of their spot volumes[a]

Spot no.	Protein identity	NCBI accession no.	Relative spot intensity % (control, 100)[b]		Experimental values (theoretical values) pI/kDa	Score
			MI	MI + SDD		
Proteins associated with energy/metabolism						
Rsp-Chain						
1[c]	NADH ubiquinone oxidoreductase (24-kDa subunit)	83305118	$68 \pm 6.2^{\#\#}$	$98 \pm 5.6^{**}$	4.8/22 (6.0/26)	126
2	ATP synthase, H(+)-transporting	57029	$123 \pm 6.9^{\#}$	$76 \pm 12.6^{**}$	7.0/25 (7.0/26)	118
3[c]	Cytochrome c oxidase subunit VA	55971	$60 \pm 14^{\#\#}$	$92 \pm 7.1^{*}$	4.7/12 (6.1/16)	88
4	NADH dehydrogenase 1 subunit alpha 10	32996721	107 ± 14	$39 \pm 12.3^{**}$	6.3/41 (7.1/40)	206
TCA-cycle						
5[d]	Malate dehydrogenase 1	15100179	$57 \pm 8.5^{\#\#}$	$78 \pm 8.9^{*}$	6.3/36 (6.2/36)	199
β-oxidation						
6[d,e]	Long-chain acyl-CoA dehydrogenase	6978431	$20 \pm 2.3^{\#\#}$	$56 \pm 7.8^{**}$	6.3/21 (7.6/48)	145
Glycolysis						
7	Glyceraldehyde 3-phosphate-dehydrogenase	56611127	$125 \pm 7.2^{\#}$	144 ± 11.9	8.0/27 (8.1/36)	91
8	Aldolase A	202837	121 ± 14	$174 \pm 15^{**}$	7.6/37 (8.3/39)	99
9	Lactate dehydrogenase B	6981146	$42 \pm 8.4^{\#\#}$	$68 \pm 4.8^{*}$	5.7/37 (5.7/37)	181
10	Triose phosphate isomerase	38512111	$194 \pm 10^{\#\#}$	$105 \pm 14^{**}$	7.0/22 (7.0/27)	85
Phosphocreatine						
11	Creatine kinase, muscle	6978661	$75 \pm 7.7^{\#}$	$145 \pm 9.3^{**}$	7.0/43 (6.6/43)	110
Stress-induced/heat shock proteins						
12	Alpha B-crystallin	57580	$902 \pm 62^{\#\#}$	$722 \pm 63^{*}$	6.8/19 (6.8/20)	178
13[f]	Heat shock protein 27	14010865	$211 \pm 22^{\#\#}$	$74 \pm 11^{**}$	5.6/21 (6.1/23)	119
14	Cu/Zn superoxide dismutase	1213217	113 ± 16	$158 \pm 14^{*}$	6.2/15 (5.7/16)	151
15	GrpE-like 1, mitochondrial	67678103	$48 \pm 5.5^{\#\#}$	$104 \pm 9.7^{**}$	6.3/20 (8.6/24)	90
16	Glucose regulated protein, 58 kDa	38382858	$147 \pm 18^{\#}$	$88 \pm 9.4^{**}$	6.2/60 (5.9/57)	293
17[c]	Peroxiredoxin 5, precursor	51261175	$61 \pm 4.8^{\#\#}$	$86 \pm 14^{*}$	7.6/17 (8.9/22)	111
Cytoskeletal proteins						
18[d]	Myosin heavy chain, polypeptide 6	554475	$65 \pm 7.7^{\#\#}$	$37 \pm 5.1^{**}$	6.2/20 (6.2/19)	80
19	Myosin, light polypeptide 3	6981240	84 ± 5.7	72 ± 7.1	4.8/20 (5.0/22)	137
20[f]	α-Actin	6671507	132 ± 17	$72 \pm 17^{**}$	5.4/21 (5.2/42)	112
Other proteins						
21	DJ-1 Protein	16924002	$62 \pm 4.6^{\#\#}$	$97 \pm 5.4^{**}$	6.3/20 (6.3/20)	90
22	Apolipoprotein A-I	2145143	90 ± 17	$242 \pm 24^{**}$	5.5/20 (5.5/30)	124
23	Phosphatidylethanolamine binding protein	8393910	$67 \pm 7^{\#\#}$	$88 \pm 5.6^{*}$	5.4/19 (5.5/21)	89

[a] Theoretical molecular mass and pI were derived from the amino acid sequences in NCBI.
[b] $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the sham-operated group.
[c] Precursor protein.
[d] Identify confirmed by MS/MS.
[e] Possible degradation product.
[f] Possible PTMs.

need for oxygen and nutrients by the ischemic myocardium. Thus, the increased level of β-oxidation and TCA-cycle related enzyme reported in our data may be attributed for NO-induced vasorelaxant effect and the partly resumed oxygen and substrates delivery. Eventually, more energy was obtained through aerobic metabolism and hence satisfying the consumption of the improved cardiac function. This was further supported by our observation that tissue ATP, TAN levels and AEC were significantly increased after SDD treatment.

As a systemic disease, ischemia heart disease also leads to differential expression of proteins associated with stress response. Abundant heat-shock proteins (HSP) and other proteins induced in ischemic stress confer cytoprotection by participating in cellular repair and restructuring during the recovery phase of ischemia [38]. In our study, two altered heat shock proteins were identified as alpha B-crystallin and Hsp27. In contrast to the control group, expression of alpha B-crystallin and Hsp27 was significantly increased in MI group, as reported that hypoxia and oxidative stress can induce the up-regulation of alphaB-crystallin and Hsp27 [39]. In the treated group, the expression of alphaB-crystallin and Hsp27 was significantly mediated compared to MI group, which suggested the oxidative stress was partially diminished after SDD treatment.

Cu/Zn superoxide dismutase can remove ROS from cellular environment for protecting organisms against oxidative damage [40]. The expression of SOD, enzymatic antioxidant was minor increased in LAD rats and significantly increased by SDD treatment. Sun et al. [33] have proved that cardiac SOD activity was increased by *Salviae Mitiorrhizae* extract in rats with acute myocardial infarction, while paeonol, the major component of *Cortex Moutan*, improved myocardial SOD activity [41]. It suggests the increased expression of SOD can be attributed to these active compounds of SDD.

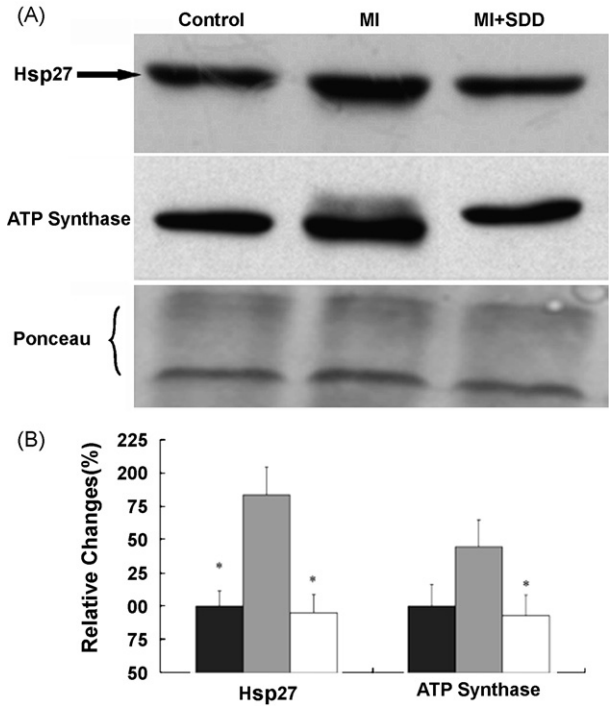

Fig. 8 – Expression of hsp27 and ATP Synthase protein in hearts of control, LAD ligation, and SDD treated rats. (A) Representative immunoblot. A portion of the membrane was stained with Ponceau S to document equitable protein sample loading. (B) Bar graph comparing mean values in Control, MI, and MI + SDD (% to control). Values are mean ± S.D. (*) $P < 0.05$ vs. MI.

Peroxiredoxins (Prxs) are a ubiquitous family of thiol-specific antioxidant enzymes and have received considerable attentions in recent years [42]. Prx5 is a mammalian cytosolic antioxidant enzyme that reduces hydrogen peroxide and other oxygen species [43]. SDD treatment significantly reversed the decline of Prx5 expression in myocardium of rats during ischemia, which may cause an alternation in the cellular redox state. Thus, the up-regulation of SOD and Prx5 suggests that promotion of antioxidant capability have been achieved after SDD treatment and it may be helpful to diminish oxidative induced injury.

The alternations of skeletal proteins in heart tissue, including various myosin and α-actin, have been reported in previous proteomic studies [44]. In our study, the expression of three skeletal proteins, i.e. myosin heavy chain 6, myosin light chain 3 and α-actin was downregulated by SDD. In ischemic preconditioned (IPC) rabbit hearts, the decrease of cytoskeletal proteins was observed [45]. We suggest these decreases to be caused by cellular recovery of infarct region after SDD treatment.

In summary, it is the preliminary effort to using comparative proteomic approach to investigate the mechanism of action of herbal medicine. The presented proteomic data suggested that the observed smaller infarct size of SDD-treated heart was due to two complementary mechanisms: preserved mitochondrial function and energy production and decreased stress-induced injury during acute ischemia. The global proteomic data can help us to better explain SDD protective effect on ischemic myocardium behind its good clinical results. Such a holistic approach will also contribute to the scientific study for other herbal medicines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.04.017.

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