Protective Effects of Shenfu Injection against Myocardial Ischemia–Reperfusion Injury via Activation of eNOS in Rats

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The aim of the present study was to investigate the protective effects of Shenfu injection (SFI) against myocardial ischemia–reperfusion injury (MIRI) in model rats and to explore its mechanism of action. Sprague–Dawley (SD) rats were pretreated with SFI and N\textsuperscript{\text{-}}-nitro-L-arginine methyl ester (L-NAME) via tail vein injection and then rats were subjected to ischemia by occlusion of the left anterior descending coronary artery for 30 min followed by reperfusion for 120 min. Left ventricular function was evaluated by echocardiography. Hemodynamic was measured by the Millar pressure–volume system; serum creatine kinase (CK), lactate dehydrogenase (LDH) and serum troponin (TNNI3) levels were determined. Myocardial infarct size was observed by 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining; p-Akt/Akt, and p-endothelial nitric oxide synthase (p-eNOS)/eNOS levels were assessed by Western blotting; nitric oxide (NO) content in serum was determined by the Griess reaction. SFI significantly decreased serum CK, LDH and TNNI3 levels in MIRI rats, while it significantly increased the level of left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVEDP), maximal rate of the increase of left ventricular pressure (+dp/dt\text{max}), maximal rate of the decrease of left ventricular pressure (−dp/dt\text{max}), left ventricle ejection fraction percentage (EF), and stroke volume (SV). In addition, SFI significantly reduced myocardial infarction area and activated the phosphorylation of eNOS via Akt. The phosphorylation of eNOS and the concurrent increase of NO production contributed significantly to the protective effects of SFI. These results demonstrate that SFI protects the rat heart against MIRI and that this effect is mediated in part by Akt/eNOS signaling.

Key words Shenfu injection; ischemia reperfusion injury; nitric oxide; left ventricular function; N\textsuperscript{\text{-}}-nitro-L-arginine methyl ester (L-NAME)

Myocardial ischemia (MI) is caused by the heart being temporarily deprived of oxygen-rich blood. Coronary thrombolysis, coronary artery bypass and other interventional treatments are used in clinic to restore the blood supply to ischemic myocardial tissue. Following blood reperfusion, the function and structure of ischemic tissue does not usually fully recover because of myocardial ischemia–reperfusion injury (MIRI). MIRI is a complex pathological process involving a variety of mechanisms. Oxygen free radicals, calcium overload, myocardial energy metabolism dysfunction, endothelial cell dysfunction, neutrophil infiltration, apoptosis and mitochondrial damage are the most prevalent reasons for the development of MIRI.

Nitric oxide (NO) is an important signaling molecule involved in many physiological and pathological processes in mammals including humans. A large number of studies have shown that NO is a vasodilator that contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation and leukocyte adhesion to the endothelium. NO is highly reactive and can diffuse across membranes freely. In MIRI, NO can act on cardiomyocytes and vascular endothelium through a variety of signaling pathways. NO also confers reperfusion injury when produced excessively during reperfusion; it can react with superoxide to produce the damaging oxidant peroxynitrite. NO is synthesized enzymatically from L-arginine, oxygen, and nicotinamide adenine dinucleotide phosphate (NADPH) by three NO synthase isoforms: inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS). The production of NO mainly depends on the activity of eNOS. After the phosphorylation of eNOS, NO is produced, which stimulates the soluble guanylate cyclase (sGC) to generate a second messenger, cyclic guanosine monophosphate (cGMP), which plays a role in cardiovascular protection.

Shenfu injection (SFI), which originated from “Ji Sheng Fang,” has been used in China for nearly 30 years to clinically treat cardiovascular diseases. The active components of SFI are extracted from Radix Ginseng and Radix Aconitum carmichaelii, which increase coronary blood flow to protect the myocardium. Recently SFI was clinically applied to treat coronary heart disease, cardiac arrhythmia and congestive heart failure. The aim of the present study was to investigate the protective effect of SFI against MIRI in model rats and to explore its mechanism of action.

MATERIALS AND METHODS

Drugs and Reagents Shenfu injection (SFI, lot. Number 150725010) was donated by YaAn Sanjiu Pharmaceutical Co., Ltd. (Sichuan, China). The main components of SFI include ginsenosides (>0.8 mg/mL) and aconitine (<0.1 mg/mL). Trimetazidine (TMZ) was purchased from Servier Pharmaceutical group (Servier, Tianjin, China). N\textsuperscript{\text{-}}-Nitro-L-arginine methyl ester (L-NAME) was from Sigma Chemical Company.
(St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

**Animals and Drug Administration** Male Sprague–Dawley (SD) rats (230±20g) were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. [Beijing, certificate id: SCXK (Jing) 2012-0001]. They were housed in the Animal House of the Institute of Radiation Medicine Chinese Academy of Medical Sciences at a controlled temperature of 22±2°C with 50±10% relative humidity and a 12-h light/dark cycle. Animals were given free access to water and food. All animal care and experimental protocols abided by the Animal Management Rules of the Ministry of Health of the People’s Republic of China. Rats were randomly assigned to six groups: sham group; MIRI+non-treated group (ischemia–reperfusion group, I/R group); MIRI+trimetazidine 5.4 mg/kg (TMZ group); MIRI+Shenfu injection (SFI) 1.5 mL/kg (SFI group); MIRI+SFI 1.5 mL/kg+t-NAME 1 mg/kg (SFI+t-NAME group); t-NAME, an inhibitor of eNOS, was administrated with SFI to evaluate the effect of eNOS), and MIRI+t-NAME 1 mg/kg (t-NAME group). Rats were intravenously treated via tail vein injection with SFI three days before ischemic insult and with t-NAME 15 min before ischemic insult. TMZ was administered intragastrically and sham and I/R groups were treated with saline once a day for three days.

**Surgical Procedure** MIRI was surgically performed according to previously described methods. Rats were anesthetized intraperitoneally using 5% chloral hydrate (6 mL/kg, Beijing Solarbio Science & Technology Co., Ltd., China). MIRI was induced by left thoracic incision to expose the heart and a 5/0 silk suture was sewn around the left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released to allow reperfusion for 120 min.

**Examination of Troponin I Type 3 (TNNI3), Lactate Dehydrogenase (LDH) and Creatine Kinase (CK)** After reperfusion, blood samples were obtained and the serum separated by centrifugation at 3000rpm for 10 min at 4°C. Serum CK and LDH levels (BioSino Bio Technology & Science Inc., Beijing, China) were determined using an Automatic biochemical analyzer 7020 (HITACHI Co., Ltd., Tokyo, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were used to determine serum TNNI3 levels (Duван USCN Business Co., Ltd., China).

**Echocardiographic Detection of Left Ventricular Function** Echocardiography was performed according to the previously described methods. A Vevo2100 high resolution ultrasound system in real time (VisualSonics Vevo 2100, Canada) with an MS-250 ultrasound scanning transducer (model CS) was used to measure the left ventricular function of rats. After reperfusion, rats were anesthetized by mask-administered 5% isoflurane. The chest of each rat was shaved and the animal then placed in the supine position on a 37°C platform. Using ultrasound coupling agent, M-mode and Doppler-mode images were recorded from the long axis of the left ventricle. The following parameters were measured and used to assess function and structure of the left ventricles: left ventricular internal diameter (LVID), left ventricular volume (LV Vol), left ventricular posterior wall (LVPW), left ventricle ejection fraction percentage (EF%), left ventricle fractional shortening percentage (FS%), and average peak velocity (pea vel). Each trial was repeated three times and the mean value was calculated.

**Measurements of Cardiac Function** After echocardiography, the right common carotid artery was exposed to a conductance catheter (PV catheter, SPR838, Millar Instruments, Houston, U.S.A.) insertion and the PV catheter was advanced, passing through the aortic valve into the left ventricle. End systolic pressure (Pes) and end diastolic pressure (Ped) were directly measured. At baseline and at the end of the experiment, 7 µL saline (20%) was intravenously injected to determine the conductance measured by the catheter of the surrounding myocardial tissue. Pressure and volume signals were continuously acquired (MPVS300, Millar Instruments, U.S.A.) and digitalized (PowerLab 16/35, AD Instruments, Australia). Stroke volume (SV), maximal rate of the increase/decrease of left ventricular pressure (+dP/dt max and −dP/dt max), isovolumic relaxation constant (TAU), stroke work (SW), and cardiac output (CO) were calculated.

**Measurement of Myocardial Infarct Size** Hearts were dissected from euthanized rats and stored at −20°C. The left ventricle was transversally cut into 1–2 mm slices and incubated in 1% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining solution (Beijing Solarbio Science & Technology Co., Ltd., China) at 37°C for 15 min. 22)

**Western Blotting Analyses** Western blotting assay was performed as previously described. After reperfusion, heart tissue was harvested. Protein was extracted in RIPA lysis buffer containing complete protease inhibitor cocktail (Beyotime biotechnology, Nanjing, China) from homogenized heart tissue. Equal amounts of total protein (40 µg) were loaded and then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to polyvinylidene fluoride membrane. The membranes were blocked for 2h with 5% non-fat milk at room temperature and incubated overnight at 4°C with primary antibodies [anti-Akt (1:1000, abcam), anti-p-Akt (1:1000, abcam), anti-eNOS (1:1000, abcam), anti-p-eNOS (1:1000, abcam)]. The membranes were then incubated with secondary antibodies (1:5000) for 2h at room temperature. All data were quantified using Image J 2x software (National Institutes of Health, U.S.A.), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the control.

**Measurement of Myocardial NO** NO detection kit (Beyotime Biotechnology, Nanjing, China) was used to determine serum NO, according to the manufacturer’s instructions.

**Statistics** All experiments were conducted a minimum of three times. Results are expressed as mean values±standard deviation (S.D.) Statistical comparisons were made using one-way ANOVA and Dunnett’s test using Prism 5.0 software (GraphPad Software, U.S.A.). p Values <0.05 were considered significant.

**RESULTS**

**Protective Effect of SFI on TNNI3, LDH and CK in Rats with MIRI** Levels of serum CK, LDH and TNNI3 in the I/R group were significantly increased compared with the sham group (p<0.01). The increased levels of LDH and TNNI3 were significantly attenuated by SFI and TMZ administration (p<0.01), but no significant decrease was observed for CK (Fig. 1, n=8). The effects of SFI were abolished by addition of the NOS inhibitor, t-NAME. There was no significant difference between the t-NAME group and the model group indicating that t-NAME had no significant effect on MIRI.
Echocardiography As shown in Fig. 2, the significant increases in LVIDs, LVIDd, LV Vols, LV Vold and decreases in EF, FS and Peak Vel in the I/R group were significantly improved by SFI treatment ($n=8$, $p<0.01$), but no significant change was observed for LV PW. The effects of SFI were abolished by addition of L-NAME. There was no significant difference between the L-NAME group and the model group indicating that L-NAME had no significant effect on MIRI.

Cardiac Function Compared with the sham group, the I/R group showed a decline in Pes, Pdev, SV, $+\text{dp}/\text{d}t_{\text{max}}$, $-\text{dp}/\text{d}t_{\text{max}}$, SW and CO. In addition, Ped and Tau were significantly increased in the I/R group, indicating a severe damage to heart function. SFI improved these impairments. The effects of SFI were partly abolished by addition of L-NAME ($n=8$). There was no significant difference between the L-NAME group and the model group indicating that L-NAME had no significant effect on MIRI.

Protective Effect of SFI on Infarct Size in Rats with MIRI Infarct size was measured by TTC staining ($n=8$). SFI significantly reduced infarct size in MIRI rats. The effect of SFI was abolished by addition of L-NAME. There was no significant difference between the L-NAME group and the model group indicating that L-NAME had no significant effect on MIRI.

SFI Unregulated Phosphorylation of Akt and eNOS SFI administration did not alter expression the levels of total Akt or eNOS; however, it significantly promoted the phosphorylation of Akt and eNOS ($n=8$). Compared with the sham group, the phosphorylation level of Akt and eNOS in the I/R group was significantly decreased ($p<0.01$). Compared with the I/R group, phosphorylation levels of Akt and eNOS were significantly increased in SFI group ($p<0.01$). There was no significant difference between the L-NAME group and the model group indicating that L-NAME had no significant effect on MIRI.

Effect of SFI on Serum NO Levels in Rats with MIRI Serum NO levels were significantly decreased after MIRI, compared with the sham group ($p<0.05$). When rats were treated with SFI, the serum NO level was significantly elevated compared with the level observed in the I/R group ($p<0.01$). The effect of SFI was abolished by addition of NOS inhibitor L-NAME ($p<0.01$, Fig. 6, $n=8$). There was no significant difference between the L-NAME group and the model group indicating that L-NAME had no significant effect on MIRI.

DISCUSSION

With the continuous development of medical technology, coronary thrombolysis, percutaneous coronary intervention and coronary artery bypass surgery have become standard treatments for coronary heart disease. These treatments effectively improve clinical efficacy.\(^{23}\) Interventional therapy as a major treatment for acute coronary syndromes contributes to the rehabilitation of patients, but it does not completely ameliorate the series of severe heart events that occur after MIRI.\(^{24}\) MIRI is a pathological process in which tissue damage is caused when the blood supply returns to the tissue after a period of ischemia. The absence of blood-born oxygen and nutrients during the ischemic period creates conditions in which the restoration of circulation results in inflammation and oxidative damage through the induction of oxidative stress rather than restoration of normal function. In recent years, traditional Chinese medicine (TCM) has made great progress in
Fig. 2. Echocardiographic Characterization of Cardiac Function in MIRI Rats

Quantitative assessment of diastolic and systolic function based on LVIDs (A), LVIDd (B), LV Vols (C), LV Vold (D), LVPWs (E), LVPWd (F), EF (G), FS (H) and Peak Vel (I). Results are expressed as mean values±S.D. *p<0.05, **p<0.01 vs. Sham group; †p<0.05, ‡p<0.01 vs. I/R group; ††p<0.05, ‡‡p<0.01 vs. SFI group.
Fig. 3. Hemodynamic Assessment Was Performed to the Left Ventricle through Right Carotid Artery to Evaluate the Role of SFI Treatment in the Management of MIRI

Quantitative assessment of hemodynamic on cardiac function based on Pes (A), Pdev (B), Ped(C), SV (D), +dp/dtmax (E), −dp/dtmax (F), TAU (G), SW (H) and CO (I). Results are expressed as mean values±S.D. * p<0.05, ** p<0.01 vs. Sham group; † p<0.05, †† p<0.01 vs. I/R group; ‡ p<0.05, ‡‡ p<0.01 vs. SFI group.
the treatment of ischemic cardiovascular disease. SFI, which is based on TCM theory, has been widely used in the treatment of heart failure, arrhythmia, acute myocardial infarction, dilated cardiomyopathy, pulmonary heart disease, and other cardiovascular diseases.\textsuperscript{12–17} Since SFI was approved by the China Food and Drugs Administration (CFDA) and applied in many hospitals and clinics, it has produced significant curative effects in patients.\textsuperscript{25} The present study evaluated the effects of SFI in a rat model of MIRI.

CK, LDH and TNNI3 levels in blood serum reflect changes to membrane integrity and the extent of myocardial injury and are used as diagnostic markers of MIRI.\textsuperscript{22} In this study, SFI reduced CK, LDH and TNNI3 levels induced by MIRI. To evaluate the effect of SFI on cardiac dysfunction following MIRI, hemodynamic parameters including Pes, Pdev, SV, \(+\Delta p/\Delta t_{\text{max}}\), \(-\Delta p/\Delta t_{\text{max}}\), CO and SW were recorded. Pretreatment with SFI significantly ameliorated heart impairment due to MIRI.

NO is a free radical produced by the NOS-catalyzed oxidation of arginine to citrulline. NO is well-established as a trigger and mediator for cardioprotection.\textsuperscript{26–28} It plays an important regulatory and protective role in vasculature by dilating blood vessels, modulating platelet aggregation and adhesion, and preventing leukocyte-endothelial adhesive interactions and angiogenesis.\textsuperscript{29–31} Many previous studies have demonstrated that reduced formation or activity of NO contributes to MIRI. The NO precursor, 1-arginine, NO donors of different chemical structures, and interventions stimulated NO production which involving endothelial receptors to protect the heart.\textsuperscript{32–37} The overexpression of eNOS and NO therapy both significantly protect the myocardium.\textsuperscript{38} NO generated by eNOS is important in blood pressure regulation and also has an impact on cardiac function and remodeling.\textsuperscript{39} The phosphorylation of eNOS is a molecular switch in vasodilation and cardioprotection.\textsuperscript{40} The relative contribution of iNOS-\textit{versus} eNOS-derived NO to NO-mediated cardioprotection is still unclear. In the present study, the protective effects of SFI were
related to the phosphorylation of eNOS. The phosphorylation of eNOS with a subsequent increase in NO production is an important effect of SFI in MI/R. However, the production of NO was blocked by l-NAME indicating that the phosphorylation of eNOS and endothelium-derived NO mediated the protective effects of SFI treatment. We have previously shown in rats that SFI dilates coronary vessels and thoracic aortic rings through NO.

Akt is a serine/threonine protein kinase that phosphorylates human eNOS at serine 1177. eNOS is an Akt target in the phosphatidylinositol 3 kinase (PI3K)-Akt/eNOS signaling pathway. In the present experiment, we demonstrated that SFI increased eNOS phosphorylation and NO production by enhancing Akt phosphorylation, which is agreement with the report from Wang et al., who showed that ginsenoside Rg3, a main component of SFI, mediated a cardioprotective effect against MIRI-induced apoptosis via Akt/eNOS signaling.

Trimetazidine is a piperazine-derived agent with protective effects that is effective in the treatment of myocardial, renal and liver ischemia/reperfusion injury in which the Akt/eNOS signaling pathway is involved. The results of the present study are similar to those reported in an in vivo rat model of IR. The cardioprotective mechanisms of SFI and trimetazidine are similar; however, SFI exerts greater protection than trimetazidine by increasing NO production, which is shown in Fig. 6.

In summary, SFI exerted protective effects against MIRI. l-NAME abolished the protective effects of SFI. SFI could activate the phosphorylation of eNOS by Akt. The phosphorylation of eNOS and the concurrent increase of NO production contributed significantly to the protective effects of SFI. In conclusion, SFI protects the rat heart against MIRI in rats and this effect is mediated in part by Akt/eNOS signaling pathway.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


