Introduction

Cardiovascular disease (CVD) remains one of the leading causes of death worldwide. More than 50% of all CVD deaths derived from sudden cardiac death, the most common cause of which is malignant arrhythmia, which also plays important roles in ischemic heart disease, myocardial infarction, cardiac failure and so on\(^1\). It is urgent to develop more drugs with high performance and less side effects\(^2\). Shenu injection (SFI), a well-known traditional Chinese herbal medicine, derived from Renshen (Radix Ginseng) and Fuzi (Radix Aconiti Lateralis Preparata), has been widely used for the treatment of CVD\(^3\). However, the cardioprotection mechanism of SFI remains to be elucidated\(^4\). Previous study showed that SFI has obvious cardioprotective effects\(^5\). It lessens the severity of heart failure when co-administered with conventional medicine in patients with myocardial infarct\(^6\) and exerts protective effects against atrioventricular block and ventricular arrhythmia caused by cardiopulmonary...
Ginsenosides, the main active component in *Radix Ginseng*, can improve ischemic myocardium metabolism, scavenge free radicals, protect myocardial ultrastructure, and reduce Ca\(^{2+}\) overload\(^{[7]}\). Higenamine, the active component in *Radix Aconiti Lateralis Preparata*, can enhance heart contractility, improve coronary circulation, and decrease the effect of acute myocardial ischemia\(^{[8]}\). Ginsenosides and alkaloids were main active components of SFI related with cardioprotection\(^{[9]}\). Twenty kinds of ginsenosides including 10 kinds of major ginsenosides Rg1, Re, Rb1, Rb2, Rb3, Rc, Rd, Rf, Rg2, and F1 and 10 kinds of rare ginsenosides Rh1, 20(R)-Rh1, Rk3, Rh4, Rg6, F4, Rg3, 20(R)-Rg3, Rk1 and Rg5 were detected in SFI\(^{[11,12]}\). Five alkaloids were identified from SFI. They are benzoylhypaconitine, benzoyldeoxyacacitine, benzoylmesaconitine,aconine and hypaconine\(^{[13-17]}\). See Figure 1.

Many hypotheses involved in oxidative stress, myocardial energy metabolism, and apoptotic cell death have been proposed, while, the cardioprotection mechanism of SFI is not exactly known. Many cytochrome P450 (CYP 450) genes have been shown to be expressed in the rat heart as well as the human heart, and their levels have been reported to be altered during cardiac hypertrophy and heart failure\(^{[18,19]}\). In the present study, we investigated whether SFI protects cardiomyocyte H9c2 from FPI-induced injury through CYP2J3 gene expression, in order to explore the underlying cardioprotective mechanisms.

**Figure 1** The plant of Renshen and Fuzi and the chemical structures of its major extracts

Renshen and Fuzi (A) are crude herbal drugs isolated from the dried root of rhizome of *Panax ginseng* C. A. Mey and *Aconitum carmichaeli* Debx., respectively. The Shenfu injection (B) manufactured by Ya’an Sanjiu Pharmaceutical Co., Ltd. (http://www.999.com/) has completed a phase II clinical trial in the USA (No. NCT00797953). Both Renshen injection and Fuqian injection are the intermediate products of Shenfu injection, which is composed of Renshen injection and Fuqian injection with a ratio of 1:2. One millimeter of Shenfu injection contains extracts of 0.1 g Renshen and 0.2 g Fupian. The chemical structures of the major components (ginsenoside Re, ginsenoside Rg1, ginsenoside Rb1 and ginsenoside Rg2) from Renshen and the major Fuzi extracts (benzoylhypaconitine and benzoyldeoxyacacitine) are shown in C and D, respectively.
2 Materials and methods

2.1 Chemicals and materials
H9c2 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). TRIzol and primers (Table 1) were purchased from Invitrogen (Carlsbad, CA, USA). The reverse transcription (RT) system and the real-time polymerase chain reaction (real-time PCR) system were purchased from Applied Biosystems (Foster City, CA, USA). Annexin V-fluorescein isothiocyanate (FITC)-propidium iodide (PI) double-stained apoptosis detection kit was purchased from Biosea Biotechnology (Beijing, China). SFI was produced by Ya’an Sanjiu Pharmaceutical Co., Ltd (No. 050304). The main components of SFI include ginsenoside Rb1, Re, Rg1, and Rg2.

2.2 Cell culture and treatment
Rat embryonic cardiomyoblast-derived H9c2 cells were cultured in DMEM supplemented with 10% (volume ratio) FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were maintained in a humidified incubator with 5% CO2 at 37 °C. The cells were subcultured after reaching 70%–80% confluence.[20,21] The cell culture cycle (progeny) of the H9c2 cell line cells used for screening studies was between 4 and 10 passages. For all experiments, the cells were plated at an appropriate density according to the experimental design and grown for 36 h before treatment. Four sets of experiments were performed: control cells, and cells treated with SFI (9, 18, 37, and 50 mg/mL). Because SFI is composed of RSI and FPI with ratio of 1:2, concentrations of RSI are 3, 6, 12, and 25 mg/mL and those of FPI are 6, 12, 25, and 50 mg/mL. Then the media were removed for experiments.

2.3 Primary cultures of cardiomyocytes
Ventricular myocytes from the hearts of neonatal Sprague-Dawley rats were cultured by previously described methods with minor modifications.[22] The tissues were washed with phosphate-buffered saline (PBS), minced and incubated for 8 min. Fresh 0.08% trysin solution was added and the incubation procedure was repeated until the tissue was totally digested. The supernatant was collected and an equal volume of DMEM containing 10% FBS was added. The cell pellet obtained by centrifugation was resuspended in fresh medium containing 10% FBS, plated in a culture dish and incubated for at least 1 h at 37 °C in a 5% CO2 incubator. Fibroblasts adhered to the dish surface while the cardiomyocytes remained unattached. The latter were replated in a gelatin-coated culture dish and incubated in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Bromodeoxyuridine (Brdu) at a final concentration of 0.1 mmol/L was added during the first 36 h to prevent proliferation of cardiac fibroblasts. They were maintained in a 5% CO2 incubator for 48 h, at which point confluent spontaneously contracting cells were observed.[22]

The cells were treated with RSI (6, 12, 25 and 50 mg/mL), FPI (12, 25, 50 and 100 mg/mL) and SFI (18, 37, 75 and 150 mg/mL) for 4 h. Cells were incubated for 48 h in complete media before spontaneous beating rate was observed with inverted fluorescence microscope.

2.4 Cell viability labeled with fluorescein diacetate
Cell viability was determined by fluorescein diacetate. Cells were seeded at a density of 5×10⁴ cells per well in 96-well microplates. The excitation and emission wavelengths of fluorescein diacetate are 488 and 530 nm, respectively. After treatment, the cells were washed in PBS containing 2.5 μg/mL fluorescein diacetate. Subsequently, the plates were maintained in the dark at room temperature for 15 min. To create a two-color image, the bright and green images were overlaid, producing purple fluorescence in areas of co-localization.[23]

2.5 Cell proliferation assay by cell counting kit-8
Cells were incubated in a 96-well plate (1×10⁴ cells each well), and four sets of experiments were performed: (1) control cells; (2) cells treated with RSI (3, 6, 12, and 25 mg/mL); (3) cells treated with FPI (6, 12, 25, and 50 mg/mL); and (4) cells pretreated with indicated concentrations (9, 18, 37 and 75 mg/mL) of SFI, followed by incubation at 37 °C in a CO2 incubator for 24 h, respectively. Adding 10 μL cell counting kit-8 (CCK-8) (Wako, Osaka, Japan) solution into each well of the plate, the cells were incubated for another 4 h. Absorbance was measured at 450 nm by the microplate reader (Perkin Elmer, Waltham, MA, USA).[24]

Table 1 Primer sequences used for real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CYP2J3</td>
<td>5’-CATTGAGCTCACAAGTGCTTTT-3’</td>
<td>5’-CAATTCCTAGGCTGATGTCG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CAAGGTCACTCCAGGACTTGG-3’</td>
<td>5’-GGGCCATCCACAGTCTTCTG-3’</td>
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CYP: cytochrome P450; GAPDH: glyceraldehydes-3-phosphate dehydrogenase.
2.6 Lactate dehydrogenase release assay
The H9c2 cells were cultured in 96-well plates at 3 × 10^4 cells/well. Four sets of experiments were performed: (1) control cells; (2) cells treated with RSI (3, 6, 12, and 25 mg/mL); (3) cells treated with FPI (6, 12, 25, and 50 mg/mL); and (4) cells pretreated with indicated concentrations (9, 18, 37, and 75 mg/mL) of SFI, followed by incubation at 37 °C in a CO₂ incubator for 24 h, respectively. Subsequently, the supernatant was collected. The lactate dehydrogenase (LDH) level was measured with the corresponding kit according to the manufacturer’s instructions.[25]

2.7 Caspase-3/7 activation assay
The caspase fluorescent assay kits specific for caspase-3/7 (Biovision, Mountain View, CA, USA) were used to detect caspase activation by measuring the cleavage of a synthetic fluorescent substrate. In brief, cells were cultured in 60 mm dishes and treated with FPI (6, 12, and 25 mg/mL), RSI (3, 6, and 12 mg/mL) and SFI (9, 18, and 37 mg/mL) for 24 h. Cell lysates were prepared with the lysis buffer provided by the assay kit and centrifuged at 10,000 × g for 1 min, and the supernatants were collected. With bovine serum albumin as the standard for protein content, equal amounts of protein were reacted with the synthetic fluorescent substrates at 37 °C for 1.5 h and the absorbance at 405 nm was read on a microplate reader. Fold-increase in caspase-3/7 activity vs control was determined.[26]

2.8 Cell apoptosis
H9c2 cells were pretreated with different concentration of FPI, RSI and SFI for 24 h. The cells were harvested at 6 h and washed twice in ice-cold PBS, then suspended in 200 μL ice-cold binding buffer and 10 μL Annexin V-FITC. The cell suspension was gently mixed and incubated in the dark for 15 min at room temperature. Then 300 μL ice-cold binding buffer and 5 μL PI were added to the cell suspension. Apoptosis was determined by flow cytometry (BD Company, Franklin Lakes, NJ, USA). In the study Annexin V-positive and PI-negative cells were defined as apoptotic cells. Both Annexin-V-FITC- and PI-negative cells were considered viable cells, while both Annexin V-FITC- and PI-positive cells were considered late apoptotic or already dead cells.[27]

2.9 RNA extraction and real-time PCR
Total RNA was isolated using TRIzol® reagent in accordance with the manufacturer’s protocol. After extraction, 5 μg of total RNA was then used as a template to synthesize cDNA using a first-strand cDNA synthesis (Invitrogen, Carlsbad, CA, USA). Quantitative analysis of specific RNA expression was performed by real-time PCR, by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Stepone System (Applied Biosystems). The 25-μL reaction mix contained 0.1 μL of 10 μmol/L forward primer and 0.1 μL of 10 μmol/L reverse primer (40 nmoL final concentration of each primer), 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water, and 1.25 μL of cDNA sample. The primers used in the current study were chosen from previously published studies and are listed in Table 1. Assay controls were incorporated onto the same plate, namely, no-template controls to test for the contamination of any assay reagents. After sealing the plate with an optical adhesive cover, the thermocycling conditions were initiated at 95 °C for 10 min, followed by 40 PCR cycles of denaturation at 95 °C for 15 s, and annealing/extension at 60 °C for 1 min. Melt curve (dissociation stage) was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product. The amount of the PCR product was measured as fluorescent signal intensity after standardizing to glyceraldehydes-3-phosphate dehydrogenase internal control.

The comparative threshold cycle method was used for the calculation of amplification fold according to a previously reported mathematical model.[28]

2.10 Statistical analysis
All data were expressed as mean ± standard deviation from at least three independent experiments. All statistical analyses were performed using one-way analysis of variance followed by post hoc analysis with a Student-Newman-Keuls test in the SPSS software package 15.0 (SPAA Inc, Chicago). Statistical significance was defined as P < 0.05.

3 Results

3.1 Effects of RSI, FPI, and SFI on H9c2 cells viability
The cytotoxicity of FPI, RSI and SFI in H9c2 cells were determined through the CCK-8 assay. Cells were treated with increasing doses of FPI, RSI and SFI for 24 h. As shown in Figure 2, the viability of cells treated with RSI (25, 50 and 100 mg/mL) and SFI (18, 37 and 75 mg/mL) was significantly higher than that of cells treated with FPI (25, 50 and 100 mg/mL) (n=3 for each, P<0.05, P<0.01, P<0.01, respectively). As shown in Figure 3, FPI impaired cell viability by a concentration-dependent manner in the tested concentration range (12, 25 and 50 mg/mL) (n=3 for each, P<0.05, P<0.01, P<0.01, respectively). The viability of cells treated with FPI (25 and 50 mg/mL) and SFI (18, 37 and 75 mg/mL) were also decreased. However, the viability of cells treated with SFI (37 and 75 mg/mL) was significantly higher than that of cells treated with FPI (25 and 50 mg/mL) (n=3 for each, P<0.05, P<0.01, respectively).

3.2 LDH release assay
LDH, which leaks from cells after plasma membrane disruption, can be used as an indicator of cell injury. As shown in Figure 4, a marked increase in LDH activity was observed after 24 h of exposure to FPI (25 and 50 mg/mL)
$n=3$ for each, $P<0.05$, $P<0.01$, respectively) and SFI (37 and 75 mg/mL) ($n=3$ for each, $P<0.05$, $P<0.01$, respectively). The LDH activity of cells treated with SFI (75 mg/mL) was significantly decreased ($n=3$ for each, $P<0.01$) compared with that of cells treated with FPI (50 mg/mL).

### 3.3 The spontaneous beating rate in primary myocardial cells

As shown in Figure 5, SFI (75 mg/mL) significantly attenuated FPI (50 mg/mL)-induced spontaneous beating rate decrease in primary myocardial cells at 4 h treatment, and increased spontaneous beating rate were found in the SFI-treated group compared with the control group ($n=3$ for each, $P<0.05$). Therefore, the protection provided by RSI against FPI-induced cell injury involved spontaneous beating rate.

### 3.4 RSI prevented FPI-induced increase of caspase-3/7

Caspase-3/7 is an executive enzyme of apoptosis and usually activated following cytochrome c release from mitochondria because of its damaged membrane. We examined the caspase-3/7 activity in H9c2 cells by monitoring the cleavage of the fluorogenic substrate Ac-DEVD-CHO. Compared with H9c2 cells treated with FPI (12 and 25 mg/mL), SFI (18 and 37 mg/mL) treatment could significantly reduce caspase-3/7 activity ($n=3$ for each, $P<0.01$, $P<0.01$, respectively) (Figure 6).

### 3.5 Apoptosis analysis of H9c2 cells by Annexin V/PI with flow cytometry

In order to determine the effects of RSI on FPI-stimulated cell death, the H9c2 cells were separately treated with FPI, RSI and SFI for 24 h. As shown in Figure 7, exposure...
of H9c2 cells to FPI (6, 12 and 25 mg/mL) led to apoptotic nuclei formation (n=3 for each, P<0.01). Apoptotic nuclear characteristics decreased significantly (n=3 for each, P<0.05, respectively) when the cells were incubated with SFI (9 and 37 mg/mL) compared with FPI (6 and 25 mg/mL), while RSI alone had no significant effect. Flow cytometry analysis using Annexin V-FITC and PI-labeling indicated that SFI caused lower rates of apoptotic cells compared with FPI-treated H9c2 cells.

3.6 Effects of RSI on CYP2J3 gene expression
To examine the effects of RSI on the expression of various CYP2J3 genes, the cells were treated with increasing concentrations of FPI, RSI and SFI for 24 h. Thereafter, the expression of different CYP2J3 genes was measured using real-time PCR. The mRNA expression of CYP2J3 was down-regulated by treatment of FPI (3, 6, 12 and 25 mg/mL, n=3 for each, P<0.05, P<0.01). However, RSI (3, 6, 12 and 25 mg/mL) and SFI (4, 8, 16, and 32 mg/mL)
could up-regulate the expressions of CYP2J3 ($n=3$ for each, $P<0.05$, $P<0.01$) (Figure 8).

4 Discussion

Consistent with the observed cardioprotection of SFI reported previously, the present study showed that RSI significantly protected cardiac H9c2 cells from cell death and apoptosis induced by FPI as determined by the CCK-8 cell viability assay and caspase-3/7 activity assay. The results demonstrated that SFI could up-regulate the CYP2J3 gene expression significantly. SFI might exert cardioprotective effect through CYP2J3 gene.

H9c2 cells are commonly used as an in vitro model to study the mechanisms involved in cardioprotection and cardiotoxicity. H9c2 cells express multiple CYP genes at comparable level to those expressed in the heart. Recently, CYP2J3 has been shown to be expressed in H9c2 cells and the rat heart at comparable levels.$^{[20]}$

CYP2J3 is important in arachidonic acid (AA) metabolism to epoxyeicosatrienoic acids (EETs)$^{[30]}$. These metabolites play a crucial role in maintaining the cardiac homeostasis, as alterations of their levels have been associated in the pathogenesis of cardiac hypertrophy and heart failure.$^{[31]}$

In the current study, RSI and SFI treatment significantly induced the gene expression of CYP2J3 to a varying degree. The differential effect of RSI and SFI on CYP2J3 genes can be explained by different regulatory mechanisms involved in their transcription. In agreement with our results, it has been reported that cardiac hypertrophy is associated with induction of several CYP genes$^{[12,13]}$. In addition, the physiological importance of these enzymes emerges from their ability to metabolize AA to various hydroxyeicosatetraenoic acid (HETEs) and EETs. Of interest, the imbalance between P450-mediated AA metabolites EETs and 20-HETE has been linked to several cardiovascular diseases$^{[14]}$. Generally, EETs, eicosanoids produced by P450-epoxygenases, are considered cardioprotective metabolites, which have beneficial effects on heart$^{[15]}$. It has been reported that EETs have a protective effect against cardiac hypertrophy and doxorubicin-induced cardiotoxicity. On the other hand, 20-HETE, an eicosanoid produced by P450-hydroxylases, has been reported to have a detrimental effect on the heart leading to cardiac hypertrophy and heart failure. Mechanistically, these opposing effects of P450-derived AA metabolites are mediated through several intracellular signaling cascades that have been implicated in development and/or progres-
sion of cardiovascular toxicity e.g., nuclear factor-kappa B (NF-kB), mitogen-activated protein kinases, and matrix metalloproteinase. As an important player affecting the balance between P450-derived AA metabolites, soluble epoxide hydrolase enzyme hydrolyzes the cardioprotective metabolites EETs to biologically less active metabolites, dihydroxyicosatetraenoic acids, which are over expressed in several heart diseases.

Studies related to SFI's direct effects on membrane ion channels have been reported. The regulatory effect of SFI on transmembrane action potential (TAP) and voltage-dependent sodium channels in ventricular myocytes was presented. SFI decreased TAP amplitude and maximum velocity of depolarizing phase without influencing TAP duration and repolarization; it diminished the current density of INa in a concentration-dependent manner with a “ceiling effect” fitted well by the Hill function, and it shifted current-voltage curve of INa upwards; it also enhanced sodium channels inactivation and prevented them from recovering. SFI could block the voltage-dependent sodium channels in ventricular myocytes, consequently change their electrophysiological characteristics. SFI antagonizes arrhythmia induced by aconitine which opens the sodium channels on cardiac myocytes. It confirms related reports that SFI could decrease \( \text{Na}^+ \) content and block extracellular \( \text{Na}^+ \) entry in nephridial tissue and provide reference to study the relationship between SFI's analgesic and protective effects on central nervous system and possible blockage on sodium channels in nervous tissue. SFI's blockage on the sodium channels in cardiac myocytes may be one of the important molecular mechanisms for its cardiac active effectiveness, including inhibition of NF-κB activity, down-regulation of proinflammatory cytokine expression, protection of mitochondria, and so on.

In summary, our study demonstrates that SFI could attenuate the delayed proliferation and apoptosis of myocardocytes H9c2 induced by FPI, and the potential molecular mechanism was involved in the CYP2J3 gene expression. To the best of our knowledge, this is the first time to investigate the effect of SFI on CYP2J3 gene expression. CYP2J3 induction would result in alteration of arachidonic acid metabolism which has been associated with cardiac hypertrophy and heart failure, therefore, this may be one of the mechanisms by which SFI provides cardioprotection. Furthermore, this study confirms the validity of H9c2 cell line as a valuable in vitro model to study the possible role of CYP2J3 genes in the pathogenesis of various heart diseases. Further research will provide us with a more complete picture of how related CYP genes interact with each other to provide cardioprotection. However, research evidence is not comprehensive enough to entirely explain cardioprotective mechanisms of Shenfu preparata, and more advanced research is necessary to further explore the mechanisms of RSI against FPI-induced cardiac injury. Further studies are in progress in our laboratory to determine other molecular mechanisms involved in cardioprotective effect of ginsenosides and alkaloids in rat cardiac cells.

5 Conclusion

These observations indicate that SFI has the potential to exert cardioprotective effects against FPI toxicity. The effect is possibly correlated with the activation of CYP2J3.

6 Funding

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7 Competing interests

The authors declare that they have no competing of interests.

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