Original Research Article

Cardioprotective effect of Shenxiong glucose injection on acute myocardial infarction in rats via reduction in myocardial intracellular calcium ion overload

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Abstract

Purpose: To explore the cardioprotective effects and potential mechanisms of Shenxiong Glucose Injection (SGI) in rat acute myocardial infarction (AMI).
Methods: AMI model was created by ligating left anterior descending coronary artery. After 7 days' consecutive intravenous administration of SGI, serum samples were used to conduct biochemical analysis while hearts were excised and processed for infraction size, enzyme activity, histopathology and qPCR studies. Intracellular Ca^{2+} {$(Ca^{2+})i$} overload in H9c2 cells was measured by laser scanning confocal microscope (LSCM).
Results: In AMI rats, pretreatment with SGI significantly ameliorated myocardial histopathologic damage. It exerted cardioprotective effect by decreasing myocardial infarct size, electrocardiogram (ECG) ST segment elevation, and CK, cTnI, BNP levels in serum. In addition, SGI significantly decreased calmodulin (CaM) and calmodulin-dependent protein kinase II (CaMK II) mRNA expression, but increased Ca^{2+}-Mg^{2+}-ATPase and Na+-K+-ATPase activities in myocardium. In doxorubicin (DOX)-induced H9c2 cells injury model, SGI reversed $(Ca^{2+})i$ overload to protect cells.
Conclusion: The results demonstrate SGI exerts cardioprotective effect by decreasing myocardial infarct size, restoring ST segment and reversing $(Ca^{2+})i$ overload. It suggests that SGI may be a new clinical candidate to treat myocardial infarction.

Keywords: Shenxiong glucose injection, Tanshinol, Ligustrazine, Myocardial infarction, Intracellular Ca^{2+} overload, Calmodulin, Calmodulin-dependent protein kinase II

INTRODUCTION

Ischemic heart disease, commonly called coronary artery disease, is a disease characterized by reduced blood supply to the heart. It has been the top one cause of life loss each year all over the world, bringing about 8.14 million fatalities in 2013 up from 5.74 million fatalities in 1990. It has been a tough global health problem to be solved according to the data analysis of cause-specific mortality for 240 causes of death during 1990 - 2013[1]. Although kinds of drugs such as aspirin, thrombolysis agents, β receptor blockers and ACE inhibitors

have been used to treat AMI [2,3], AMI is still one of the major cause of ischemic heart diseases, and with high rate of morbidity and mortality [4]. Therefore, the safer and more effective drugs still need to be explored for clinical use.

SGI is also known as *Bai Se Tong*, and its components are originated from danshen (*Salvia miltiorrhiza* Bge.) and chuanxiong (*Ligusticum chuanxiong* Hort.). It has been widely used in cerebral ischemia disease and other ischemic diseases in Asia. Tanshinol and ligustrazine hydrochloride, the active ingredients of SGI, have been confirmed to exhibit powerful protective effects on cardio-cerebrovascular disease *in vitro* and *in vivo*, and they have shown various pharmacological activities such as anti-oxidation, anti-apoptosis, and anticoagulation in the treatment of ischemic and infarction disease [5-7].

Previous studies have shown that SGI possesses protective effects on cerebral, and its potential mechanisms are involved in anti-inflammation, anti-oxidation and anticoagulation [8]. And what's more, clinical applications and animal experimental studies have demonstrated that there are seldom adverse effects after administration of SGI [8,9]. However, the cardioprotective effect and mechanisms of SGI has been rarely reported.

The main objective of our study is to investigate the cardioprotective effects and potential mechanisms of SGI on AMI injury.

EXPERIMENTAL

Materials

SGI was provided by Guizhou Jingfeng Injection Co., Ltd. (Guiyang, China). Panax notoginseng saponins (PNS) was bought from Guangxi Wuzhou Pharmaceutical Co., Ltd. (Wuzhou, China). Kits for creatine kinase (CK), Ca^{2+}-Mg^{2+}-ATPase and Na^+-K^+-ATPase were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Cardiac troponin I (cTnI) and brain natriuretic peptide (BNP) Elisa kits were obtained from Wuhan Cusabio Biotech Co., Ltd. (Wuhan, China). Fluo-3-AM was supplied by Beijing Fanbo biochemical pharmaceutical Co., Ltd. (Beijing, China). N-acetylcysteine (NAC) and DOX were provided by MP biochemical (California, USA). And kits for PrimeScript™ RT reagent Kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were gained from Takara(Shenyang, China). H9c2 cells were got from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

Animals and AMI injury model

Adult specific pathogen free (SPF) male Sprague-Dawley (SD) rats (180-220 g, certificate: NO. SCXK (Guangdong) 2013-0034) were obtained from the Medical Laboratory Animal Research Center of Guangdong Province. All rats were kept in a SPF level laboratory animal room in the institution of Laboratory Animal Science of Jinan University with normal temperature and 12 h light-dark cycle. Rats were provided with standard laboratory diet and water. The experiments were approved by the Committee on Animal Care of Jinan University (no. 20140615231359) and were performed according to the instructions of the National Institute of Health (OLAW/NIH 2002) [10].

SD rats were randomly assigned into 6 groups with 14 rats in each group: sham-operated (control), AMI, PNS (22.30 mg/kg), Low-dose SGI (0.714 mg/kg tanshinol and ligustrazine hydrochloride 3.57 mg/kg), Middle-dose SGI (3.57 mg/kg tanshinol and ligustrazine hydrochloride 17.85 mg/kg), High-dose SGI (tanshinol 17.85 mg/kg and ligustrazine hydrochloride 89.25 mg/kg). PNS has been widely used in cerebral and myocardial ischemia disease in clinics. In this study, it was treated as a positive control. The rats of control and AMI groups were administered with 5 % of glucose injection via caudal vein for 7 days consecutively, while the rest of rats were administered for 7 days respectively with the drugs accordingly.

AMI injury model was created according to our previous study with slight modifications [11]. The rats were anesthetized with 25 % of urethanum (1 g/kg, intraperitoneally). Following intubation and artificial ventilation, the surgical operation was performed to expose heart. The heart was rapidly exposed and the left anterior descending coronary artery was ligated with a suture, control group just exposed heart without ligating. Electrocardiography was recorded by ECG2206B system (Nanjing Hengteng electronic technology Co., Ltd., Nanjing, China) before and 5, 15, 30, 60, 120 min after the surgery, the ECG recording speed was 50 mm/s at a voltage of 10 mm/mV. The ST segment elevation of limb lead (II) ECG was used to determine the AMI injury. After the last time ECG recording, blood samples were collected from abdominal aorta to separate out serum by centrifugation at 3000 rpm for 10 min for further biochemical analysis. Successively, the hearts of all rats were got and performed for infraction size, pathology, and qPCR researches.

Measurement of serum biomarker of AMI

The serum content of CK, cTn-I and BNP was measured by corresponding commercial enzyme-linked immunosorbent assay kits described above. Spectrophotometry method was used to detect the kits by multi-detection microplate reader (Synergy™ HT, BioTek, USA). All protocols were performed according to the kit manufacture recommendations.

Histopathological analysis

Left ventricular heart tissues were fixed in paraformaldehyde and embedded in paraffin, and then slice into serial very thin pieces using microtome. The pieces were stained with H&E to observe the change of myocardium s by a microscope (Olympus CX31, Japan).

Determination of infarct size

The infarct size was determined as previously described [11]. The TTC-stained areas (red staining) and TTC-stained negative areas (myocardial infarction) were isolated and the infarct size was expressed as the percentage of the myocardial weight.

Determination of Ca^{2+}-Mg^{2+}-ATPase and Na^+-K^+-ATPase activities

Myocardial tissues were weighed and made into homogenates with normal saline. After centrifugation, the supernatant was obtained and used to determinate the protein concentration and the enzyme activity. The procedure used were strictly in accordance with the instruction of ATPase assay kits and Bradford Protein Assay Kit.

Measurement of mRNA expression of *CaM* and *CaMK II*

RNA was obtained from the myocardial tissues. Myocardial tissues were grinded with liquid nitrogen. Later, mRNA was extracted by RNA Trizol reagent and RNA integrity was determined by 1 % of agarose gel. cDNAs were reversely transcribed using each RNA sample and were amplified by PCR. The primers sequences for rat *CaM* were: F:5' GATAAGGATGGCAATGGC TACA3' R:5'CGATGTCTGCTTCCCTGATCAT3', *CaMK II* were: F:5'GTGATGGGAAGTGGCA GAATA3' R: 5'CGTGGCACTGTTGACAATTAG A3' and *β-actin* were: F:5'CCTGTGGCA TCCATGAAACTAC3' R: 5'CTTCTGCATCCTG TCAGCAAT3'. β-actin was used as a reference gene. The mRNA relative expression levels of

CaM and *CaMK II* were normalized to β-actin and calculated using the $2^{-\Delta\Delta CT}$ method.

Measurement of intracellular calcium concentration

Changes in $(Ca^{2+})i$ concentration were measured by Ca^{2+} sensitive fluorescent indicator Fluo-3-AM and LSCM (LSM 510 META, Zeiss, Germany). H9c2 cells were cultivated with or without SGI (0.08, 0.28 and 0.48 µmol/L, calculated by ligustrazine hydrochloride), NAC(1 mmol/L) for 2 h except control group, then added 1 µmol/L DOX cultivated for 48 h. The cells were quickly flushed and incubated with 5 µM Fluo-3-AM in a glass-bottom dish for 30 min at 37 °C and quickly flushed three times with PBS buffer and incubated for 15 min. $(Ca^{2+})i$ was measured as described previously[12]. Fluorescence was excited at a wavelength of 490 nm and emitted light was observed at 525 nm. Zeiss LSM image browser, a data processing system, was used to calculate intracellular fluorescence intensity.

Statistics

All numerical results are presented as mean ± SEM and statistical significance was analyzed with one-way analysis of variance (ANOVA) using SPSS 17.0 software. Differences were considered significant at $p < 0.05$.

RESULTS

SGI ameliorates ECG and decreases biomarkers of AMI injury in AMI rats

The myocardial injury can lead to abnormal ST segment change, so ECG can reflect the myocardial injury directly. In our present study, AMI injury resulted in significant ST segment elevation in the AMI group, which demonstrated that the AMI injury model of rats was successfully established. In addition, compared with AMI group, the ST segments of other groups were significantly reduced in a dose and time-dependent manner (Figure 1A). More importantly, the ST segment elevation of PNS and SGI (H) was significantly reduced within 120 min of the experiment ($p < 0.01$ or 0.05). The ECG of rats at 120 min are shown in Figure 1B. What's more, we also measured the content of CK, cTnI and BNP to examine the extent of myocardial damage and the protective effects of SGI. As shown in Table 1, the CK, cTnI and BNP contents of AMI group rats were higher than those for control group ($p < 0.05$). However, after being treated with SGI or PNS, the serum CK, cTnI and BNP contents were significantly decreased ($p < 0.01$ or 0.05).
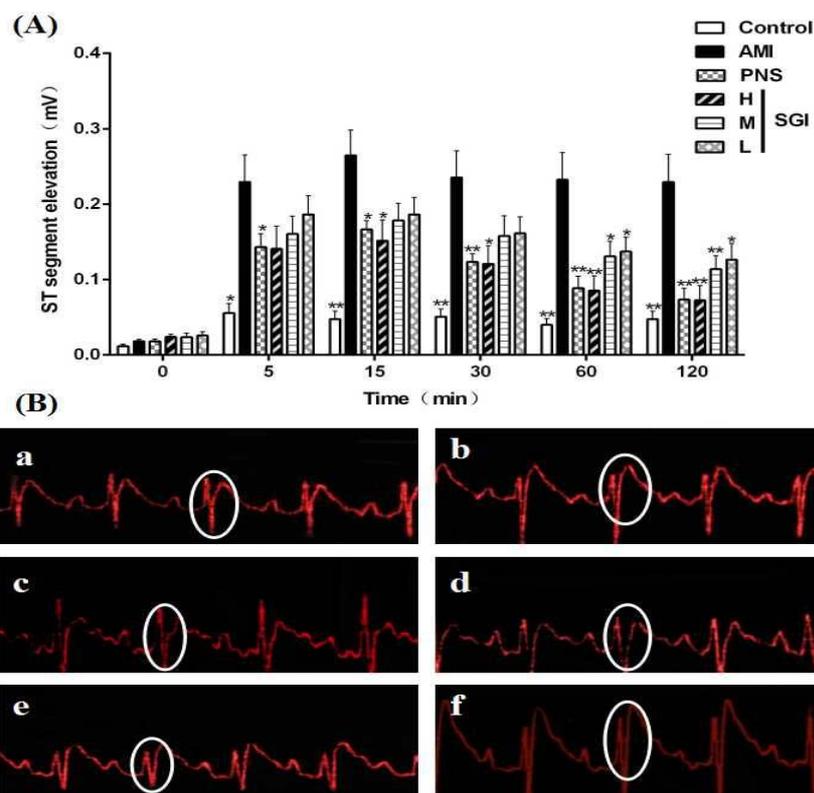
Figure 1: Effect of SGI on ECG in AMI injury rats. ST segment elevation in AMI injury rats during 120 min (A). ECG of AMI injury rats at 120 min (B). Compared with AMI group; $^*p$ <0.05, $^{**}p$ < 0.01, (n ≥ 12). The letters a to f are represented for control group, AMI group, PNS group, SGI(H) group, SGI(M) group and SGI(L) group, respectively

Table 1: Effect of SGI on serum CK, cTnI and BNP content in AMI injury rats

Group	CK (serum, U/mL)	cTnI (serum, µg/L)	BNP (serum, pg/mL)
Control	0.90±0.06*	217.21±26.73*	1088.32±89.14*
AMI	1.11±0.07	293.66±24.16	1364.94±79.80
PNS	0.85±0.07*	317.23±42.13	1331.29±134.82
SGI (H)	0.82±0.08*	168.11±13.19**	1259.32±131.70
SGI (M)	0.88±0.06*	176.66±35.73*	1081.37±111.03*
SGI (L)	0.72±0.06**	214.19±35.80	996.14±47.40**

Compared with AMI group, $^*p<0.05$, $^{**}p<0.01$, (n ≥ 7)

SGI decreases infarct size and protects myocardium structure in AMI rats

As shown in Figure 2B, myocardial infarct size in AMI group was significantly larger than that of control group (p < 0.01). When treated with SGI or PNS, myocardial infarct size was significantly reduced (p < 0.01 or p < 0.05), except SGI (L). TTC staining of myocardium intuitively revealed the degree of AMI injury of rats (Figure 2A, a–f). Meanwhile, histopathological analysis showed that myocardium structure of control group rats were normal with clear boundaries, but AMI injury in AMI group caused myocardium to be remarkably loose and disordered. Rats treated with PNS or SGI can ameliorate irregular arrangement of myocardium (Figure 2C, a–f).

SGI regulates Ca^{2+} related enzyme activity and mRNA expression

As shown in Table 2, rats suffered from AMI injury induced to significant decline of the Ca^{2+}-Mg^{2+}-ATPase and Na$^+$-K$^+$-ATPase activities (p < 0.01). PNS and SGI pretreatment protected their activities decline from AMI injury (p < 0.01 or p < 0.05). Meanwhile, *CaM* and *CaMK II* expressions were significantly improved in AMI injury rats (p < 0.01). On the contrary, pretreatment of PNS and SGI significantly decreased *CaM* and *CaMK II* mRNA expression in some degree (p < 0.01 or p < 0.05).

SGI decreases DOX-induced H9c2 cells (Ca^{2+})i overload

In order to confirm the effect of SGI on (Ca^{2+})i

overload, we investigated it in DOX-induced H9c2 cells injury model. Intuitively, the fluorescence intensity of DOX group was more severe than other groups (Figure 3A). Then, 35 cells were randomly selected in each group to calculate the relative intracellular calcium concentration. The results show that $(Ca^{2+})i$ of the DOX group was higher than that of the control group ($p < 0.01$). Similarly, $(Ca^{2+})i$ of NAC group, all the SGI groups had a significantly decrease compared with DOX group ($p < 0.01$) (Figure 3B).
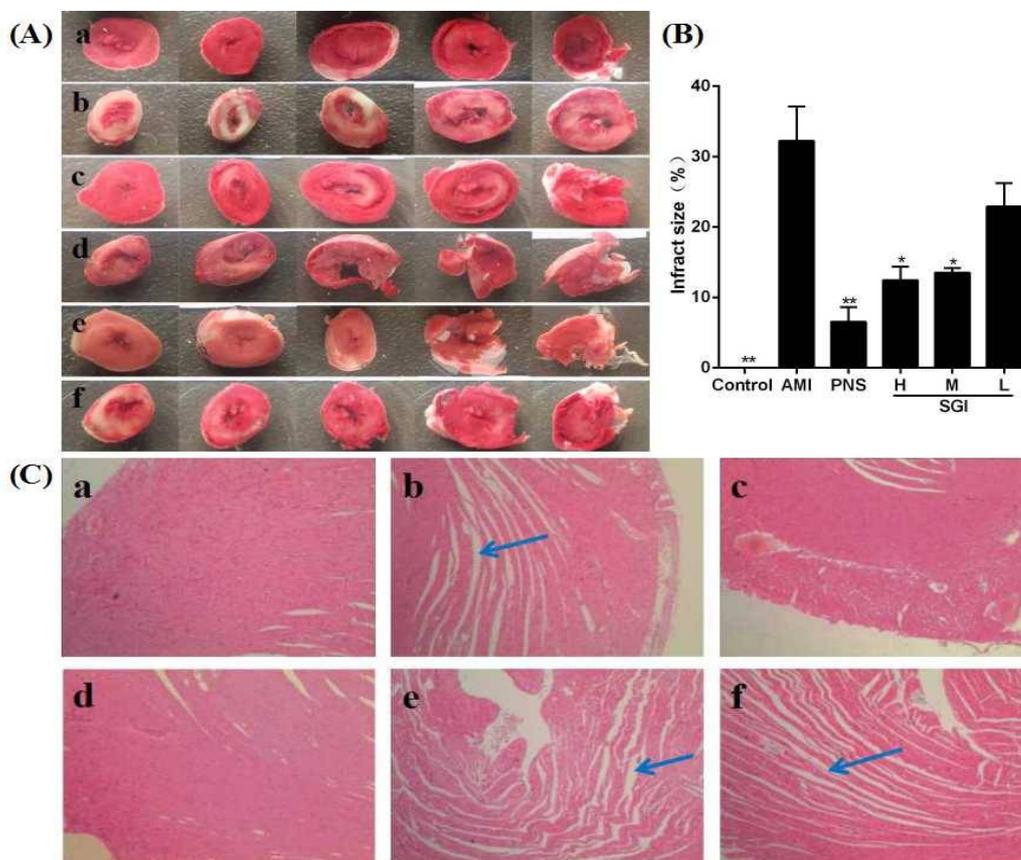
Figure 2: Effect of SGI on infarct size and microscopic observation of cardiac tissue. The myocardial infarct size was measured by TTC staining (A). Quantitative analysis the myocardial infarct size of the AMI injury rats (B). Myocardium stained with H&E to observe myocardial injury (C) (×100). Compared with AMI group; $^{*}p < 0.05$, $^{**}p <$ 0.01, (n ≥ 4). The letters a to f are represented as describe above

Table 2: Effect of SGI on Ca^{2+}-Mg^{2+}-ATPase, Na^+-K^+-ATPase activities and *CaM* , *CaMK II* mRNA relative expression in AMI injury rats

Group	Na^+-K^+-ATPase (µmolPi/mgprot/h)	Ca^{2+}-Mg^{2+}-ATPase (µmolPi/mgprot/h)	*CaM*	*CaMK II*
Control	1.93±0.17**	2.01±0.14**	0.29±0.02**	0.66±0.03**
AMI	0.89±0.17	1.14±0.09	1.00±0.24	1.00±0.03
PNS	1.69±0.14**	1.85±0.16**	0.35±0.09**	0.80±0.01*
SGI(H)	1.66±0.13**	1.84±0.22**	0.35±0.02**	0.80±0.17*
SGI(M)	1.38±0.14**	1.57±0.24*	0.65±0.03**	1.00±0.08
SGI(L)	1.33±0.09**	1.54±0.12*	0.75±0.07**	1.01±0.02

Compared with AMI group, $^{*}p < 0.05$, $^{**}p < 0.01$, (n = 4)

DISCUSSION

SGI, consisted of two effective ingredients ligustrazine hydrochloride and tanshinol, has shown multiple effects on cerebral disease, but the cardioprotective effect and mechanisms of SGI has been rarely reported [8]. The objective of our present study is to examine whether SGI plays a protective role in AMI injury and explore its potential mechanisms.

ECG and the content of CK, cTnI and BNP have been widely used as biomarkers of myocardial injury. Consistent with the previous studies [11],
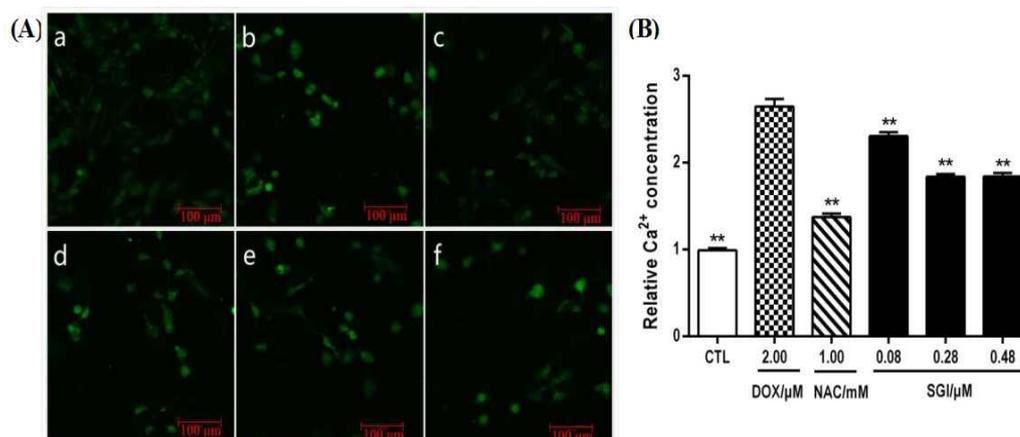
Figure 3: Treatment of SGI inhibited DOX-induced $(Ca^{2+})i$ overload. $(Ca^{2+})i$ was determined with Fluo-3-AM probe by LSCM (scale bar: 100 µm) (A). Quantitative analysis of relative $(Ca^{2+})i$ concentration (B). Compared with DOX group, $^*p < 0.05$, $^{**}p < 0.01$. a: Control group, b: DOX group, c: 1 mmol/L NAC group, d: 0.08 µmol/L SGI group, e: 0.28 µmol/L SGI group, f: 0.48 µmol/L SGI group

we observed significant increase of CK, cTnI and BNP content and elevation of typical ECG ST segment in rats subjected to acute myocardial infarction. The pretreatment of SGI can decline the high level of CK, cTnI, BNP and the elevation of ECG ST segment. Meanwhile, we also found that the area at risk was significantly reduced and myocardial structure was better-preserved in SGI pretreatment rats. All in all, these results have demonstrated the cardioprotective effects of SGI.

As an important second messenger, Ca^{2+} signal is a leading mediator in the management of many physiological and cellular activities, such as muscle contraction, metabolism, cell necrosis, apoptosis and autophagy and so on [13,14]. In ischemic and infarcted myocardium, the deficiency of ATP triggers the lower activity of Ca^{2+}-Mg^{2+}-ATPase and Na^+-K^+-ATPase, which induces Na^+ overload and secondary Ca^{2+} influx. $(Ca^{2+})i$ overload can cause arrhythmia and necrosis during myocardial infarction [15]. The overwhelming $(Ca^{2+})i$ combines with CaM and further activates CaMK II, which can regulate Ca^{2+} concentration by changing the function of ryanodine receptor (RyR), phospholamban (PLB) and L type calcium channel (LTCC) [16,17]. On the one hand, CaMK II can improve the $(Ca^{2+})i$ concentration by prolonging the opening time of RyR and LTCC. On the other hand, it can phosphorylate PLB, further relieve the inhibition on Ca^{2+}-ATPase and enhance the Ca^{2+} reuptake of the sarcoplasmic reticulum (SR). As outlined above, CaMK II, as a significant regulator of $(Ca^{2+})i$, has been confirmed as a therapeutic target in new drug research and development, and studies have demonstrated that blocking CaMK II with inhibitor KN93 can significantly diminish ischemic contracture *in vivo*, prevent cardiomyocyte mortality, and make SR Ca^{2+}

release lower to ameliorate myocardial infarct size in ischemia-reperfusion rats [18,19]. The overload of $(Ca^{2+})i$ concentration also causes the dysfunction of mitochondrial, increases concentration of oxygen free radicals and leads to myocardial cell death. Furthermore, clinical AMI cause sympathetic activation and catecholamine levels to rise, while catecholamine can increase the influx of Ca^{2+} through PKA pathway by activating α and β receptors [20,21].

Some studies have demonstrated that Danshen and its components have effects on calcium channels in ventricular myocytes and coronary artery [22,23]. Similarly, ligustrazine, defined as a calcium antagonist, not only will it block the entry of extracellular calcium but it will also inhibit the release of intracellular calcium. Moreover, it can also open potassium channel to make calcium influx lower [24,25]. According to the results, we found that SGI can reduce DOX-induced H9c2 cells damage by decreasing Ca^{2+} overload *in vitro*. In order to verify such effect *in vivo*, we investigated its myocardial protective effect in the left anterior descending coronary artery occlusion-induced AMI injury experiment. The results shown that SGI significantly improved the activities of Ca^{2+} related enzymes and decreased the mRNA expression of *CaM* and *CaMK II* in a dose-dependent manner.

The results indicate that SGI might protect cardiomyocytes from AMI injury by decreasing $(Ca^{2+})i$ overload through inhibiting CaM/CaMK II pathway and improving the activities of Ca^{2+}-Mg^{2+}-ATPase and Na^+-K^+-ATPase.

CONCLUSION

SGI protects cardiomyocytes against AMI by decreasing the infarction size, ameliorating

myocardial histopathological damage, improving the activities of Ca^{2+}-Mg^{2+}-ATPase and Na^+-K^+-ATPase, and reducing $(Ca^{2+})i$ overload through inhibition of CaM/CaMK II pathway, This suggests that SGI may be a new clinical candidate for the therapy of myocardial infarction.

DECLARATIONS

Acknowledgement

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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