Danshensu protects against ischemia/reperfusion injury and inhibits the apoptosis of H9c2 cells by reducing the calcium overload through the p-JNK-NF-κB-TRPC6 pathway

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Abstract. Ischemia-reperfusion (I/R) plays an important role in myocardial injury. In the present study, we aimed to examine the protective effects of Danshensu (DSS) against I/R injury and to elucidate the underlying mechanisms. For this purpose, H9c2 cells were cultured in hypoxic solution in a hypoxic incubator for 2 h, and then cultured in a high oxygen incubator for various periods of time and pre-treated with or without DSS, ammonium pyrrolidine dithiocarbamate (PDTC) or SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor]. Cell apoptosis and cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) levels were analyzed by flow cytometry. The protein expression levels of JNK, phosphorylated (p-)JNK, nuclear factor-κB (NF-κB) and transient receptor potential cation channel, subfamily C, member 6 (TRPC6) were measured by western blot analysis. The mRNA expression levels of JNK were measured by RT-qPCR. The results revealed that TRPC6 protein expression, the cell apoptotic rate and the $[Ca^{2+}]_i$ levels increased in a time-dependent manner in the H9c2 cells following the induction of I/R injury. The apoptotic rate and TRPC6 protein expression decreased when the cells were treated with DSS prior to the induction of I/R injury. The knockdown of JNK expression by siRNA decreased the p-JNK and TRPC6 protein expression levels in the H9c2 cells subjected to I/R injury. The protein expression levels of p-JNK and NF-κB in the nucleus increased significantly when the H9c2 cells were subjected to I/R injury, whereas NF-κB expression in the cytoplasm decreased in a time-dependent manner. However, p-JNK, NF-κB and TRPC6 protein expression, the $[Ca^{2+}]_i$ level and cell apoptosis decreased when the H9c2 cells were pre-treated with DSS or SP600125. Therefore, our data suggest that DSS prevents myocardial I/R injury by inhibiting p-JNK activation and NF-κB translocation, which potentially upregulate TRPC6 expression, increase the $[Ca^{2+}]_i$ level, and result in the apoptosis of H9c2 cells.

Introduction

Coronary heart disease (CHD) is the leading cause of morbidity and mortality worldwide, accounting for an estimated 7.3 million deaths in 2008 according to the World Health Organization (1). Myocardial ischemia, which is commonly observed when arteries supplying the heart become occluded, results when inadequate blood perfusion affects the cardiac tissues. To minimize cardiac damage, ischemic tissue must be sufficiently reperfused. However, reperfusion has the potential to exacerbate severe tissue injury, a process termed 'reperfusion injury'. The main pathological manifestation of CHD is myocardial damage due to ischemia/reperfusion (I/R) injury (2). Clinically, myocardial I/R injury occurs during or following coronary angioplasty, thrombolytic therapy, coronary revascularization and heart transplantation (3). Myocardial I/R injury may lead to the expansion of the myocardial infarct area, cardiac arrhythmias, contractile dysfunction and even sudden death (4).

Myocardial I/R injury has been studied for over 50 years. Jennings *et al* first described myocardial I/R injury using a canine heart coronary artery ligation model in 1960 (5). The authors observed that reperfusion accelerated the development of myocardial necrosis, and the degree of myocardial necrosis following I/R for 30-60 min was similar to that observed 24 h after coronary occlusion. To date, various studies have shown that I/R injury is associated with calcium overload (6-8), the production of free radicals (9,10) and mitochondrial alterations (10,11). Cytosolic calcium overload is now well known as an essential pathophysiological mechanism which is involved in reperfusion injury, although the source and origin of the calcium remains to be determined (12).

Calcium transport in cells has been reported to be governed by non-selective cation channels referred to as 'transient

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receptor potential (TRP) channels', which are highly permeable to calcium. At the present time, >30 mammalian TRP channels have been identified, cloned and characterized. These channels are grouped into the following 6 subfamilies based on their amino acid sequence homology: i) TRPC, ii) TRPM, iii) TRPV, iv) TRPA, v) TRPML and vi) TRPP (13). TRPC proteins are widely expressed in cardiac, pulmonary and vascular tissues and partially regulate cellular Ca^{2+} flux either by acting as Ca^{2+} entry channels or by altering membrane potential (14,15). As mentioned above, I/R injury is associated with calcium overload. As a calcium channel, we wished to determine whether TRPC participates in the development of I/R injury. Certain studies have previously examined this idea. The study by Zhang*et al* demonstrated that interleukin (IL)-17A contributes to brain I/R injury via the calpain-transient receptor potential cation channel, subfamily C, member 6 (TRPC6) pathway in mice (16). Similarly, it has been demonstrated that the activation of TRPC6 channels is essential for lung I/R-induced edema in mice (17). However, whether TRPC6 is associated with myocardial I/R injury remains unknown. Thus, in the present study, we aimed to investigate this matter.

Nuclear factor-κB (NF-κB) is one of the key factors regulating cell gene transcription. Known as a nuclear transcription factor, NF-κB is involved in the regulation of the expression of several genes. As a key regulator of cardiac genes, NF-κB regulates the expression of multiple downstream signal transduction cascades in a variety of physiological and pathophysiological states (18). Studies have shown that NF-κB is associated with myocardial I/R injury (19). The nuclear translocation of NF-κB upregulates TRPC6 expression and enhances Ca^{2+} influx (20,21). The c-Jun N-terminal kinase (JNK) signaling channel, as a member of the mitogen-activated protein kinase (MAPK) family, plays an important role in the cellular stress response. Also known as the stress-activated protein kinase (SAPK), research has indicated that JNK is closely related to myocardial I/R injury (22,23). In a model of myocardial I/R injury, Shimizu *et al* found that myocardial ischemia first activated MAPK activity, and then activated the transcription factors, activator protein-1 (AF-1) and NF-κB (24). We thus wished to determine whether TRPC6 participates in myocardial I/R injury, and whether this is associated with the activation of the JNK signaling pathway through the translocation of NF-κB. In this study, we conducted a preliminary investigation into this matter.

Danshensu (DSS) [(R)-3-(3, 4-dihydroxyphenyl)-2-hydroxy-propanoic acid; CID 11600642] is a water-soluble component of phenolic acid from the widely used Chinese herb, Danshen. Previous studies have confirmed that DSS has biological activities in that it improves microcirculation and exerts cardiovascular protective effects. For example, DSS has been shown to restore endothelium-dependent vasorelaxation via the prostacyclin pathway by increasing cyclooxygenase (COX)-2 gene expression and prostacyclin production (25), and thus it suppresses the formation of reactive oxygen species (ROS) and protects the myocardium against ischemia (26), protecting endothelial cells against injury induced by inflammation and inhibiting apoptosis (27,28), as well as inhibiting cardiac fibrosis through the negative regulation of ROS-/p38 MAPK signaling (29).

It has previously been suggested that DSS exerts a number of protective effects associated with NF-κB and JNK. DSS has also been shown to reduce lipopolysaccharide (LPS)-induced inflammatory responses in murine RAW264 macrophages by decreasing the nuclear translocation of NF-κB p65 (30). It has also been shown to partly inhibit the expression of the receptor for advanced glycation endproducts (RAGE), phosphorylated (p-)p38 and COX-2, and inhibit NF-κB activation in diabetic mice (31). Other studies have confirmed that DSS exerts obvious inhibitory effects on JNK and NF-κB p65 expression in hepatic stellate cells (HSCs) stimulated with IL-1β and may thus be used to inhibit hepatic fibrosis (32).

It has been suggested in previous research that DSS protects against myocardial I/R injury (26). However, whether DSS is associated with TRPC6 remains unknown. Therefore, in this study, we examined the association between the protective effects of DSS and TRPC6 following myocardial I/R injury *in vitro* and the intermediary roles of JNK and NF-κB.

Materials and methods

H9c2 cell culture and treatment. Rat H9c2 cells provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) were cultured in DMEM supplemented with 10% FBS at 37˚C in a humidified incubator with 5% CO_2. The cells were fed every 3 days and subcultured upon reaching 90% confluence. The cells were plated at an appropriate density according to each experimental design. To induce I/R injury, the H9c2 cells were cultured in hypoxic solution in a hypoxic incubator (95% N_2, 5% CO_2) for 2 h. The hypoxic solution was subsequently replaced with reoxygenation solution, and the cells were cultured in a high oxygen incubator (95% O_2, 5% CO_2) for various periods of time. The inhibitors, SP600125 (10 μM) and ammonium pyrrolidine dithiocarbamate (PDTC, 10 μM) (Sigma, St. Louis, MO, USA) were administered 30 min prior to the induction of I/R injury. DSS (Shanxi, China) (98% purity) was provided by Xi'an Hongxing Biotechnology Co., Ltd, Xi'an, China. DSS was added to double distilled water and prepared in 5, 25, 50 and 100 mg/l concentrations, separately.

In this study, the H9c2 cells were divided into 6 groups as follows: normal cultured H9c2 cells (Con), 2 h of ischemia (I), 2 h of ischemia and then 0.5, 1, 2 and 3 h of reperfusion (I/R 0.5 h, I/R 1 h, I/R 2 h and I/R 3 h). For DSS treatment the cells were divided into 7 groups as follows: 1) the untreated control (Con) group; ii) the ischemia (I) group; iii) the cells subjected to 2 h of ischemia and 3 h of reperfusion (I/R 3 h) group; iv) cells exposed to I/R 3 h and treated with 5 mg/l DSS (D5+I/R 3 h) group; v) cells exposed to I/R 3 h and treated with 25 mg/l DSS (D25+I/R 3 h) group; vi) cells exposed to I/R 3 h and treated with 50 mg/l DSS (D50+I/R 3 h) group; and vii) cells exposed to I/R 3 h and treated with 100 mg/l DSS (D100+I/R 3 h) group.

Apoptosis assay. An Annexin V-FITC Apoptosis Detection kit (BD Biosciences) was used to evaluate H9c2 cell injury. The H9c2 cells were harvested and washed twice with cold phosphate-buffered saline (PBS), and then centrifuged at 1,000 x g for 5 min. Subsequently, the cell pellets were resuspended in 200 μl binding buffer with 10 μl Annexin V (1 μg/ml), and then incubated for 15 min in the dark at room temperature. Following the addition of 300 μl binding buffer with 5 μl propidium iodide (PI; 50 μg/ml), the cells were analyzed by flow

cytometry with a BD FACSCalibur. The positive staining of Annexin V indicated early apoptotic cells, and double staining of Annexin V and PI indicated late apoptotic cells. The statistical data are presented as the percentage of apoptotic cells.

Western blot analysis. At various time points during the experiment, the cells were harvested and lysed in cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) at 4˚C. Protein samples were separated by 8-10% SDS-PAGE, transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA) and blocked in 5% non-fat milk in TBST (150 mM NaCl, 50 mM Tris pH 7.5, 0.1% Tween-20) for 1 h at room temperature. The membranes were incubated with one of the following primary antibodies at the appropriate concentrations: i) rabbit anti-TRPC6 (T6442; Sigma), ii) mouse anti-JNK (sc-827) and anti-p-JNK (sc-12882) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), iii) mouse anti-NF-κB p65 (SAB4502609; Sigma), iv) mouse anti-β actin (A2228; Sigma), or v) mouse anti-lamin B (SAB1306342-40TST; Sigma) overnight at 4˚C. Finally, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or -mouse IgG (both from Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. An enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Inc.) was used to detect the bound antibodies, and the blots were developed with a Supersignal chemiluminescence detection kit. Relative protein levels were normalized to those of either β-actin or lamin B.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the TRIzol RNA kit (Sigma) according to the manufacturer's instructions. cDNA was prepared from total RNA using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania). The reverse transcription reaction was performed under the following conditions: i) 37˚C for 15 min, ii) 85˚C for 5 sec, and iii) 42˚C for 2 min. PCR was performed using a primer specific for either JNK or β-actin. The following primer pairs were used for PCR: i) JNK sense, 5'-GGUUAUGUACGGAUUGUGGtt-3' and antisense, 5'-CCACAAUCCGUACAUAACCtt-3' and ii) β-actin sense, 5'-GGGAAATCGTGCGTG ACATTAAGG-3' and antisense, 5'-CAGGAAGGAAGGCTG GAAGAGTG-3'.

qPCR was run on a 7300 PCR system (Applied Biosystems, Foster City, CA, USA) using a SYBR® Premix Ex Taq™ kit (Takara Bio, Inc., Shiga, Japan). Each reaction consisted of a 20-μl sample containing 2 μl cDNA, 10 μl 2X SYBR Green mixtures, 2 μl of each primer and 4 μl of RNase-free water. The following cycling conditions were used: i) 1 cycle of denaturation at 95˚C for 5 min, ii) 60˚C for 30 sec, and iii) 40 two-segment cycles from amplification (95˚C for 5 sec and 60˚C for 30 sec). Calculations were made using the $\Delta\Delta Ct$ method.

Knockdown of JNK by siRNA. Specific siRNA targeting JNK and negative control siRNA were purchased from the Ambion (Austin, TX, USA). Cell transfection was performed according to the manufacturer's instructions (Ambion). First, the cells were trypsinized and diluted to 1×10^5 cells/ml medium. The transfection reagent (Lipofectamine™ 2000, 11668-027; Ambion) and siRNA were diluted separately in serum-free mediuma, mixed and incubated at room temperature for 10 min to allow the siRNA/lipid complex to form. The siRNA/lipid complex was then added to each well at a final siRNA concentration of 60 pmol/well. At 48 or 72 h following transfection, the cells were harvested for RT-qPCR or western blot analysis to determine the JNK mRNA and protein levels. The H9c2 cells were subjected to I/R injury following transfection with the JNK siRNA for 48 h. si-con represented the H9c2 cells that were transfected with the negative control siRNA, and OR represented the oligofectamine reagent group without siRNA.

Calcium flux assay. The H9c2 cells were trypsinized and diluted to 1×10^5 cells/ml medium. Subsequently, the cells were collected and loaded with 5 μM of the fluo-3AM calcium indicator (Invitrogen, Carlsbad, CA, USA) in Hank's balanced salt solution (HBSS) for 30 min at 37˚C. After the baseline ofcytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was recorded, the receptor-operated channels were activated by the addition of 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) (Sigma) for 1 min, followed by changing the extracellular buffer to 2 mM Ca^{2+} ($CaCl_2$). The $[Ca^{2+}]_i$ level was detected at 5 time points at 1-min intervals using an FLX 800 spectrophotofluorometer (BioTek, Winooski, VT, USA) with a filter for 480 nm excitation and 510 nm emission wavelengths.

Statistical analysis. The data are presented as the means ± SD of 5 independent experiments performed in quintuplicate. Differences were evaluated using one-way ANOVA. Statistical analyses were performed using SPSS version 10.0. Values of $p<0.05$ were considered to indicate statistically significant differences.

Results

I/R injury increases the apoptosis of H9c2 cells. In this study, the H9c2 cells were divided into 6 groups as follows: Con, I, I/R 0.5 h, I/R 1 h, I/R 2 h and I/R 3 h. The results revealed that there was no significant difference in the apoptotic rates between the cells in the I group and the Con group, although the apoptotic rate was slightly increased in the I group. However, when the ischemic cells underwent reperfusion, cell apoptosis was not terminated, but rather increased in a time-dependent manner, with a noticeable difference ($P<0.05$, n=5). The difference between the cells exposed to 2 h of ischemia followed by 3 h of reperfusion (I/R 3 h) and those in the I group was the most significant ($P<0.01$, n=5; Fig. 1A).

TRPC6 protein expression increases over the course of I/R. Western blot analysis was used to measure the TRPC6 protein expression levels in H9c2 cells; β-actin was used as a protein standard. The results revealed that TRPC6 protein expression increased in a time-dependent manner over the reperfusion time following 2 h of ischemia ($P<0.05$, n=5). The cells in the I/R 3 h group exhibited the most significant difference compared with the cells in the I group ($P<0.01$, n=5; Fig. 1B).

The $[Ca^{2+}]_i$ level increases following reperfusion. The $[Ca^{2+}]_i$ level was measured using the fluorescence indicator, fluo-3AM, which reflects the TRPC6 channel activity under basal and OAG/$CaCl_2$ stimulation conditions. A significant increase in the basal $[Ca^{2+}]_i$ level was detected in the cells in the I/R 0.5, 1, 2 and 3 h groups compared with the cells in the the Con
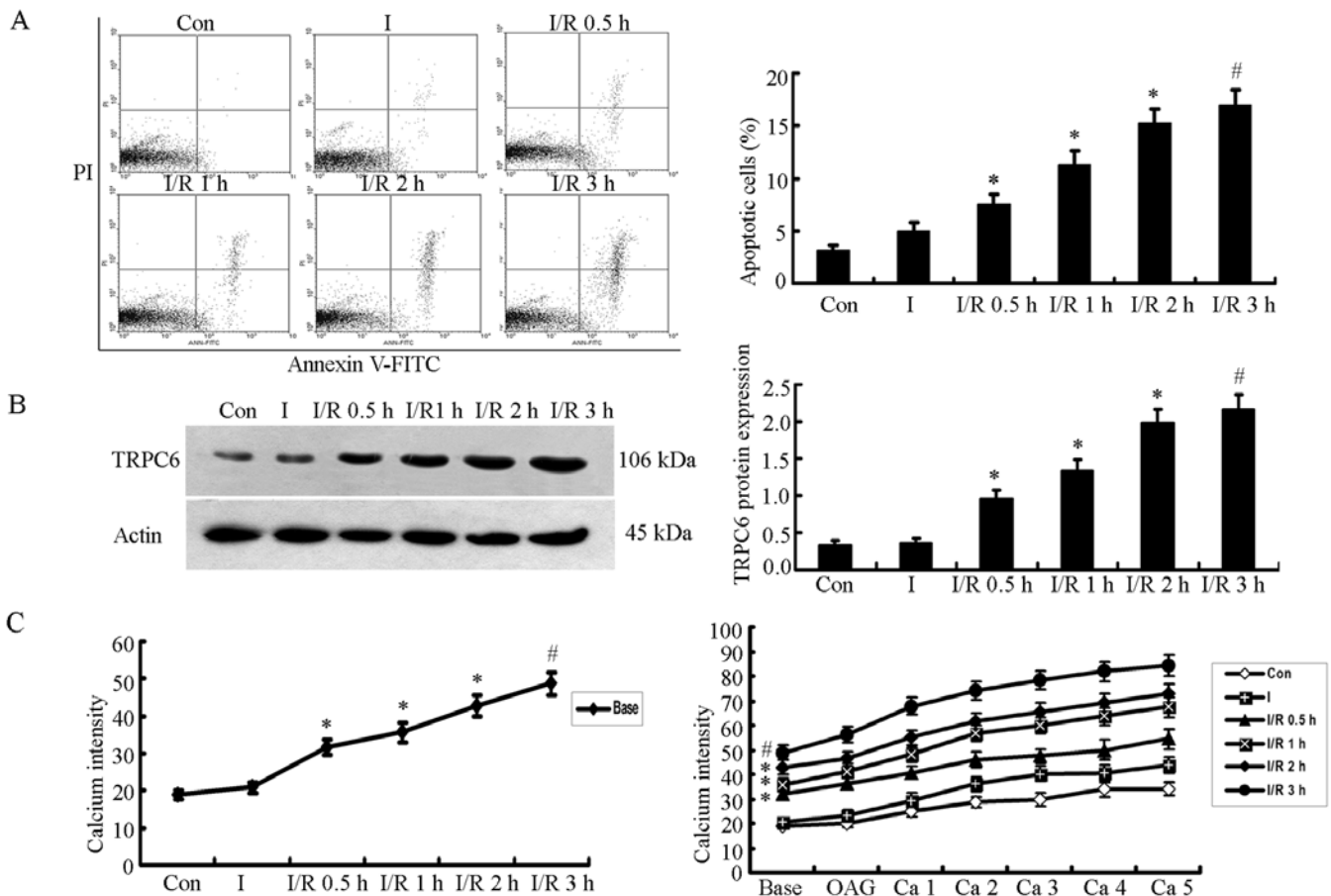
Figure 1. H9c2 cells subjected to ischemia-reperfusion (I/R). The cell apoptotic rate, transient receptor potential cation channel, subfamily C, member 6 (TRPC6) protein expression and the $[Ca^{2+}]_i$ level increased with the increasing duration of reperfusion. *P<0.05 vs. normal cultured H9c2 cells (Con) or ischemia (I) groups; most significant difference was observed in the I/R 3 h group; #P<0.01 vs. Con or I group (n=5).

or I groups (P<0.05), and the difference between the I/R 3 h group and the I group was most significant (P<0.01, n=5). Furthermore, following the activation of TRPC6 by OAG and CaCl₂, the $[Ca^{2+}]_i$ level was slightly higher at each time point (Ca 1-5) than the basal level. This was similar to the basal $[Ca^{2+}]_i$ level, and the level between the Con group vs. the I group (P<0.05), and the level between the I/R 3 h group vs. the Con or I group (P<0.01; Fig. 1C).

In summary, the apoptosis of the H9c2 cells, the protein expression of TRPC6, and the $[Ca^{2+}]_i$ levels increased significantly over the course of I/R, with I/R 3 h being the most significant time point.

Cell apoptotic rate, and TRPC6 protein and mRNA expression are reduced when the H9c2 cells are treated with DSS prior to the induction of I/R injury. The H9c2 cells were treated with various concentrations of DSS (5, 25, 50 and 100 mg/l) for 2 h prior to being subjected to 2 h of ischemia and 3 h of reperfusion. The apoptotic rate of the H9c2 cells was detected by flow cytometry. There was a significant difference between the 7 groups, and the rate of apoptosis decreased in a dose-dependent manner in the cells treated with DSS, with the most significant difference being observed between the cells in the I/R 3 h group (P<0.05, P<0.01, n=5) and the cells treated with 100 mg DSS (Fig. 2A). The TRPC6 protein and mRNA levels

both decreased significantly when the H9c2 cells were treated with 100 mg/l DSS prior to being subjected to I/R injury, compared with the cells exposed to I/R 3 h not treated with DSS (P<0.01, n=5; Fig. 2B and C).

Knockdown of JNK prior to the induction of I/R injury decreases TRPC6 protein expression, cell apoptosis and the $[Ca^{2+}]_i$ level in H9c2 cells. We used siRNA to selectively inhibit JNK protein expression. The JNK mRNA level decreased significantly in the siRNA group 48 h following transfection (P<0.05, n=5; Fig. 3A). The JNK protein level decreased by 71.20% at 72 h following transfection (0.87±0.07 in the siRNA group compared to 3.03±0.35 in the control group, P<0.05, n=5). The difference in JNK expression between the OR and si-con groups and the Con group was not significant (Fig. 3B).

Subsequently, we analyzed the changes in the JNK, p-JNK and TRPC6 protein levels, cell apoptosis, and the $[Ca^{2+}]_i$ level in the H9c2 cells undergoing I/R 3 h with or without JNK siRNA transfection. p-JNK and TRPC6 protein expression increased significantly after the H9c2 cells were subjected to I/R injury (P<0.05 vs. Con or I group), whereas there was no significant difference observed in JNK protein expression between the Con and I groups. The H9c2 cells were subjected to I/R injury following transfection with JNK siRNA, and we found that JNK, p-JNK and TRPC6 protein expression
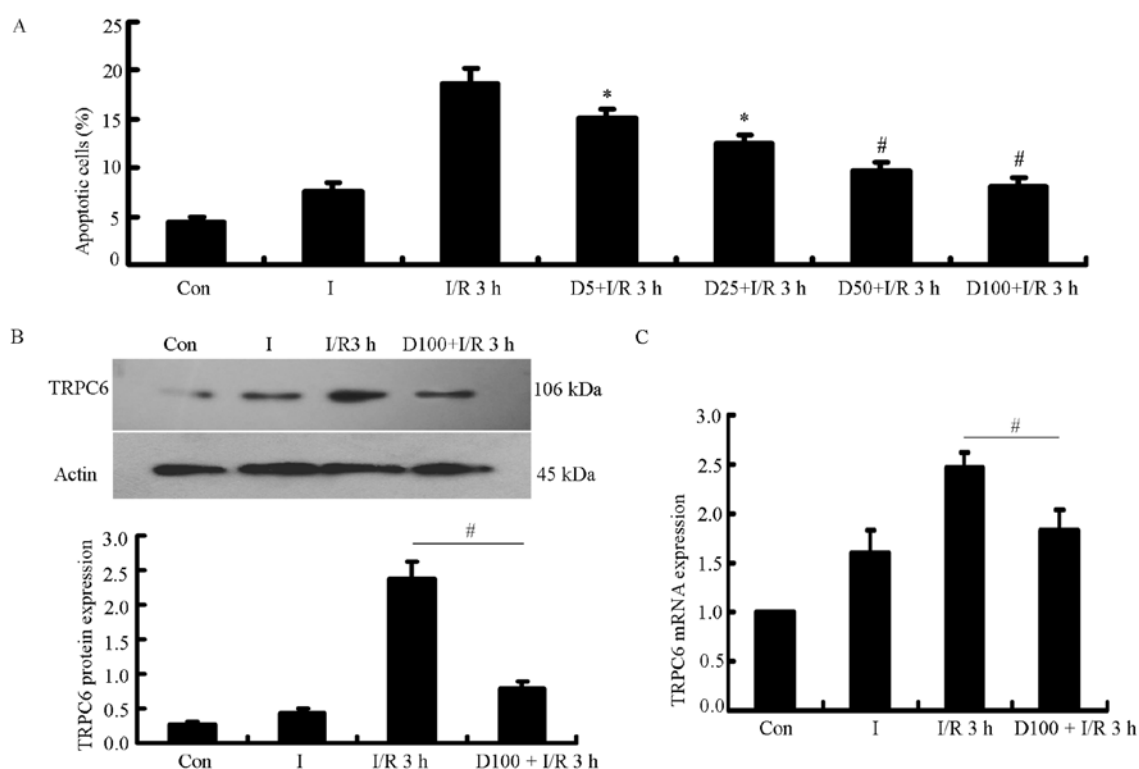
Figure 2. H9c2 cells were treated with Danshensu (DSS) prior to being subjected to ischemia-reperfusion (I/R) injury. (A) H9c2 cell apoptotic rate decreased in a dose-dependent manner in the cells in the D5+I/R 3 h, D25+I/R 3 h, D50+I/R 3 h and D100+I/R 3 h groups, *P<0.05 vs. I/R 3 h group, #P<0.01 vs. I/R 3 h group (n=5). (B and C) Transient receptor potential cation channel, subfamily C, member 6 (TRPC6) protein and mRNA levels decreased significantly, #P<0.01 I/R 3 h vs. I/R 3 h+D100 group (n=5).
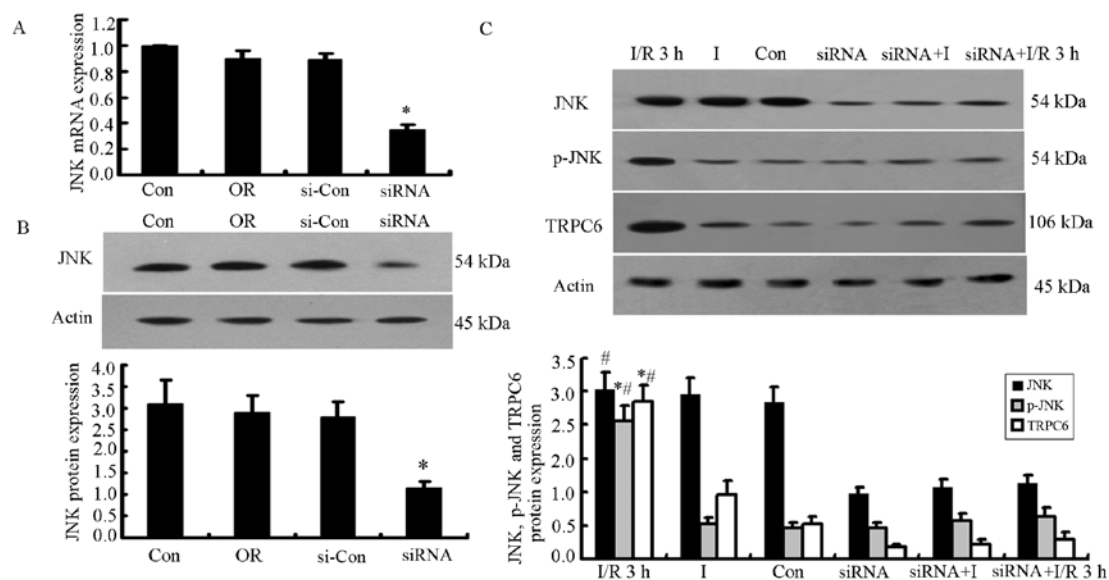

Figure 3. Knockdown of c-Jun N-terminal kinase (JNK) expression by siRNA attenuates p-JNK and transient receptor potential cation channel, subfamily C, member 6 (TRPC6) protein expression in H9c2 cells subjected to ischemia-reperfusion (I/R) injury. (A) JNK mRNA expression decreased significantly after 48 h when the H9c2 cells were transfected with siRNA. *P<0.05 siRNA vs. normal cultured H9c2 cells (Con), oligofectamine reagent-treated (OR), negative control, or siRNA (si-Con groups) (n=5). (B) The protein level of JNK decreased by 71.20% at 72 h following transfection with siRNA. *P<0.05 siRNA vs. Con, OR or si-Con group (n=5). (C) p-JNK and TRPC6 protein expression increased significantly in H9c2 cells during I/R, while there no significant difference observed in JNK protein expression. *P<0.05 I/R 3 h vs. I or Con groups. JNK, p-JNK and TRPC6 protein expression decreased significantly when the H9c2 cells were subjected to I/R following transfection with JNK siRNA. #P<0.05 I/R 3 h vs. siRNA, siRNA+I or siRNA+I/R 3 h group (n=5).

decreased significantly after the knockdown of JNK (P<0.05 vs. siRNA group, siRNA+I or siRNA+I/R 3 h group; n=5; Fig. 3C).

NF-κB p65 is activated during the I/R process and translocates from the cytoplasm into the nucleus. Western blot analysis demonstrated that the protein expression of NF-κB p65 in
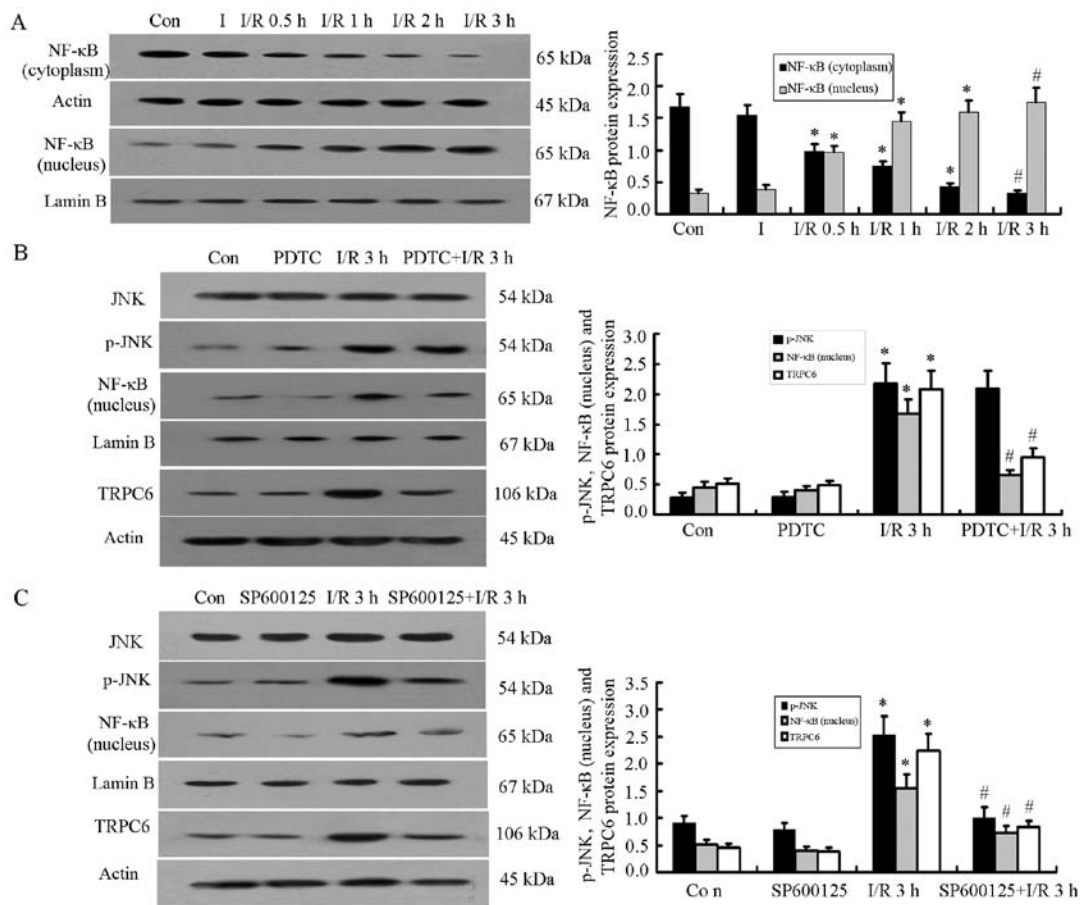
Figure 4. Changes in protein expression changes of phosphorylated (p-)c-Jun N-terminal kinase (p-JNK), nuclear factor-κB (NF-κB) (nucleus) and transient receptor potential cation channel, subfamily C, member 6 (TRPC6) in H9c2 cells subjected to ischemia-reperfusion (I/R) injury and pre-treated with or without PDTC or SP600125. (A) NF-κB protein expression in the cytoplasm decreased gradually with the increasing duration of reperfusion, and NF-κB expression in the nucleus increased. *P<0.05 vs. normal cultured H9c2 cells (Con) or ischemia (I) group; I/R 3 h group exhibited the most significant difference; #P<0.01 vs. Con or I group (n=5). (B) NF-κB (nucleus) and TRPC6 protein expression decreased markedly when the H9c2 cells were treated with PDTC (10 µM) prior to being subjected to I/R injury; JNK and p-JNK expression did not differ significantly between the I/R 3 h group and PDTC+I/R 3 h group. *P<0.05 I/R 3 h group vs. Con, I or PDTC+I/R 3 h group; #P<0.05 I/R 3 h vs. PDTC+I/R 3 h (n=5). (C) p-JNK, NF-κB (nucleus) and TRPC6 protein decreased, but no difference was observed in the JNK levels when H9c2 cells were treated with SP600125 (10 µM) prior to being subjected to I/R injury. *P<0.05 vs. Con, I or SP600125+I/R 3 h group, #P<0.05 I/R 3 h group vs. SP600125+I/R 3 h (n=5).

the nucleus increased significantly when the H9c2 cells were subjected to I/R injury, with the highest level observed in the I/R 3 h group. At the same time, the protein content of NF-κB p65 in the cytoplasm decreased, with the level decreasing gradually during reperfusion; the protein level of NF-κB p65 in the cytoplasm was the lowest in the I/R 3 h group (P<0.05 vs. Con or I group; P<0.01 vs. Con or I group; n=5; Fig. 4A).

Inhibition of NF-κB reduces NF-κB and TRPC6 protein expression. To further examine the association between p-JNK, NF-κB and TRPC6 during I/R in H9c2 cells, the NF-κB pathway inhibitor, PDTC (10 µM), was added 30 min before the H9c2 cells were subjected to I/R injury. The results revealed that the protein expression of p-JNK and NF-κB in the nucleus and the expression of TRPC6 increased significantly in the I/R 3 h group compared with the Con or PDTC groups (P<0.05, n=5); the JNK levels were not altered significantly between the groups. Subsequently, when the cells were treated with PDTC prior to being subjected to I/R injury, the protein expression of NF-κB in the nucleus and the expression of TRPC6 decreased significantly (P<0.05, n=5), whereas JNK and p-JNK protein

expression were not altered significantly following treatment with PDTC compared to the I/R 3 h group (Fig. 4B).

Inhibition of p-JNK affects NF-κB translocation and TRPC6 activation. We then examined the role of p-JNK in the translocation of NF-κB and the activation of TRPC6. We treated the H9c2 cells for 30 min with the p-JNK inhibitor, SP600125 (10 µM), prior to the induction of I/R injury; this reduced the increase in the protein expression of p-JNK, NF-κB (in the nucleus) and TRPC6. We noted significant differences between the I/R 3 h group and the Con or SP600125 group (P<0.05), while the levels in the I/R 3 h and SP600125+I/R 3 h groups also differed significantly (P<0.05, n=5; Fig. 4C).

H9c2 cells treated with DSS or SP600125 prior to the induction of I/R injury. The protein expression of JNK, p-JNK, NF-κB (in the nucleus) and TRPC6 was detected when the H9c2 cells were treated with DSS (5, 25 and 100 mg/l) for 2 h or with SP600125 for 30 min prior to the induction of I/R injury. p-JNK, NF-κB (nucleus) and TRPC6 protein expression decreased significantly in a dose-dependent manner
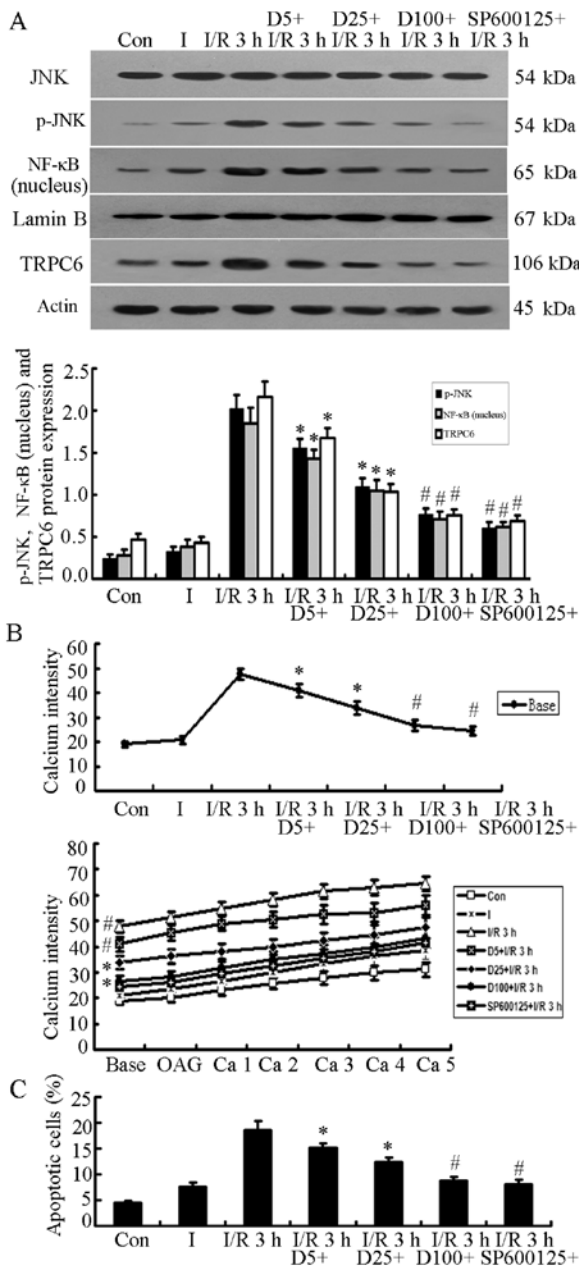
Figure 5. Changes in phosphorylated (p-)c-Jun N-terminal kinase (p-JNK), nuclear factor-κB (NF-κB) (nucleus) and transient receptor potential cation channel, subfamily C, member 6 (TRPC6) protein expression, cell apoptotic rate and the $[Ca^{2+}]_i$ level when the H9c2 cells were treated with Danshensu (DSS) or SP600125 prior to being subjected to I/R injury. (A-C) p-JNK, NF-κB (nucleus) and TRPC6 protein expression, cell apoptotic rate and $[Ca^{2+}]_i$ level decreased significantly in a dose-dependent pattern when the H9c2 cells were treated with DSS or SP600125 prior to being subjected to I/R. $^*P<0.05$ vs. I/R 3 h; $^{\#}P<0.01$ vs. I/R 3 h (n=5). There was no significant difference observed between the D100+I/R 3 h group and SP600125+I/R 3 h group.

in the cells treated with DSS compared with the cells in the I/R 3 h group (P<0.05), while the cells in the D100+I/R 3 h and SP600125+I/R 3 h groups exhibited the most significant differences compared with the cells in the I/R 3 h group (P<0.01, n=5; Fig. 5A). Similarly, the $[Ca^{2+}]_i$ level and the cell apoptotic rate decreased significantly in a dose-dependent manner when the H9c2 cells were cultured with DSS or SP600125 prior to being subjected to I/R injury (P<0.05 or P<0.01 vs. the I/R 3 h group; n=5; Fig. 5B and C).

Discussion

Calcium influx leads to the development of various diseases of the cardiovascular system (33,34); the discovery of mammalian TRP channels was a new starting point for examinations of the molecular basis of Ca^{2+} entry, and the discovery greatly promoted the development of cardiovascular disease research (35-37). Previous studies have indicatd that TRPC6 is widely expressed in the cardiovascular system and participates in cardiac hypertrophy and remodeling (20,38), arrhythmia (39), heart failure (40) and pulmonary arterial hypertension (41). TRPC6 has previously been shown to accelerate the deterioration of cardiac function (42); however, the role of TRPC6 in myocardial I/R injury remains unclear.

It is well known that calcium overload is an important event for cellular apoptosis (6-8). The TRP superfamily is highly permeable to calcium. Previous studies have confirmed that the TRPC subfamily is involved in calcium overload and apoptosis. It has been previously shown that the overexpression of TRPC3 and the increased Ca^{2+} influx due to TRPC3 resulted in the apoptosis of mouse cells (43). Satoh et al confirmed that TRPC7 acts as a Ca^{2+} channel, leading to myocardial apoptosis induced by AngII (44). Similarly, enhancing the expression of TRPC6 has also been shown to increase Ca^{2+} influx mediated by TRPC6 channels and contribute to podocyte apoptosis (45). These data suggest that the calcium overload resulting from the transient change in the calcium level is an important event for apoptosis. However, the association between myocardial I/R injury, the TRPC6 protein content, calcium influx and apoptosis was not clear until this study was undertaken.

In this study, our results suggested that TRPC6 was expressed in H9c2 cells, and during I/R it increased over the course of reperfusion. At the same time, the apoptotic rate and the $[Ca^{2+}]_i$ level also increased, which is similar to the results of Shen et al (6) and Assayag et al (8). We noted an increase in the basal $[Ca^{2+}]_i$ level, and we also noted that the continuous Ca^{2+} influx increased slightly following stimulation with OAG, an activator of TRPC6, following the addition of $CaCl_2$. This suggested that the Ca^{2+} influx mediated by TRPC6 was involved in I/R-induced cell injury, but not all TRPC6 channels were activated in the I/R process in H9c2 cells.

Traditional Chinese medicine (TCM) has been practiced for thousands of years; however, the specific mechanisms involved are not so well known, thus restricting its clinical use. DSS, the water-soluble active component of danshen, is abundant and accessible and is known for its cardioprotective properties. Thus, in this study, we used a series of experiments to examine the protective effects of DSS against I/R injury and to elucidate the potential mechanisms involved, in order to provide a sufficient basis for its clinical use.

In the present study, H9c2 cells were treated with various concentrations of DSS for 2 h prior to the induction of I/R injury. This study confirmed that pre-treatment with DSS reduced H9c2 cell apoptosis in a dose-dependent manner. These data indicated that DSS exerted cardioprotective effects on myocardial I/R injury in vitro, and that pre-treatment with 100 mg/l DSS exerted the most prominent protective effect. Further studies are required to further explore the underlying mechanisms. In this study, we also showed that the TRPC6 protein and mRNA levels both decreased significantly in a

dose-dependent manner when the H9c2 cells were treated with DSS prior to being subjected to I/R injury, which suggests that DSS exerts protective effects against I/R injury by reducing TRPC6 expression.

Studies have demonstrated that JNK and NF-κB are closely related to myocardial I/R injury (19,22,23). The p65 and p50 subunits of NF-κB are activated in response to numerous stimuli and translocate into the nucleus by the degradation of the inhibitor protein IκB. In previous research, it has been demonstrated that NF-κB participates in I/R injury. The activation of NF-κB induces the upregulation of fibrinogen-like 2 (FGL2) expression through tumor necrosis factor (TNF)-α during myocardial I/R (46). The levels of hepatic c-JUN, NF-κB expression and the apoptotic rate have been shown to be decreased during I/R in Toll-like receptor 4 (TLR4)-deficient mice compared with wild-type mice (47).

In this study, the specific knockdown of JNK prior to the induction of I/R injury using siRNA reduced JNK, p-JNK and TRPC6 protein expression; p-JNK and TRPC6 protein expression increased significantly in the H9c2 cells following I/R injury, which suggests that the increase in TRPC6 protein expression is mediated by the JNK signaling pathway. NF-κB p65 expression in the nucleus increased significantly following I/R injury, whereas the NF-κB protein content in the cytoplasm decreased. This suggests that more and more NF-κB was activated and then transferred to the nucleus during I/R. Previous studies have demonstrated that NF-κB regulates the expression of the TRPC family. Hai *et al* reported that NF-κB acts as an important positive transcriptional regulator of TNF-α-induced COX-2-dependent prostaglandin E2 (PGE2) production downstream of TRPC1-associated Ca^{2+} influx in colonic myofibroblasts. Inhibitors of NF-κB (curcumin and SN-50) attenuated the TNF-α-induced enhancement of TRPC1 expression and store-dependent Ca^{2+} influx (48). Yu *et al* also provided evidence that transforming growth factor (TGF)-β1 induces podocyte damage by upregulating TRPC6 protein expression most likely through the Smad3-ERK-NF-κB pathway (49). In the present study, we found that the I/R-induced TRPC6 upregulation was reduced when the cells were pretreated with PDTC, an NF-κB pathway inhibitor, but JNK and p-JNK protein expression was not altered significantly. Thus, NF-κB may be an essential upstream pathway that is able to block the expression of TRPC6 protein but that of p-JNK during I/R.

We further examined the association between the p-JNK signaling pathway, NF-κB and TRPC6. We found that the protein expression of NF-κB in the nucleus and TRPC6 was suppressed when the H9c2 cells were treated with the p-JNK inhibitor, SP600125, prior to being subjected to I/R injury. Our results revealed that p-JNK was involved in an upstream pathway that activates NF-κB and results in its translocation into the nucleus, and increased TRPC6 protein expression in H9c2 cells during I/R. Previous research supports this hypothesis: Pan *et al* reported that MAPKs, particularly JNK, play important roles in the high glucose (HG)-induced NF-κB activation in NRK-52E cells. The administration of the JNK specific inhibitor, SP600125, markedly decreased NF-κB activation, which emphasizes that JNK is a critical upstream protein of NF-κB and plays an important role in HG-induced renal inflammation (50).

In this study, the H9c2 cells were cultured with or without DSS (5, 25 and 100 mg/l) for 2 h or SP600125 for 30 min prior to the induction of I/R injury, and the results revealed that p-JNK, NF-κB and TRPC6 protein expression decreased markedly in the cells treated with DSS compared with cells in the I/R 3 h group. Similarly, the cell apoptotic rate and the $[Ca^{2+}]_i$ level significantly decreased in a dose-dependent manner when H9c2 cells were treated with DSS or SP600125 prior to being subjected to I/R. All these results suggest that the DSS-induced cardioprotective effects are mediated through the p-JNK, NF-κB and TRPC6 signaling pathways.

In conclusion, our data demonstrate that DSS exerts significant protective effects against myocardial I/R injury, possibly by inhibiting the phosphorylation of JNK, reducing the production of p-JNK, inhibiting the NF-κB translocation into the nucleus, and decreasing the protein expression of TRPC6, as well as decreasing Ca^{2+} influx and reducing cell apoptosis. Ultimately, DSS is an important component of Danshensu, a widely used herb in traditional Chinese medicine, and it may prove to be a promising therapeutic agent for reducing myocardial I/R injury in clinical settings.

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