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Shenfu injection suppresses apoptosis by regulation of Bcl-2 and caspase-3 during hypoxia/reoxygenation in neonatal rat cardiomyocytes in vitro

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Abstract Shenfu injection (the major components of which are ginsenosides compound, extract of Panax ginseng shown to have antioxidant properties) is a well-known important Chinese traditional medicine used for the treatment of various diseases especial for cardiac diseases. The precise mechanism of the biological actions of this plant is not fully understood, in order to elucidate the protection of cardiomyocytes. The aim of the present study was to investigate the effect of Shenfu injection on hypoxia/reoxygenation (H/R)-induced apoptosis and the expression of bcl-2 and caspase-3 in cultured neonatal rat cardiomyocytes in vitro. Ventricular myocytes were isolated from neonatal rat hearts and were exposed to 4 h of hypoxia followed by 16 h of reoxygenation. The results indicated that treatment with different doses of Shenfu injection protected cardiacmyocyte cultures from hypoxia/reoxygenation-induced apoptosis. Caspase-3 activation was decreased in hypoxic/reoxygenationed cardiomyocytes co-treated with Shenfu injection when compared to hypoxia/reoxygenation alone treated cultures. Expression of the Bcl-2 proteins was increased in Shenfu injection-treated cardiomyocytes subjected to hypoxia/reoxygenation. In conclusion, ginsenosides compound has obviously protective effects on cardiacmyocytes against apoptosis induced by hypoxia/reoxygenation injury, whose mechanisms probably involve the inhibition of down-regulation of Bcl-2 protein levels and sequential activation of caspase-3.

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Introduction

Myocardial ischemia causes depression of myocardial function and associated deleterious morphologic alterations that lead to heart failure. Myocardial ischemia and the following reperfusion can also cause the same or even more serious injury than pure ischemia that is called myocardial ischemia/reperfusion injury (MIRI). This injury is a pathologic process that results in extensive cell death, a significant portion of which can be attributed to apoptosis [1]. Recent studies have indicated that apoptotic death occurs in cardiac cells exposed to a variety of damaging stimuli especially including continuous ischemia or transient ischemia followed by reperfusion both in vitro and in the intact heart in vivo [2–5].

Although the identities of the molecular signaling pathways that mediate ischemia-induced apoptosis are largely unknown, a common and critical event in the execution phase of apoptosis is the activation of a family of aspartate-specific proteases termed caspases [6, 7], which participate in a cascade where initiator caspases activate effector caspases and ultimately cleave a set of proteins, causing disassembly of the cell. Activation of caspases may be regulated directly or indirectly by members of Bcl-2 family proteins [8, 9]. Caspases and Bcl-2 family proteins are involved in apoptotic cell death in cardiomyocytes [10–12], but nothing is known about molecules able to block the death of ischemic cardiomyocytes [13]. So, it is important to investigate apoptosis in myocardial ischemia /reperfusion (I/R) injury and to find effective drugs to prevent it.

Shenfu injection (SFI), an extract of traditional Chinese herbs, is a preparation mainly consists of ginsenoside and aconitine. SFI is applied routinely in clinical practice in treating cardiac diseases including coronary heart disease, cardiac arrhythmia and congestive heart failure. Moreover, some studies have proved that SFI has significantly protective effects against I/R injuries on many organs such as the brain, spinal cord, kidney, intestines, liver and especially on heart [14–17]. To date, studies of Shenfu injection (SFI) have focused on molecular mechanisms of protection on cardiacmyocytes. In the present study, we therefore investigated the effects of SFI on hypoxia/reoxygenation (H/R)-induced apoptosis and the expression of the apoptosis-related protein bcl-2 and caspase-3 in cultured cardiomyocytes in order to explore the cardioprotective mechanisms of SFI.

Methods

Drugs and reagents

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were from hyclone (Hyclone, America), trysin (Amersco, America). Antibodies used were as follows: mouse monoclonal antibodies, anti-β-actin (Sigma), rabbit polyclonal antibody, anti-rat caspase-3, anti-rat Bcl-2 (Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from Santa Cruz Biotechnology. All other chemicals used in the present study were of analytical grade.

Shenfu injection was produced by Ya'an Sanjiu Pharmaceutical Co., Ltd (No.050304). The main components of Shenfu injection include ginsenoside (>0.8 mg/m1) and aconitine (<0.1 mg/m1).

Primary cultures of cardiomyocytes

Ventricular myocytes from the hearts of neonatal Sprague–Dawley rats (1- to 2-day old) were cultured by previously described methods with minor modifications [18, 19]. The tissues were washed with phosphate-buffered saline (PBS), minced and incubated with 0.08% trysin (Amresco, USA) at 37°C for 8 min. Fresh 0.08% trysin solution was added and the incubation procedure 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% CO_2 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 mM was added during the first 36 h to prevent proliferation of cardiac fibroblasts. They were maintained in a 5% CO_2 incubator for 48 h, at which point confluent spontaneously contracting cells were noted.

Hypoxia and reoxygenation

Cardiomyocytes were cultured for 2–3 days until they were confluent and then serum-starved for 24 h before use. Select conditions that may be observed in hypoxia were simulated by serum and glucose deprivation of the culture medium and by H/R. Cells were washed with serum-free DMDM and placed in serum-free and glucose-free medium and then incubated in a sealed, hypoxic GENbox jar fitted with a catalyst (BioMérieux, Marcy l'Etoile, France) to scavenge free oxygen. Oxygen tension in the medium was measured using a blood gas analyzer and was found to be 33.5 mm Hg within 0.5 h after being transferred into the hypoxic chamber and maintained at approximately 22–24 mm Hg over the experimental time. The cardiomyocytes were exposed to different concentrations of SFI for 30 min and immediately replaced in the hypoxic chamber to maintain hypoxia. After 4 h of hypoxia, the cells were subjected to reoxygenation by changing the medium into a DMEM base medium with 5.5 mM glucose and 10% FBS followed by incubation under normoxia at 37°C for 16 h. SFI was present during the hypoxic period.

There were four experimental groups: (i) Control group, cardiacmyocytes were kept in 5%CO_2–95% air, (ii) H/R group, cardiacmyocytes were exposed to 4 h of hypoxia followed by 16 h of reoxygenation, (iii) L-SFI + H/R group, cardiacmyocytes were pretreated with a low dose (50 μl/ml) of SFI for 0.5 h followed by H/R, (iv) H-SFI + H/R group, cardiacmyocytes were pretreated with a high dose (100 μl/ml) of SFI for 0.5 h followed by H/R.

Assessment of morphological changes

Chromosomal condensation was assessed using the chromatin dye Hoechst 33342 (Sigma, America). Cells were fixed for 30 min in phosphate-buffered saline (PBS) containing 1% glutaraldehyde. After being fixed, the cells were washed twice with PBS and then exposed to 5 μg/ml Hoechst 33342 in PBS for 30 min at room temperature. All samples were observed using a fluorescence microscope. Apoptotic cells were characterized by morphological alteration such as condensed nuclei and cell shrinkage.

Detection of apoptosis

Annexin V-FITC/propidium iodide staining was performed after 16 h reoxygenation according to the manufacturer's instructions. In brief, cells were rinsed with ice-cold PBS and then resuspended in 200 μl of binding buffer. Ten micro liters of Annexin V stock solution was added to the cells and incubated for 30 min at 4°C. The cells were then further incubated with 5 μl propidium iodide (PI) and were immediately analyzed on a FACSC-LSR equipped with CellQuest software; approximately 1 to 2 $\times$ 10^4 cells were analyzed in each of the samples.

Cell extract preparation and Western blot analysis

Harvested myocytes were lysed at 4°C with ice-cold lysis buffer (50 mM HEPES pH 8.0, 150 mM Nacl, 1% NP40, 10^5 U Aprotion,10 mM PMSF,0.1 M DTT, 0.5 M EDTA). Cell lysates were centrifuged and protein concentrations in the supernatants were determined by BCA Protein Assay Kit (Beyotime Biotechnology). Equal amounts of proteins (50 μg) were loaded onto and separated by 10% SDS–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred to nylon membranes by electrophoretic transfer system (Bio-Rad). The membranes were blocked in 5% skim milk for 1 h, and incubated with primary antibody (anti-caspase-3, anti-bcl-2 antibody at 1:500 dilution, anti-β-action at 1:5,000 dilution) overnight at 4°C. Excess antibody was then removed by washing the membranes in PBS–0.05% Tween-20, and the membranes were incubated in secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or mouse antibodies, 1:1,000 dilution) for 2 h. After washes in PBS–0.05% Tween 20, the bands were detected by enhanced chemiluminescence (ECL) and exposed to radiography film.

Statistical analysis

All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was performed followed by Student–Newman–Keul test using SSPS11.5 software. A P value less than 0.05 were considered as statistically significant.

Results

Effect of SFI on nuclear morphology

The cardiomycytes were staining of the nuclear configuration typical of apoptosis using nuclear dyes, e.g., Hoechst 33342. And the cells became more evident in cells exposed to 16 h of reoxygenation after 4 h of hypoxia (4H/16R). As shown in Fig. 1, most cells from the control group had big, regular nuclei, with only a few showing apoptotic nuclei with condensed chromatin. In the cells exposed to 4H/16R, there was a clear condensation of chromatin together with a clear decrease in cell size, with both features being characteristic of apoptosis. After SFI treatment with two different dose (50 μl/ml, 100 μl/ml, respectively), the development of these apoptotic features were almost completely inhibited, most cells approached to normal morphology, with only a few cells showing the changes similar to those in the H/R group.

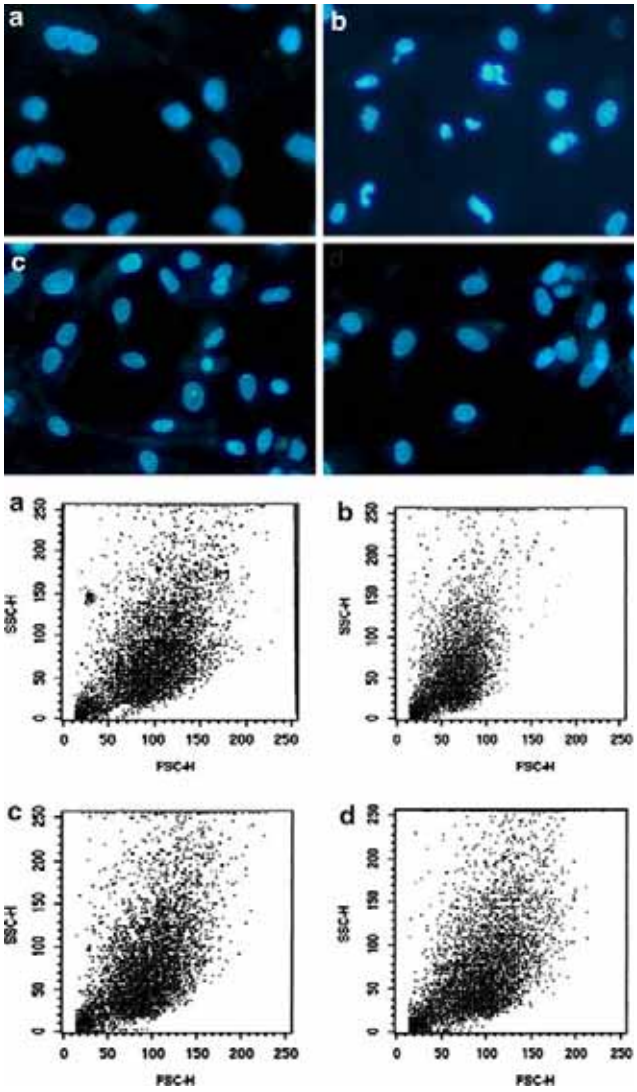

Fig. 1 Suppression of apoptotic morphology in reoxygenated cells by SFI. H/R induce significant morphological changes in cells. Cardiomyocytes were subjected to normoxia or 4 h hypoxia/16 h reoxygenation with and without SFI pretreatment. Following incubation, cells were stained with 10 μg/ml Hoechst 33342 for 30 min at room temperature. Cell morphology and nuclear staining of the same fields were recorded by phase contrast and fluorescence microscopy, respectively. (**a**) The control group, (**b**) the H/R group, (**c**) the L-SFI + H/R group, (**d**) the H-SFI + H/R group

Effect of SFI on apoptosis rate

Apoptosis was quantified by using fluorescent dye Annexin V-FITC, which binds to phosphatidylserine residues that are redistributed from the inner to the outer leaflet of the cell membrane as an early event in apoptosis. After loss of membrane integrity, PI can enter the cell and intercalate into DNA. Viable cells are Annexin V^-/PI^-. The Annexin V^+/PI^- cells are in early stage in the apoptotic process, whereas the Annexin V^+/PI^+ cells have lost cell membrane integrity and have taken up PI that are in late stage in the apoptotic process. Necrotic cells show Annexin V^-/PI^+. Figure 2 show the percentages of apoptosis at every stage significantly increased in H/R group. H/R cells have a large population of Annexin V^+/PI^-, Annexin V^+/PI^+ and Annexin V^-/PI^+ cells. However, with the treatment of SFI, those cells' population decreased significantly. SFI reduced the occurrence of cardiomyocytes apoptosis induced by H/R. And the results showed that cells treated with 50 μl/ml or higher dose (100 μl/ml) of SFI had levels of apoptosis similar to those cells under normoxia, and cells treated with 50 μl/ml SFI exhibited three times higher apoptosis than normoxic cells, but that was still significantly lower than that of cells subjected to H/R.

Effect of SFI on protein level of Bcl-2 and caspase-3 activities of cardiomyocytes

Caspases activation, especially caspase-3 activation, has been reported in the programmed cell death (PCD) and in the pathological conditions such as H/R. Some researches suggest that casapse-3 may be the main executor of cells apoptosis. So we determined the caspase-3 activation in protein level by Western blot analysis. Activation of caspase-3 is a useful parameter to be employed in Western blot where activation of caspase-3 is recognized by the occurrence of a 17 kDa band indicating cleavage of the 32 kDa molecules. Figure 3 shows that under control conditions, only the uncleaved proforms of endogenous caspases were detected by immunoblotting. Exposure of H/R cells were induced proteolytic cleavage of caspases, as revealed by the appearance of the characteristic fragment at 17 kDa of caspase-3. This proteolytic activation was nearly complete with difference concentration SFI incubation.

Bcl-2 family proteins play a critical role in the decision of the cell to die or survive by acting at multiple levels with a prompt impact on caspase activation [5, 6]. Therefore we also investigated the expression of the anti-apoptotic protein Bcl-2 in cardiomyocytes. In order to define additional mechanisms by which SFI inhibits H/R-induced cardiomyocytes apoptosis, the amount of Bcl-2 family proteins expressed by H/R-treated cardiomyocytes was quantified

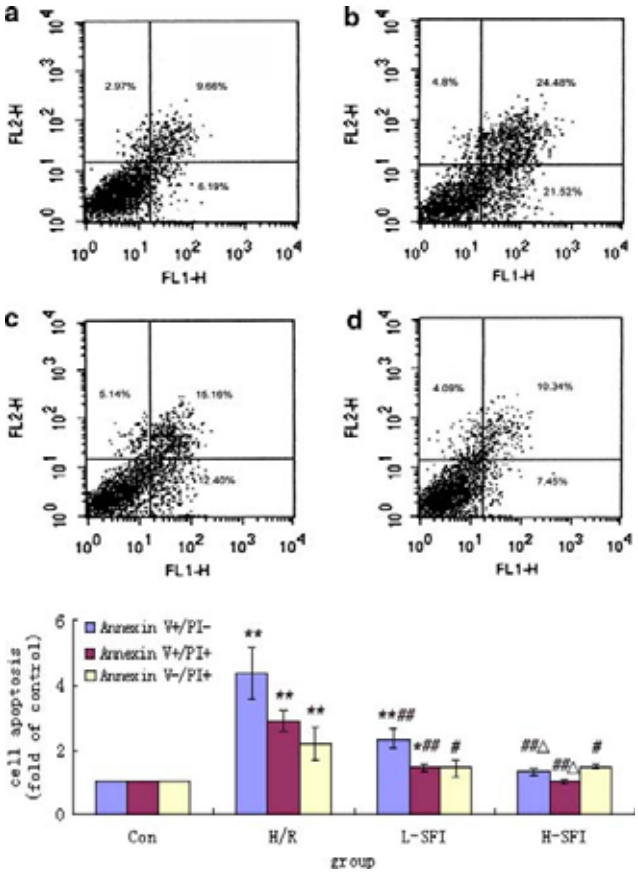

Fig. 2 Apoptosis induced by H/R in cardiomyocytes is inhibited by SFI treatment. Cardiomyocytes were subjected to normoxia or 4 h hypoxia/16 h reoxygenation with and without SFI pretreatment. Apoptosis was quantified by FACS analysis after being stained with Annexin V and propidine iodide (PI). Viable cells (Annexin V^-/PI^-). early apoptosis (Annexin V^+/PI^-). late apoptosis (Annexin V^+/PI^+). Necrotic cells (Annexin V^-/PI^-). The results are presented as fold changes compared with corresponding control cells. Data are the mean ± SEM of three independent experiments. $^*P<0.05$, $^{**}P < 0.01$ compared with the control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the H/R group, $^{\Delta}P < 0.05$ compared with the L-SFI group

by Western blot analysis. It was found that Bcl-2 expression was decreased in cardiomyocytes subjected to 4H/16R and this decrease was significantly reversed by co-treatment with different dose of SFI. Since decreased Bcl-2 expression is associated with increased apoptosis, these results suggested that SFI attenuate the apoptotic effects of H/R, in part, by up-regulation Bcl-2 protein level.

Discussion

Thrombotic occlusion of a coronary artery often leads to myocardial ischemia. Reperfusion to restore blood flow to the site of injury is critical to outcome [20]. However, reperfusion also appears to facilitate a series response, which may result in additional tissue damage. Increasing

evidence demonstrates that apoptosis in myocardial ischemia/reperfusion injury plays an important role in reversible and irreversible tissue injury and organ dysfunction [21]. Many studies have been designed to identify therapeutic agents that can improve tissue perfusion, yet attenuate reperfusion injury [22].

The present study examined the effects of SFI on cardiomyocytes apoptosis using the cultured neonatal rat cardiomyocytes H/R model. Apoptosis is a critical cellular event involved in the pathogenesis of myocardial ischemia/reperfusion injury. However, investigation of the significance of the contribution of apoptosis to overall myocardial injury in vivo is technically difficult, which is affected by many various factors including cell types, neurohormonal systems et al. In this context, primary cultures of cardiomyocytes provide advantages that overcome these complications to study the detailed mechanisms. Numerous recent reports have documented that either hypoxia alone or in combination with reoxygenation can trigger cardiomyocyte apoptosis [23, 24]. Therefore, in the present study, we used primary cultures of neonatal rat cardiomyocytes to define the significance of apoptosis in cardiomyocytes loss due to H/R, an in vitro model of ischemia/reperfusion.

It was known that nuclear dye Hoechst 33342 and annexin V-FITC binding assay are two kinds of measuring apoptosis methods, the former with a special ability showing dye nuclear and the cell morphology change, the latter being a more apoptosis-specific and early detection procedure. With using these two measuring methods, the results showed that most cells exposed to 4H/16R demonstrated a clear evidence of chromatin condensation together with a distinct decrease in cell size, as well as a significantly increased rate of different apoptotic process in cardiomyocytes. It testified that H/R injury could induce cardiomyocytes apoptosis, which is in accordance with results of previous studies. In SFI group most cells approached normal morphology and the apoptotic rate of different apoptotic process decreased, which demonstrated that SFI obviously has an anti-apoptotic effect on cardiomyocytes exposed to 4H/16R.

Bcl-2 family proteins play important roles in the regulation of apoptosis and are important modulators of cardiacmyocytes apoptosis [12, 25]. Constitutive expression of high levels of Bcl-2 protein enhances survival of many kinds cells including cardiomyocytes on exposure to various adverse stimuli. The main site of action of Bcl-2 family proteins appears to be the mitochondrion. Homodimers of Bcl-2 or Bcl-xL associates with the mitochondrial membrane and affects membrane permeability. The protective effect of Bcl-2 or Bcl-xL on mitochondria permeability is lost when Bcl-2 or Bcl-xL homodimers are sequestered by the formation of Bcl-2/Bax heterodimers. A family of caspases is a key regulator of apoptotic signaling pathway. Considering that Caspases especially are key mediators of the apoptotic process, identifying any regulatory modifications of these proteases is necessary to elucidate mechanisms of cellular balance between survival and death. Bcl-2 has been shown to prevent apoptosis by functioning upstream of caspase-3 [26]. Therefore, Bcl-2 and caspase-3 protein levels were examined by Western blot analysis to determine whether these regulators of apoptosis were involved in the mechanism of cardiomyocytes death induced by H/R insult. β-actin protein levels as internal control confirmed that equal amounts of the protein were loaded in each well of the gel (Fig. 3). The results of the present study suggest that down-regulation of Bcl-2 expression may trigger activation of caspase-3 in cardiomyocytes during H/R, resulting in cell death.

Since apoptosis represents an active, gene-directed mechanism, it should be possible to control this process for therapeutic purposes. SFI have received increasing attention because of its unique biological properties. The main components of SFI include Ginsengoside and Aconitine. It has been shown that SFI, produced by the improved traditional method, exhibits multiple pharmacological actions: enhance the ability of clearing away oxygen free radicals, alleviate the lipid peroxidation, antagonize the poisonous effect of free radicals, relieve Ca^{2+} overload in cells, stabilize the membrane structure, and is the first choice among traditional Chinese medicines for the treatment of shock. SFI has been reported to have a protective effect of multiorgans, which contribute to protection of ischemia myocardium [27].

According to the studies related to organ protection of SFI, Shenfu injection can act via different pathways, levels and targets simultaneously. In addition, one of the components of SFI is able to modulate apoptosis in different models [28, 29]. And in this research it is shown that SFI attenuates apoptosis induced by H/R in cardiomyocytes, inhibiting

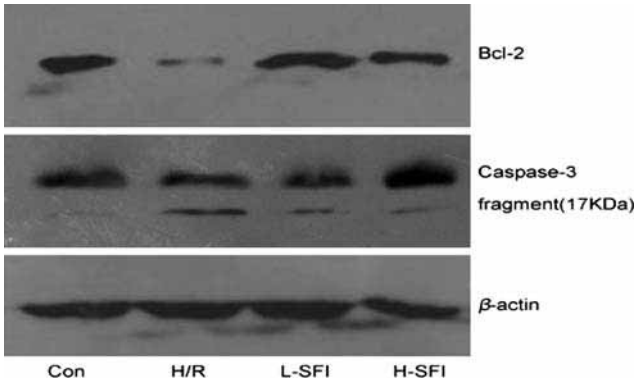

Fig. 3 SFI modulates apoptosis-related gene protein expression. The apoptosis-related gene is regulated by SFI treatment in cardiomyocytes subjected to H/R Neonatal rat cardiomyocytes were pretreated with or without SFI before being subjected to hypoxia/reoxygenation. Cell protein extracts were immunoblotted with anti-Bcl-2 antibody and caspase-3 antibody. Western blot analysis for Bcl-2, Caspase-3 and β-actin (used as a loading control). Results are representative of three independent experiments

caspase-3 activation and reducing cytotoxicity. This result suggests that the cell survival signal imparted by SFI could interfere with the apoptotic signal pathway activated by H/R in cardiomyocytes. After 16 h reoxygenation following 4 h hypoxia, it was detected a strong down-regulation of Bcl-2 protein level that was inhibited by SFI treatment, which identifies SFI have the effect on the down-regulation of Bcl-2 that follows exposure to H/R. This effect on Bcl-2 family proteins level may be an important component of the anti-apoptotic action of SFI. Elucidation of the details by which SFI can inhibit apoptosis in cardiomyocytes may suggest novel strategies for the treatment of ischemic heart disease.

In conclusion, this investigation demonstrated H/R-induced apoptosis of cardiomyocytes were almost completely suppressed by SFI treatment. The protective effect of SFI correlated with a marked up-regulation of the anti-apoptotic protein Bcl-2 and inhibited the activation of subsequent effector caspase-3. Moreover, SFI can inhibit apoptosis in vitro in a dose-dependent manner. This research affords the theory basis for SFI clinical application, whereas the further detailed study is needed in these fields.

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Reference

1. Kang PM, Izumo S (2000) Apoptosis and heart failure: a critical review of the literature. Circ Res 86:1107–1113
2. Cheng W, Kajstura J, Nitahara JA et al (1996) Programmed myocyte cell death affects the viable myocardium after infarction in rats. Exp Cell Res 226:316–327
3. Narula J, Pandey P, Arbustini E et al (1999) Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. Proc Natl Acad Sci USA 96:8144–8149
4. Gottlieb RA, Burleson KO, Kloner RA et al (1994) Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest 94:1621–1628
5. Umansky SR, Shapiro JP, Cuenco GM, Foehr MW, Bathurst IC, Tomei LD (1997) Prevention of rat neonatal cardiomyocyte apoptosis induced by simulated in vitro ischemia and reperfusion. Cell Death Differ 4:608–616
6. Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. Science 281:1312–1316
7. Cohen GM (1997) Caspases: the executioners of apoptosis. Biochem J 326:1–16
8. Desagher S, Martinou JC (2000) Mitochondria as the central control point of apoptosis. Trends Cell Biol 10:369–377
9. Reed JC, Zha H, Aime-Sempe C, Takayama S,Wang HG (1996) Structure–function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 406:99–112
10. Kirshenbaum LA, de Moissac D (1997) The bcl-2 gene product prevents programmed cell death of ventricular myocytes. Circulation 96:1580–1585
11. Fortuño MA, Ravassa S , Etayo JC , Díez J (1998) Overexpression of Bax protein and enhanced apoptosis in the left ventricle of spontaneously hypertensive rats: effects of AT1 blockade with losartan. Hypertension 32:280–286
12. Yamamura T, Otani H, Nakao Y, Hattori R, Osako M, Imamura H (2001) IGF-I differentially regulates Bcl-xL and Bax and confers myocardial protection in the rat heart. Am J Physiol Heart Circ Physiol 280:H1191–H1200
13. Postma G., Nemec W, Kleinspehn KL (1999) Large floating clasts in turbidites—a mechanism for their emplacement. Sediment Geol 58(1):47
14. Zhu ZH, Xiong LZ, Dong HL et al (2000) Dose response effects of Shenfu injection on ischemic reperfusion injury of spinal cord in rabbit. Chin J Anesthesiol 20:664–668
15. Xia ZY, Zhan LY, He YH, Liu XY (2003) The effect of Shenfu on gastrointestinal tract injury and its potential mechanism during cardio-pulmonary bypass in patients undergoing cardiac surgery. Chin J Traumatol 6:245–248
16. Liu XY, Zou HD, Yu JF et al (1997) Protective effect of Shenfu injection on multiple organ damage in rabbit during ischemia reperfusion. Chin J Anesthesiol 17:430–432
17. Zhang BJ, Wang YL Wang CY, Ke JJ (2005) Effect of Shenfu injection on nuclea r factor-κB during myocardial ischemia/reperfusion injury in rats. Chin J Traumatol 8:200–204
18. Buja LM, Hagler HK, Parsons D, Chien K, Reynolds RC, Willerson JT (1988) Alterations of ultra structure and elemental composition in cultured neonatal rat cardiac myocytes after metabolic inhibition with iodoacetic acid. Lab Invest 53:397–412
19. Bo HS, Seung HC, Eun YC et al (2004) Thiamine attenuates hypoxia-induced cell death in cultured neonatal rat cardiomyocytes. Mol Cells 18:133–140
20. Carden DL, Granger DN (2000) Pathophysiology of ischemia-reperfusion injury. J Pathol 190:255–266
21. Anderson HV, Willerson JT (1993) Current concepts: thrombolysis in acute myocardial infarction. N Engl J Med 329:703–709
22. Vermeiren GLJ, Claeys MJ, Van Bockstaele D et al (2000) Reperfusion injury after focal myocardial ischemia: polymorphonuclear leukocyte activation and its clinical implications. Resuscitation 45:35–61
23. Long X, Boluyt MO, Hipolito ML et al (1997) p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. J Clin Invest 99:2635–2643
24. Wang G-W, Zhanxiang Z, Klein JB, Kang YJ (2001) Inhibition of hypoxia/reoxygenation-induced apoptosis in metallothionein-overexpressing cardiomyocytes. AJP—Heart 280:2292–2299
25. Cook SA, Sugden PH, Clerk A (1999) Regulation of bcl-2 family proteins during development and in response to oxidative stress in cardiac myocytes: association with changes in mitochondrial membrane potential. Circ Res 85:940–949
26. Chinnaiyan AM, Orth K, Orourke K, Duan HJ, Poirier GG, Dixit VM Molecular ordering of the cell death pathway-Bcl-2 and Bcl-xL function upstream of the Ced-3-like apoptotic proteases. J Biol Chem 271:4573–4576
27. Cao J, Zhang CD, Zhang GX, Zhang YJ, Min S (2005) Protective effect of Shenfu injection on myocardial mitochondria injured by ischemia-reperfusion in rabbits. Chin Med J 118:505–507
28. Xia ZY, Meng QT, Zhang F, Chen XD (2004) Effect of Shenfu injection (ginesenoside and aconite alkaloid) on the apoptosis of intestinal mucosal epithelial cells and its mechanism during ischemia reperfusion in rats. Chin J Traumatol 7:363–367
29. Kim E-H, Jang M-H, Shin M-C, Shin M-S, Kim C-J (2003) Protective effect of aqueous extract of ginseng radix against 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 Cells. Biol Pharm Bull 26:1668–1673