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Original Article

Proteomic studies on the protective effects of Shenfu decoction in chronic heart failure

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Abstract: Objective: Shenfu decoction has been shown to improve left ventricular function following chronic heart failure (CHF). However, the mechanisms underlying these cardioprotective effects are incompletely understood. In the present study, the heart ventricle was subjected to proteomic analysis to identify possible cellular targets. Methods: Isobaric tag for relative and absolute quantification (iTRAQ) was used to investigate the expression of different proteins in heart tissue after ligation of the left anterior descending coronary artery with or without administration of Shenfu decoction. Results: Treatment with Shenfu decoction conferred cardioprotective effects in myocardial infarction-induced CHF. Proteins which had a 3-fold change in abundance, compared to the sham control sample, were selected for identification. Gene ontology analysis linked these proteins to specific biological processes, including energy metabolism, mitochondrial function, the cytoskeleton and others. Conclusion: The protective effects of Shenfu decoction on the myocardium in CHF may be related to the recovery of energy supply and the promotion of cell recovery. These findings suggest that Shenfu decoction may be a potential therapeutic agent in the treatment of CHF and may be a strong candidate for future research due to its actions on individual proteins.

Keywords: Chronic heart failure, proteomics, Shenfu decoction

Introduction

Myocardial infarction (MI) is a major cause of heart failure. According to the latest estimates, 610,000 new attacks and 325,000 recurrent attacks of MI are reported in the USA annually. Chronic heart failure (CHF) is a complex syndrome and one of the main causes of morbidity and mortality worldwide. However, its underlying mechanisms are elusive [1]. Previous evidence demonstrated that heart failure is a dynamic process involving cell death, oxidative stress, fibrosis, inflammation, and a complicated underlying regulatory network [2]. However, current pharmacological treatments are limited, thus an effective method is needed to elucidate these mechanisms. Traditional Chinese medicines (TCM) have protective effects in ventricular remodeling. These provide alternative methods for the prevention of ventricular remodeling to ameliorate the prognosis of MI. Determining the underlying mechanism of effective TCM formulae on ventricular remodeling, especially the classical formulae, would not only provide a better understanding of these formulae, but also provide a translational opportunity for the ancient formulae. During heart failure, the heart undergoes early- and late-phase changes [3] in both structure and left ventricular dysfunction (systolic and diastolic) [4]. Effective prevention is crucial for the control of CHF-related events; however, current pharmacological treatments are limited. New insights into the underlying mechanisms are needed for the identification of novel therapeutic targets. As western medicine is more effective in the early stages of heart failure, the present study focuses on the effects of Shenfu decoction during post-infarct remodeling. Traditional Chinese Medicines (TCM) have been used for many centuries, and are still widely used in South and East Asian countries for the treatment of CHF. To some extent, TCM can improve symptoms during the heart failure process.

Proteomics offers an essential strategy for large-scale research into alterations in protein profiles. Not only does it enable the identification of changes in protein profiles at specific time points, but it could also help us understand the molecular mechanism of heart failure [3, 5]. In the present study, we used a novel proteomic method known as iTRAQ (isobaric tag for relative and absolute quantification). Other methods include two-dimensional differential in-gel electrophoresis (2D-DIGE) and isotope-coded affinity tagging (ICAT). iTRAQ is more appropriate and sensitive than 2D-DIGE and ICAT, especially for comparative studies in which more than two samples are evaluated in parallel [6, 7]; as it enables all samples to be treated simultaneously [8]. Moreover, it can identify low-abundance proteins [9] as well as integral membrane proteins [10]. In the iTRAQ method, four isotope tags (with reporter group m/z 114, 115, 119, 121) are used to label peptides. The reporter groups released from the chemical tags after ion fragmentation are used for quantification by measuring the relative peak area contributed by each sample to the total peptides present.

The aim of this study was to assess the proteomic changes and the effects of Shenfu decoction in CHF, which may be a strong candidate for future research due to its actions on individual proteins. The findings of this study could also provide novel evidence for the value of TCM in improving cardiac function.

Methods

Animals

Forty-five adult male Sprague-Dawley (SD) rats (220±10 g) were purchased from the Experimental Animal Center of the Vital River Laboratory Animal Technology Co. Ltd, China. These rats were randomized into three groups, each comprising 15 animals: Sham-operated plus 0.9% NaCl, CHF plus 0.9% NaCl and CHF plus Shenfu decoction. One week later, myocardial infarction (MI) was induced by permanent ligation of the left anterior descending coronary artery, as described previously [11]. During the operation, electrocardiograms showed ST-elevated MI. Rats in the sham-operated group underwent the same surgical procedure, including thread-drawing, but not ligation at the same site. Shenfu decoction or 0.9% NaCl was then injected intragastrically once per day for four weeks. The Shenfu decoction contained Radix Ginseng (RG) and Radix Aconitum Carmichaeli (AC) in a 1:1 ratio and each rat received 0.25 g/mL (1 ml/100 g).

Echocardiographic studies

A noninvasive transthoracic echocardiography method was used to evaluate the morphology and function of the left ventricle (LV). Transthoracic echocardiographic determinations were conducted in the lateral decubitus position using a commercially available echocardiograph (Philips iU22 Ultrasound System with a 6-15 MHz transducer, Bothell, WA, USA). Echocardiography was performed in anesthetized animals using sodium pentobarbital (80 mg/kg, intraperitoneal, Fluka, Sigma-Aldrich, Inc., St. Louis, MO, USA). It consisted of a two-dimensional mode, that is, time-motion (TM) mode and blood flow measurements in pulsed Doppler mode. TM mode measurements were performed in the parasternal long-axis view. The measured parameters included diastolic septal wall thickness, diastolic posterior wall thickness of the LV, and end-diastolic and end-systolic diameter of the LV. We calculated LV shortening fraction using the following formula: [(end-diastolic diameter-end-systolic diameter)/end-diastolic diameter × 100]. End-systolic wall stress was measured according to the method described by Boissiere et al.

Measurement of hemodynamic parameters

Four weeks after the operation, the rats were anesthetized with 10% chloral hydrate and a 2 mm diameter cardiac catheter was inserted into the LV via the right carotid artery and connected to a polygraph (Biopac Systems Inc., USA). Thus, the left ventricular systolic pressure (LVSP), the left ventricular end-diastolic pressure (LVEDP), and the maximal left ventricular pressure increase/decrease ratio (± dp/dt_{max}) were recorded. Following these hemodynamic measurements, the animals were killed and the heart was rapidly removed and divided into the RV, LV, right atrium, left atrium and septum. The tissue from within 2.0 mm outside the infarct zone (border zone) was dissected out, weighed and stored in liquid nitrogen. iTRAQ was used to identify differentially expressed proteins in specimens from the failing (n = 30) and non-failing (n = 30) rat hearts.

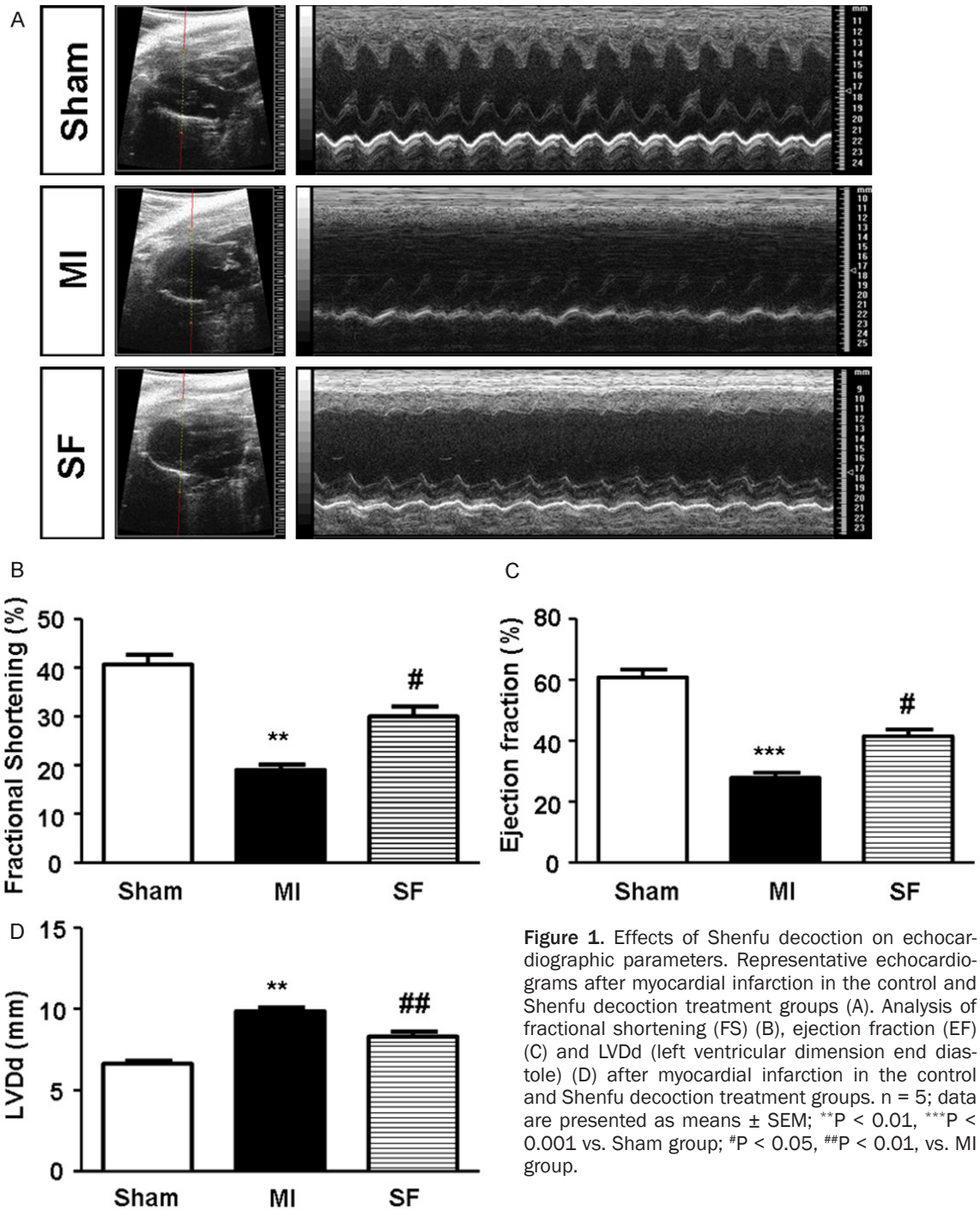

Figure 1. Effects of Shenfu decoction on echocardiographic parameters. Representative echocardiograms after myocardial infarction in the control and Shenfu decoction treatment groups (A). Analysis of fractional shortening (FS) (B), ejection fraction (EF) (C) and LVDd (left ventricular dimension end diastole) (D) after myocardial infarction in the control and Shenfu decoction treatment groups. n = 5; data are presented as means ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, vs. MI group.

Protein extraction

Before protein extraction, the samples were ground with liquid nitrogen. Total proteins were then extracted using the cold-acetone method. After 10% trichloroacetic acid (TCA) in acetone was added, each sample was incubated at -20°C for 2 h and then centrifuged at 20,000 g for 30 min at 4°C. The supernatant was discarded without disturbing the white pellet. To reduce acidity, the pellets were washed with acetone and centrifuged again at 20,000 × g for 30 min at 4°C. This acetone washing step was repeated three times. The dried pellets were lysed with 1 ml protein extraction reagent [8 M urea, 4% (w/v) CHAPS, 30 mM HEPES, 1

Table 1. The hemodynamic parameters measured in anesthetized rats including HR, LVSEP, LVEDP, +dp/dt$_{max}$ and -dp/dt$_{max}$ in the different groups

Groups	Sham	Model	Model + SF
Number	10	10	10
HR	419.41±59.70	399.09±68.83	475.57±63.64*
LVSEP	113.98±11.20	81.76±8.79*	108.19±13.66Δ
LVEDP	6.35±4.53	10.73±8.16*	5.48±2.03Δ
+dp/dt$_{max}$	2799.06±545.30	1616.44±247.17*	2739.82±525.77Δ
-dp/dt$_{max}$	-2943.17±440.67	-1641.71±235.88*	-2671.93±461.61Δ

mM PMSF, 2 mM EDTA and 10 mM DTT] and then dissolved using ultrasound. The resulting solution was centrifuged at 20,000 × g for 30 min at 4°C to remove insoluble impurities. The protein content was determined using a 2-D Quant Kit and the sample was stored at -80°C for future use.

iTRAQ labeling

After quantification using the Bradford method, a total of 100 μg proteins from each sample were denatured and cysteines were blocked as described in the iTRAQ protocol. The protein samples were then digested with 5 μg sequence grade modified trypsin (Promega, Madison, WI, USA) at 37°C for 36 h. The digested samples were dried in a centrifugal vacuum concentrator. The protein pellets were dissolved in 30 μL of 50% TEAB (Sigma, St. Louis, MO, USA) with 70 μL isopropanol and labeled with iTRAQ reagents according to the 8-plex iTRAQ labeling kit protocol (Applied BioSystems). The trypsin-digested samples were analyzed using MALDI-TOF-TOF to ensure complete digestion. During labeling, iTRAQ tags 114, 115, 119, and 121 were added to digest the protein samples. The iTRAQ-labeled samples were then pooled and subjected to strong cation exchange (SCX) fractionation.

SCX fractionation

The labeled samples were fractionated using a high performance liquid chromatography (HPLC) system (Shimadzu, Japan) connected to an SCX column (Luna 5 u column, 4.6 mm I.D. × 250 mm, 5 μm, 100 A; Phenomenex, Torrance, CA, USA). The retained peptides were eluted using buffer A (10 mM KH_2PO_4, in 25% ACN, pH 3.0) and buffer B (2 M KCl, 10 mM KH_2PO_4 in 25% ACN, pH 3.0), and the fractions were collected in 1.5 mL microfuge tubes. The flow rate was set at 1 mL/min. The following gradient was applied: 30 min 100% buffer A; 30~31 min increasing to 5% buffer B; 31~46 min increasing to 30% buffer B; 46-51 min increasing to 50% buffer B, maintained for 5 min; 55-61 min decreasing to 100% buffer B. All solutions used were freshly prepared and filtered through a 0.22 μm pore membrane. The collection of fractions began at 31 min following the injection and continued every minute to yield a total of 38 fractions. For the high salt concentration fractions, an additional step was included to remove the salt using a Strata-X 33 u. Polymeric Reverse Phase column (Phenomenex). The eluted fractions were dried in a vacuum concentrator, and each fraction was redissolved in 0.1% formic acid prior to reverse-phase LC-tandem mass spectrometry.

Reverse-phase nano liquid chromatography/tandem MS (LC-MS/MS)

In order to equalize the amount of peptides in each fraction before injecting it into the Nano-LC system, we used MALDI-TOF/TOF for analysis. Therefore, the SCX peptide fractions were pooled to produce 17 fractions, reducing the peptide complexity. A 10 μL portion of each fraction was injected twice into the Proxeon Easy Nano-LC system. Peptides were separated on a C18 analytical reverse phase column at a flow rate of 300 nL/min solvent (solution A: 5% acetonitrile, 0.1% formic acid; solution B: 95% acetonitrile, 0.1% formic acid) for 120 min. A linear LC gradient profile was used to elute peptides from the column. The gradient started with 5% solution B. After equilibration in 5% solution B, a multi-slope gradient was started 10 min after the injection signal as follows: 45% solution B at 80 min, 80% solution B at 85 min, maintained for 15 min, 5% at 105 min and held for 15 min before ramping down to the initial solvent condition. The fractions were analyzed using a hybrid quadrupole/time-of-flight MS (MicroTOF-Q II, Bruker, Germany) with a nano electrospray ion source. The data were collected and analyzed using Data Analysis Software (Bruker Daltonics, Bremen, Germany). The MS/MS scans from 50 to 2000 m/z were recorded. Nitrogen

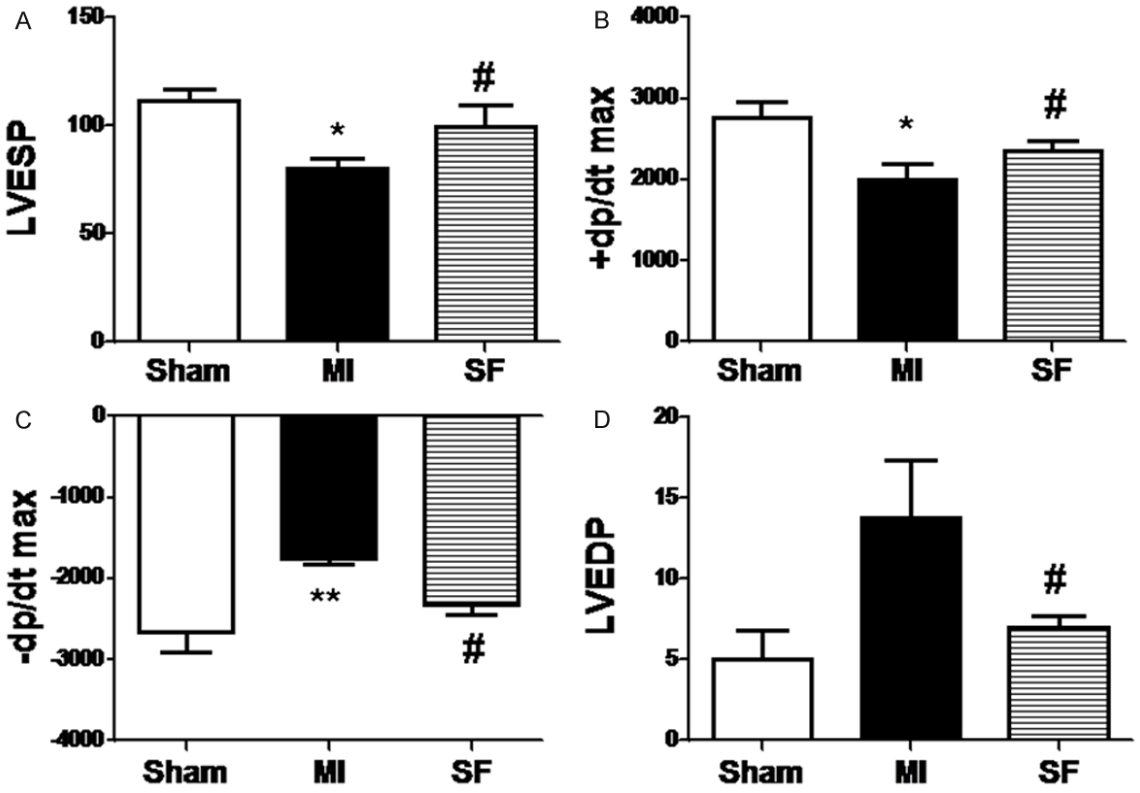

Figure 2. Effects of Shenfu decoction on hemodynamic parameters. A-D. Analysis of left ventricular systolic end pressure (LVSEP), left ventricular end-diastolic pressure (LVEDP), minimum (dp/dtmin) and maximum rate (dp/dtmax) of pressure in the ventricle after myocardial infarction in the control and Shenfu decoction treatment groups. n = 5; data are presented as means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Sham group; $^{\#}P < 0.05$, vs. MI group.

was used as the collision gas. The ionization tip voltage and interface temperature were set at 1250 V and 150°C, respectively.

Data analysis

All the mass spectrum data were collected using a Bruker Daltonics microTOF control, and processed and analyzed using Data Analysis Software. The uniprot_rat database was downloaded and integrated into the Mascot search engine version 2.3.01 by its database maintenance unit. All parameters were set as follows: specifying trypsin as the digestion enzyme, cysteine carbamidomethylation as the fixed modification, iTRAQ 8 Plex on the N-terminal residue, iTRAQ 8 Plex on tyrosine (Y), iTRAQ 8 Plex on lysine (K), glutamine as pyroglutamic acid, and oxidation on methionine (M) as the variable modification. The tolerance settings for peptide identification in Mascot searches were 0.05 Da for MS and 0.05 Da for MS/MS. Finally, the Mascot search results were exported into a DAT FILE, normalized and quantified using Scaffold version 3.0 Software. Protein fold change (iTRAQ ratio for individual proteins) was calculated automatically using Protein Pilot software. Data are presented as mean ± S.E.M. Differences between groups were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of Shenfu decoction on echocardiographic parameters

Changes in cardiac function after MI were determined by echocardiography. **Figure 1** shows an M-mode trace of LV function in a normal heart, an untreated infarcted heart (**Figure 1**, middle panel), and an infarcted heart treated with Shenfu decoction (**Figure 1**, lower panel). The loss of LV free wall motion, and the increase in LVEDD (distance during diastole between the upper and lower wall), 4 weeks after MI was moderated as a result of targeted Shenfu decoction compared with the untreated MI group. Rats with MI had significant sys-

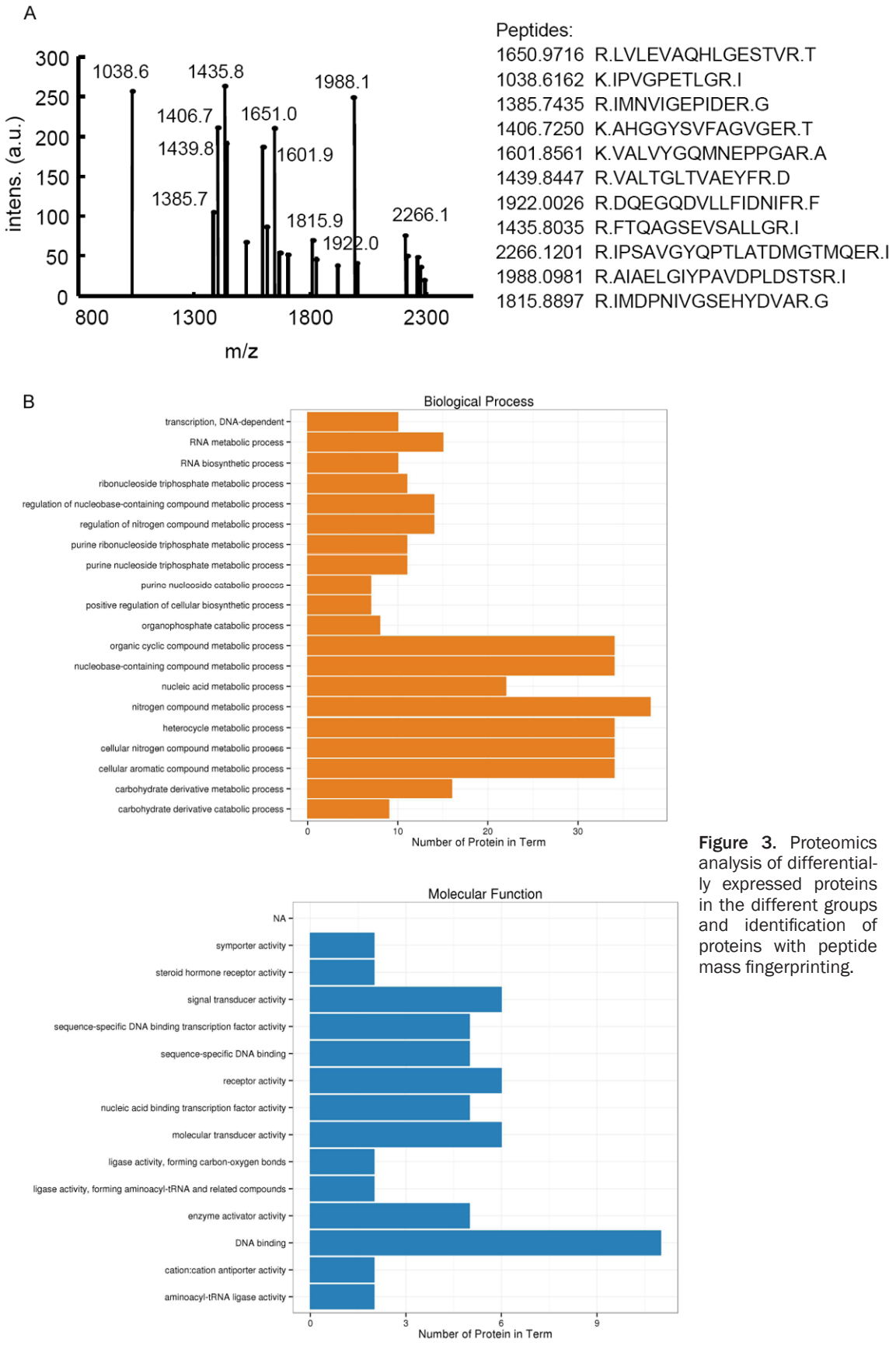

Figure 3. Proteomics analysis of differentially expressed proteins in the different groups and identification of proteins with peptide mass fingerprinting.

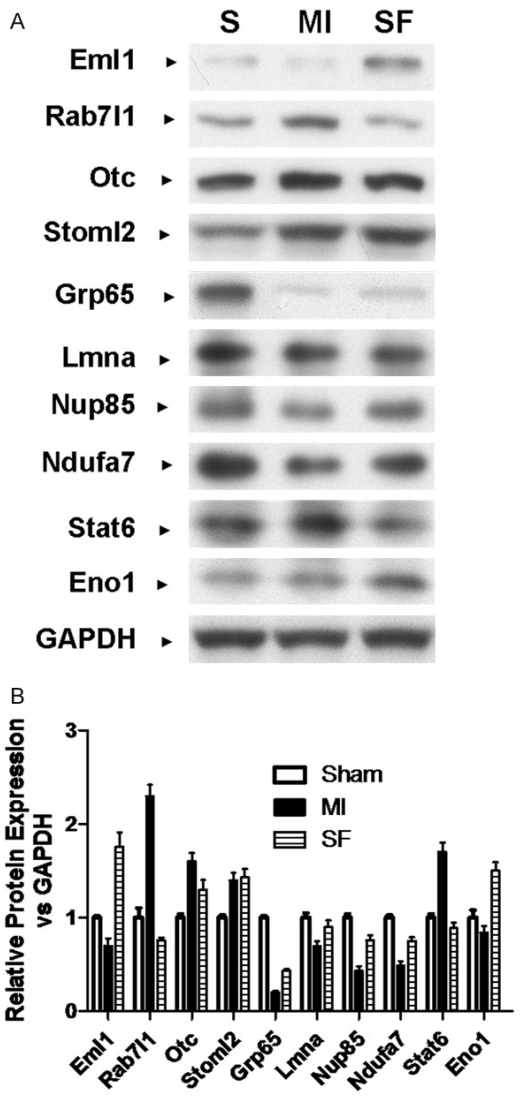

Figure 4. Representative immunoblots (upper panel) and averaged immunoblot data (lower panels) for detection of the expression of Eml1, Ndufa7 and others.

tolic dysfunction, as demonstrated by decreased fractional shortening in the 4th week, which was reversed by Shenfu decoction (**Figure 1B**). In addition, other functional parameters including fractional shortening (FS) and LVDd (left ventricular dimension end diastole) were improved by treatment with Shenfu decoction (**Figure 1C**, **1D**).

Effects of Shenfu decoction on hemodynamic parameters

Table 1 summarizes the hemodynamic parameters measured in anesthetized rats. As shown in **Figure 2**, Shenfu decoction significantly lowered left ventricular systolic end pressure (LVSEP) in rats with MI. Left ventricular end-diastolic pressure (LVEDP) was significantly higher in rats with MI at 4 weeks than in controls. In Shenfu decoction-treated rats, LVEDP decreased to a similar extent (**Figure 2D**). The minimum (dp/dt_{min}) and maximum rate (dp/dt_{max}) of pressure in the ventricle were significantly depressed after MI, and were reversed by treatment with Shenfu decoction (**Figure 2A**, **2B**).

Proteomics analysis of differentially expressed proteins in the different groups and identification of proteins with peptide mass fingerprinting

A total of 1755 proteins which varied between the different treatment groups were identified in the samples prepared from rat heart. Approximately 369 proteins which demonstrated at least a 1.5-fold change were selected by comparing the four groups. These proteins were selected by fold change, score, function, and other information. Compared with the sham group, 42 proteins were up-regulated and 99 were down-regulated in the MI group. In the control MI group, 86 proteins were strongly expressed and 38 were weakly expressed compared to the MI group treated with Shenfu decoction. The expression of these proteins were statistically significantly different ($P < 0.05$). We divided these proteins according to their location or function. The biological processes and molecular functions of the proteins are shown in **Figure 3**.

Detection of the expression of eml1, ndufa7 and others

Western blotting (**Figure 3**) analysis further confirmed the results of the proteomics analysis. Semiquantitative analysis of immunohistochemistry (data not shown) and western blotting showed that Shenfu treatment significantly decreased the expression of Rab71l and stat6 ($P < 0.01$). It also increased the expression of SORBS2, Ano9, Eml1, Ndufa7 and Eno1 which are involved in energy metabolism ($P < 0.05$). Most of the altered proteins were located within the mitochondria or were implicated in processes involved in energy production such as electron transport and catabolism. Moreover,

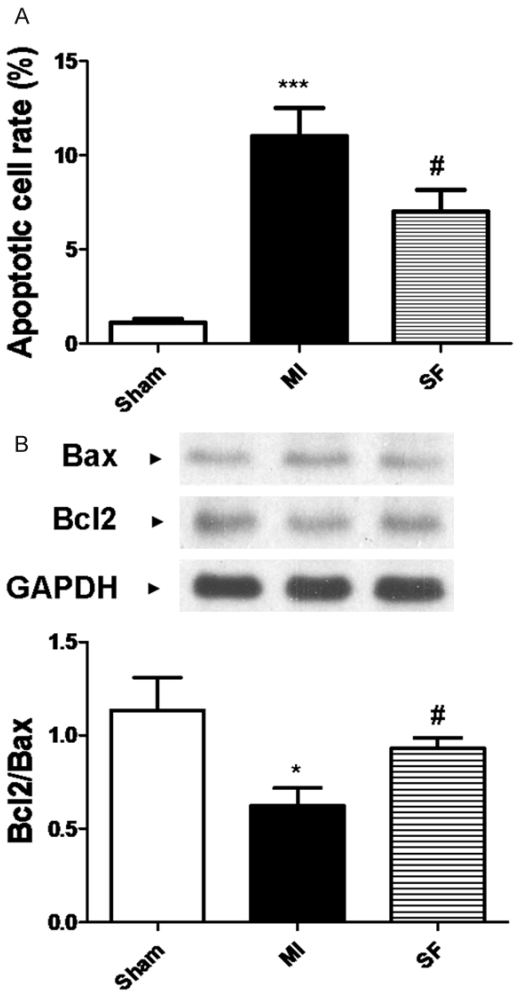

Figure 5. Effects of Shenfu decoction on apoptosis and expression of Bcl-2 and Bax. A. TUNEL analysis of apoptosis in left ventricular area at risk. B. Representative immunoblots (upper panel) and averaged immunoblot data (lower panels) of Bcl-2 and Bax expression. Data are presented as means ± SEM; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Sham group; $^{\#}P < 0.05$, vs. MI group.

ontology analysis linked these proteins to specific biological processes, including energy metabolism, mitochondrial function, the cytoskeleton and others. Western blot analysis suggested that the protective effects of Shenfu decoction on the myocardium in CHF may be related to the recovery of energy supply and the promotion of cell recovery (**Figure 4**).

Effects of Shenfu decoction pretreatment on myocardium apoptosis

The expression of Bcl-2, Bax and TUNEL staining suggested that a higher number of brown stained cells were found in the model group, as compared with the sham group ($P < 0.01$). Compared with the model group, Shenfu decoction significantly decreased the number of apoptotic cells ($P < 0.05$) (**Figure 5A**). Western blotting showed that the expression of Bcl-2 and Bax ($P < 0.01$; $P < 0.01$) (**Figure 5B**) in the model group increased significantly compared with the sham group. Compared with the model group, Shenfu decoction significantly decreased the expression of Bax ($P < 0.01$), but not Bcl-2. Compared with the sham group, the Bcl-2/Bax ratio in the model group decreased significantly ($P < 0.05$). Compared with the model group, Shenfu decoction significantly increased the Bcl-2/Bax ratio ($P < 0.01$) (**Figure 5B**).

Discussion

In this study, we determined the long-term effects of Shenfu decoction treatment in the setting of chronic MI. This is the first report of a proteomic analysis specifically using myocardial tissue from the border zone in relation to treatment with Shenfu decoction.

Following arterial ligation, our rat model demonstrated the pathophysiological process of heart failure induced by MI [12, 13]. The heart gradually evolved into three regions: infarct zone, border zone, and remote zone [14]. The border zone, located between the infarct and remote zones, has the greatest significance. This region is critical for the progression of ventricular remodeling following an acute MI [14]. Therefore, we chose to analyze this area. Four weeks after MI was induced, the hemodynamic parameters in rats were significantly impaired, consistent with previous reports [15]. These are crucial indices of heart failure. It is worth mentioning that more extensive changes were observed, such as changes in metabolic enzymes, contractile protein expression, and structures. Our results indicated that most of the proteins detected were related to metabolism. This finding is consistent with previous reports and indicated a high proportion of metabolic proteins in the rat cardiac proteome [16-18]. Most of the altered proteins were located within the mitochondria or were implicated in processes involved in energy production such as electron transport and catabolism [19].

Proteomic analysis also indicated marked changes in metabolic enzymes. Some meta-

bolic proteins were down-regulated in the MI group compared with the sham group, but after four weeks of intragastric administration of Shenfu decoction these proteins were up-regulated. Other enzymes which showed changes included those involved in oxidative phosphorylation, the principal process by which (adenosine triphosphate (ATP) is formed, and most superoxide is generated at complexes I and II of the electron transport chain. The changes observed in NADH dehydrogenase (ubiquinone) 1 alpha sub complex 8, NADH dehydrogenase (ubiquinone) flavoprotein 3, and testis-specific cytochrome c could be pivotal in oxidative stress. Enolase mediates the penultimate step in glycolysis, converting 2-phosphoglycerate to phosphoenolpyruvate, and is thus important in glycolytic energy production. Early elevated expression is likely to be a response to increased metabolic needs. Down-regulation of proteins such as enolase, involved in glycolysis, causes glucose accumulation, and together with decreased pyruvate dehydrogenase, decreases the rate of transformation of pyruvate to acetylCoA for entry into the TCA cycle. Increased glucose concentrations can also be due to increased glucose uptake. However, the glucose level declined after Shenfu decoction treatment, which acted on glycolysis. Modern pharmacological research has shown that Ginseng delays the impairment of heart mitochondria, improving oxidative phosphorylation [20], inhibiting mitochondrial swelling, and thus improving energy metabolism [21].

Substantial changes occurred in contractile protein expression in the MI group compared with the sham group. Myosin light chain 3 was the only protein that initially increased (0.729-fold), and later declined (0.804-fold). Myosin light chain proteins are essential components for generating contractile force and are linked to the development of contractile dysfunction in heart failure models [22]. Moreover, changes in these proteins could indicate a shift in phosphorylation or deamidation rather than an overall increase in expression. Troponin-I was also notably down-regulated in the MI group compared with the sham group (1.276-fold), but increased after four weeks in the MI group treated with Shenfu decoction (0.803-fold). These alterations are relevant to the effects of Shenfu decoction, as ginsenosides have a similar molecular structure, affecting cardiac contractility and ameliorating heart dysfunction [23].

In addition to these proteins, we also found immunity and defense proteins such as peroxiredoxin 6 (Prx6) in the MI group compared with the sham group. Prdx6 overexpression decreases susceptibility to ischemia-reperfusion injury [24] and confers cardioprotective effects [25]. Previous studies have shown an association between oxidative stress and the progression of heart failure [26].

In addition, the following proteins are reported for the first time in heart failure: lymphocyte cytosolic protein 2, Lmna, DnaJ (Hsp40) homolog, multi-drug resistance-associated, and *Rattus norvegicus* utrophin. Heat shock proteins were not found in our model of CHF, but this does not preclude their presence.

Shenfu decoction is a traditional Chinese formulation comprising Ginseng (Radix Ginseng, RG) and Fuzi (Radix Aconitum Carmichaeli, AC). It has long been used to treat the loss of essence-energy and excessive body fluid. Moreover, it is prescribed for coronary disease in China. Fuzi is still used to treat coronary heart disease. Its excitation of the heart muscle is related to stimulation of the β receptor [27]. However, because of its toxic effect, Fuzi is not often used alone. Shenfu decoction not only ameliorates acute myocardial ischemia and reperfusion injury, but also prevents anoxia and lipid peroxidation [28], thus improving the balance between oxidation and antioxidation during reperfusion of the myocardium [29].

There are some limitations to this study. First, there was no clear boundary between the infarct and normal zones. Second, the effect of Shenfu decoction is multi-targeted, in contrast to western medicine, which is single-targeted. Thus, some changes caused by Shenfu decoction may have eluded detection. Third, although we extracted the same area of myocardial tissue, the border zone is a dynamic area, thus we cannot exclude internal differences in protein expression.

As previously mentioned, this is the first study to analyze the changes in ventricular protein expression using iTRAQ technology in an animal model of CHF. There is evidence that these protein changes are significant determinants of

the pathophysiology of CHF [30]. Cardiac performance relies on energy production in the form of fatty acid and carbohydrate oxidation in the mitochondria to produce ATP. Accordingly, we found changes in many metabolic proteins. Shenfu decoction is a staple of Chinese medicine used to cure heart disease. It is considered an effective formula to protect the heart by blocking sodium channels and other functions [31]. Therefore, we carried out full-scale screening of the protein changes involved in cardiac dysfunction in rats with heart failure, which may be critical for future use. Our findings may help identify patients at greater risk of heart failure associated with such an event.

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Disclosure of conflict of interest

None.

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