Effects of phytoestrogen genistein on myocardial ischemia/reperfusion injury and apoptosis in rabbits

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ABSTRACT

AIM: To study the effect of genistein (GST) on rabbit heart ischemia/reperfusion (I/R) injury. **METHODS:** Rabbit heart I/R injury was induced by occluding the left anterior descending coronary artery for 45 min and reperfusing for 180 min. GST (1.0 mg/kg) was intravenously injected 5 min before heart ischemia. Hemodynamic data, infarct size, and cardiomyocytic apoptosis were measured. The pathologic changes of I/R myocardium were observed. **RESULTS:** During the I/R, heart rate, mean arterial blood pressure, myocardial oxygen consumption, left ventricular (LV) $-\mathrm{d}p/\mathrm{d}t_{max}$ and $+\mathrm{d}p/\mathrm{d}t_{max}$ were decreased progressively. The infarct size was occupied 60.23 %±3.97 % (% of area at risk) in vehicle +I/R group while GST reduced the infarct size to 39.62 %±4.30 % ($P<0.01$). DNA ladder patter in myocardium was revealed by agarose gel electrophoresis in vehicle +I/R group while was not found in GST+I/R group. Apoptotic cardiomyocytes were sparse within ischemic myocardium at risk in GST+I/R group as compared with that in vehicle +I/R group (TUNEL stain). Apoptosis rate in ischemic myocardium from vehicle +I/R and GST+I/R groups detected by flow cytometry were 15.33 %±1.31 % and 3.88 %±0.33 %, respectively. Fas and Bax protein expressions in ischemic myocardium of vehicle +I/R group were higher than that in GST+I/R group ($P<0.01$). Bcl-2/Bax ratio in vehicle +I/R group was lower than that in nonischemic myocardium ($P<0.01$), while in GST+I/R group, the Bcl-2/Bax ratio was higher than that in vehicle +I/R group ($P<0.01$). **CONCLUSION:** GST reduced infarct size and apoptosis of myocytes in I/R rabbit heart.

INTRODUCTION

Ischemia/reperfusion (I/R) injury is often seen in clinics which pathogenesis has not been elucidated clearly. It is generally believed that its mechanism is related to lipid peroxide induced by oxygen free radicals and to irreversible damage caused by intracellular calcium overload. The Ca^{2+} overload is induced mainly by the action of sarcolemmal Na^+/H^+ and Na^+/Ca^{2+} exchangers[1,2], which then activates several intracellular process, resulting in myocyte death[3-5].

Genistein (GST), a naturally occurring plant estrogen, mainly derived from soybean. This compound is in fact estrogen agonist for bone, liver and the cardiovascular system but does not appear to have agonist effects on the female reproductive system[6-8]. It was already demonstrated that GST had a hypocholesterolemic effect in animals and humans and was able to inhibit LDL oxidation, endothelial cell proliferation and angiogenesis[9], and to enhance the dilator response to acetylcholine of atherosclerotic arteries[10], all effects may predict a favorable impact on the cardiovascular system. Furthermore, GST was able to inhibit L-type Ca^{2+} channel[11]. These beneficial actions of GST suggest its important

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value theoretically and practically.

Several lines of evidences showed that cardiomyocyte apoptosis played a significant role in myocardial damage induced by I/R or permanent ischemia, as well as in end-stage heart failure[12-14]. Here we aimed to study the influence of GST on I/R injury of rabbit heart so as to further understand its cardiovascular effect.

MATERIALS AND METHODS

Experimental materials Adult male New Zealand white rabbits were provided by the Experimental Animal Center of Hebei Province (grade II, certificated No 04037). GST was purchased from Sigma. The antibodies for immunofluorescence detection of Fas, Bcl-2, and Bax protein were as follow: 1) Monoclonal antibodies Bcl-2 (C-2, Santa Cruz, US, working concentration 1:100) and Bax (B-9, Santa Cruz, US, working concentration 1:100) are mouse against rabbit protein; 2) Polyclonal antibody Fas is mouse against rabbit protein (M-20, Santa Cruz, US, working concentration 1:50); 3) The second antibody is used with FITC-conjugated goat anti-mouse/anti-rabbit IgG (Tackson immunoresearch laboratories Inc, code number 115-095-003/115095, working concentration 1:100).

General surgical preparation Male rabbits, weighting 2.0-3.0 kg, were used in this study. The rabbits were anesthetized via intravenously administration of urethane (1 g/kg) and placed on a heated operating table. A tracheotomy was performed. A polyethylene catheter was inserted into the right common carotid artery for the measurement of arterial blood pressure (ABP) with a pressure transducer (MPU-0.5) and a carrier amplifier (AP-620G) as reported previously[15]. A left thoracotomy was performed from the second to fifth intercostals space, and the pericardium was opened to expose the heart. A 3.0 silk ligature was placed under the left anterior descending coronary artery. The ends of the suture were threaded through a polyethylene tube to form a snare. Pull the ends of the suture taut and clamp the snare with a hemostat to occlude the coronary artery. Coronary artery occlusion was verified by epicardial cyanosis and subsequent decrease in arterial blood pressure. Reperfusion of the heart was initiated via unclamping the hemostat and loosening the snare and was confirmed by visualizing an epicardial hyperemic response. In addition, the apex of heart was canalized to measure the left ventricular $\pm dp/dt_{max}$ and heart rate (HR) derived from the left ventricular pressure signal by a differentiator (ED-600G). Experiment

was allowed to stabilize for 30 min before the following protocols were initiated.

Experimental protocol and groups Rabbits were divided randomly into 4 groups: I, sham operation control group-the animals underwent all the previously described procedures apart from the fact that the suture passing around the left coronary was not tied; II, I/R model group-the animals underwent a 45-min coronary artery occlusion and 180-min reperfusion period; III, GST+I/R group-the animals received genistein (1.0 mg/kg iv) 5 min before the coronary occlusion; IV, vehicle +I/R group-the animals received vehicle of GST (saline containing 2.5 % dimethylsulfoxide) 5 min before the coronary occlusion.

Determination of infarct size On completion of the above protocols, the coronary artery was reoccluded and the heart was perfused with 3 mL of Evans blue to delineate myocardial area at risk. The atria, right ventricle and great vessels were removed before the left ventricle was sectioned into 3 mm slices from the apex to the base. The slices were incubated in 1 % nitro-blue tetrazolium chloride (NBT, pH 7.4) for 15 min (37 ℃), which stains vital tissue to blue but leaves infarct tissue unstained. Finally the different areas of the ventricle were weighed separately. Myocardial ischemic area (area at risk) was expressed as the percentage of the left ventricle. Infarct size was expressed as the percentage of the area at risk.

DNA extraction and gel electrophoresis Myocardial specimens from ischemic and nonischemic regions (100 mg) were used for DNA extraction. Myocardial DNA was extracted as Itoh *et al*[16]. Identical-size aliquots of DNA were loaded on 2 % agarose gel containing ethidium bromide (0.5 μg/mL) in TAE buffer (40 mmol/L TRIS-acetate, 2 mmol/L EDTA, pH 8.0). Electrophoresis was conducted at 100 V in a flatbed gel apparatus.

TdT-mediated *in situ* nick end labeling (TUNEL) To evaluate apoptotic activity, the TdT-mediated *in situ* nick end labeling (TUNEL) method was used. Each section was deparaffinized and rehydrated with serial changes of xylene and ethanol. Proteinase K (20 mg/L) was applied to the section for 15 min with the intention of producing optimal proteolysis. The endogenous peroxidase was inhibited with 3 % hydrogen peroxide for 5 min. A commercial apoptosis detection kit (*In Situ* Cell Apoptosis Detection Kit I, POD) was used. The TdT reaction was carried out for 1 h at 37 ℃, and then antidigoxigenin-peroxidase was applied at room tem-

perature for 30 min. Hematoxylin-Eosin was used as a counterstain.

Determination of apoptosis with flow cytometric measurements Ischemic and nonischemic cardiac myocytes were collected and fixed in 70 % ethanol at 4 ℃ overnight. Cell suspension (1×10^9 cells/L) were centrifuged (5 min, 1000 r/min) and washed twice with 0.9 % NaCl solution. After centrifugation the cells were stained in one million of propidium iodide (PI) solution (PI 50 mg/L with Triton-X-100 and RNase) for 30 min and filtered through a 47-μm nylon mesh to remove cellular fragment and cluster. Chicken red blood cells were added to the sample before staining as an internal standard for calibration of the Flow Cytometry (FCM) instrument.

The sample fluorescence staining was performed using indirect immufluoresence labling method. Each sample (1×10^9 cells/L) was washed twice with PBS and incubated in 37 ℃ water-bath with 100 μL antibody (Fas, Bcl-2 and Bax) for 30 min. The samples were then washed twice with PBS and incubated in 37 ℃ water-bath with 100 μL of the second antibody of FITC-conjugated goat anti-mouse/rabbit IgG for 30 min. The cell suspension was washed, resuspended in 1.0 mL PBS, filtered through 47-μm nylon mesh, analyzed by flow cytometry.

Three control samples were used. One sample was used as negative control with PBS replacing the first/second antibody. One sample was used as positive isotype control with only first antibody incubated. The other sample was incubated only with the second fluorescence antibody as positive isotype control.

The stained samples were analyzed in a FACS 420 flow cytometer (FACS 420 Fluorescence Activated Cell Sorting, Becton Dickinson, Sunnyvale, California, USA). Single parameter was measured respectively in DNA (with a liner mode) and each protein (with a lg mode). Usually, 1×10^4 cells for each sample were analyzed. The analytic data were processed with a HP-300 consort 300 computer. The coefficient of variation (CV) of the instrument was adjusted within 5 % using PI staining chicken red blood cell. Fluorescence index (FI, FI=(average fluorescence intensity of sample protein expression-average fluorescence intensity of isotype control)/average fluorescence intensity of normal control was used to describe Fas, Bcl-2 and Bax protein expression. The sample was considered positive when FI is above 1.0.

Statistical analysis All data were expressed as mean±SD. Statistical analysis was carried out by analysis of variance. Unpaired *t*-test was used to assess statistical significance of differences between GST and various related control groups. $P < 0.05$ was considered to be significant.

RESULTS

Hemodynamics The hemodynamic changes were summarized in Tab 1. During 45 min of myocardial ischemia and 180 min of reperfusion, MAP, HR and myocardial oxygen consumption (Rate pressure product) decreased steadily in all the groups and there are no significant differences among them. Compared with sham group, the LV $+dp/dt_{max}$ 45 min after ischemia and 180 min after reperfusion were lowered by 44.9 % and 35.8 %, while LV $-dp/dt_{max}$ lowered by 27.4 % and 24.6 %. Compared with vehicle +I/R group, administration of GST before ischemia increased LV $+dp/dt_{max}$ at 45 min after ischemia and 180 min after reperfusion by 39.5 % and 22.9 %, and augmented LV $-dp/dt_{max}$ by 17.5 % and 16.7 %, respectively (Tab 1).

Myocardial area at risk and infarct size The myocardial ischemic area (area at risk) showed no difference among I/R, vehicle +I/R and GST+I/R groups. The myocardial infarct size (% of area at risk) also showed no difference between I/R group and vehicle +I/R group, implying vehicle had no effect on this I/R injury. The infarct size (% of area at risk) in vehicle +I/R group was 60.23 %±3.97 %, while that in GST+I/R group was significantly reduced to 39.62 %±4.30 % ($P < 0.01$) (Fig 1).

DNA agarose gel electrophoresis No ladder pattern of DNA was observed in the nonischemic myocardium. In contrast, DNA ladder was observed in the ischemic myocardium of vehicle +I/R group and it was less apparent in GST+I/R group (Fig 2). Each lane contains a same fraction of DNA extracted from identical amount of myocardium.

TUNEL stains TUNEL-positive cells showed typical apoptosis. The cell size was obviously reduced, cytoplasm shrinked but plasma membrane was integral. The nuclei were pyknosis and marginated to the periphery of cell membrane which indicated condensation of chromatin. In vehicle +I/R group, TUNEL-positive cells were found more frequently in the area at risk (Fig 3), but in the GST+I/R group, the TUNEL-positive cells were significantly decreased.

Determination of apoptosis rate with flow cytometry The quantitative assessment of sub-G_1 cells

Tab 1. Effect of GST on HR, MAP, myocardial oxygen consumption and $\pm \mathrm{d}p/\mathrm{d}t_{max}$ in 4 groups of rabbits. *n*=12. Mean±SD. $^bP <0.05$, $^cP <0.01$ *vs* sham group; $^eP <0.05$, $^fP <0.01$ *vs* vehicle +I/R group.

Group	Before ischemia	Ischmia (45 min)	Reperfusion (180 min)
		Heart rate/bpm	
Sham	258±20	260±18	257±15
I/R	283±15	265±14	257±19
Vehicle+I/R	279±18	264±14	267±15
GST+I/R	282±23	264±20	263±23
		MAP/kPa	
Sham	12.9±1.1	12.9±1.0	12.8±0.8
I/R	13.3±1.5	10.4±1.1^b	10.4±1.1^b
Vehicle+I/R	12.7±1.0	10.1±1.0^b	10.0±0.8^b
GST+I/R	13.6±1.5	10.5±1.3^b	10.6±1.1^b
		Rate pressure product/kPa·min^{-1}	
Sham	3328.2±472.3	3354.0±463.3	3289.6±428.2
I/R	3763.9±625.4	2756.0±553.2^b	2672.8±562.3^b
Vehicle+I/R	3543.3±421.2	2666.4±512.3^b	2670.0±517.2^b
GST+I/R	3835.2±562.3	2772.0±557.4^b	2787.8±653.4^b
		LV $+\mathrm{d}p/\mathrm{d}t_{max}$/kPa·s^{-1}	
Sham	565.2±99.2	546.8±81.2	538.1±76.0
I/R	591.1±99.4	318.2±76.1^c	351.1±83.2^c
Vehicle+I/R	498.3±48.8	301.0±48.7^c	345.7±69.8^c
GST+I/R	548.6±91.7	497.5±94.3^f	448.2±80.4^f
		LV $-\mathrm{d}p/\mathrm{d}t_{max}$/kPa·s^{-1}	
Sham	474.8±92.3	462.3±82.9	431.2±97.2
I/R	460.6±85.3	360.3±79.5^b	321.8±91.3^b
Vehicle+I/R	478.8±46.0	335.4±34.9^c	325.0±53.1^b
GST+I/R	446.4±90.7	406.5±76.0^f	390.3±44.0^e

by flow cytometry was used to estimate the number of apoptotic cells. The apoptosis rate was 15.33 %±1.31 % in vehicle +I/R group and 3.88 %±0.33 % in GST+I/R group. There was significantly difference between them (*P*<0.01), while in sham group the apoptosis rate was 2.48 %±0.17 % (Fig 4).

Determination of Fas, Bcl-2 and Bax protein expression with flow cytometry Tab 2 showed that FI values of Fas and Bax protein expressions in ischemic myocardium of vehicle +I/R group were increased. Bcl-2/Bax ratio was increased in GST +I/R group (*P*<0.01).

DISCUSSION

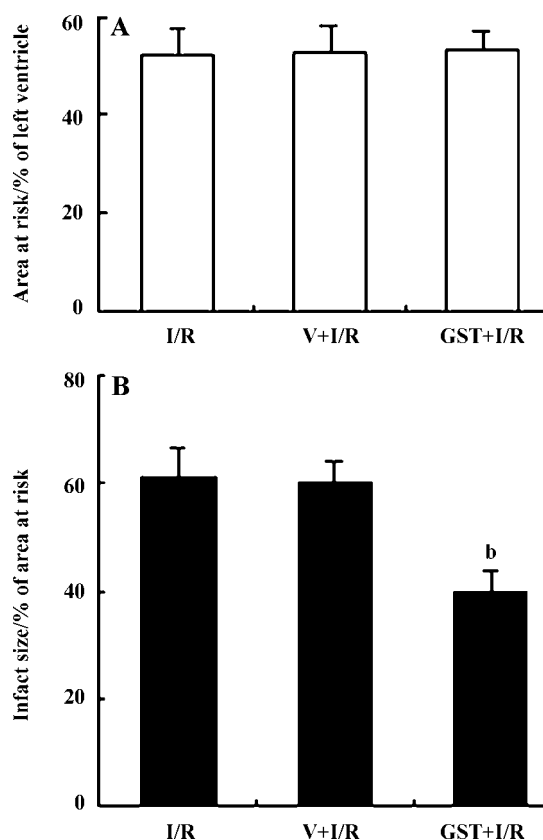Experimental and clinical evidences suggest that

Fig 1. Changes of myocardial area at risk and infarct size in anesthetized rabbits. A) Ischemic myocardium (area at risk) as a percentage of the left ventricle; B) Necrotic myocardium (infarct size) as a percentage of area at risk. I/R: I/R group; V+I/R: vehicle +I/R; GST+I/R: genistein +I/R. $^cP<0.01$ *vs* vehicle +I/R group.

Tab 2. Changes of Fas, Bcl-2 and Bax protein expression in ventricular myocardium. *n*=6. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* sham group. $^eP<0.05$, $^fP<0.01$ *vs* vehicle + I/R.

Group	Fluorescence index Fas	Bcl-2	Bax	Bcl-2/Bax
Sham	1.00±0.05	1.00±0.03	1.00±0.11	1.00±0.06
Vehicle+I/R	1.21±0.06^c	0.86±0.06^b	1.63±0.16^c	0.53±0.08^c
I/R	1.23±0.08^c	0.87±0.03^b	1.61±0.11^c	0.54±0.05^c
GST+I/R	0.97±0.04^f	1.07±0.12^e	1.02±0.12^f	1.05±0.10^f

brief coronary occlusion followed by reperfusion leads to reversible myocardial dysfunction[17], whereas cardiomyocyte death during I/R is partially mediated by apoptosis[18-20]. In the present study, we have demonstrated that GST pretreatment before 5 min of coronary occlusion followed by 180 min reperfusion reduced myocardial infarct size combined with the reduction of
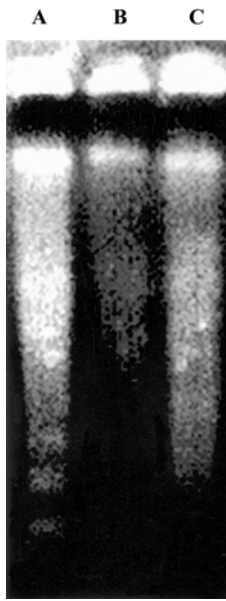
Fig 2. DNA electrophoresis in agarose gel from rabbit ventricular myocardium. A) Ischemic myocardium following vehicle +I/R with ladder pattern of DNA; B) Non-ischemic myocardium following I/R; C) Ischemic myocardium following GST (1.0 mg/kg).

Fas, Bax expression and increased Bcl-2/Bax ratio and decreased apoptosis ratio in rabbit hearts. Our findings suggest that GST attenuated the ischemia/reperfusion injury. This result is partially in agreement with previous *in vivo* experiment showing that GST reduced the infarct size[21]. Our data also showed that cardioprotection by GST pretreatment was also accompanied by the improvement of left ventricular function. Although the

precise mechanisms of myocardial injury following ischemia with reperfusion are not fully understood, experimental studies have led to the idea that oxygen free radical formation, calcium overload, neutrophil-mediated myocardial and endothelial injury, progressive decline in microvascular flow to the reperfused myocardium and depletion of high energy phosphate stores might be involved[22,23]. GST, as the principal isoflavone found in soy, inhibited the myocardial L-type calcium channel[24]. So we hypothesize that the mechanisms of action of GST are probably associated with blockade of Ca^{2+} influx, and inhibition of Fas-Fas ligands, which are capable of reducing Ca^{2+}-overload in cytoplasm evoked by I/R[25], maintaining balance between intra- and extra-cellular calcium, remaining vasodilatation and stability of mitochondria[26], and finally, leading to decrease cardiomyocyte apoptosis.

The present findings are in disagreement with previous reports showing that GST, by inhibiting also tyrosine kinase attenuates or abolishes the cardioprotective effects of ischemic preconditioning[27,28]. The cause responsible for this discrepancy may be related to the dose of GST used. The dose of GST used in their experiments is higher (5 mg/kg) than that in our experiment. Under our experimental conditions, the lower dose of GST (1.0 mg/kg) was used which caused a marked reduction in infarct size. It was reported that GST behaves as a tyrosine kinase inhibitor only at higher doses while at lower doses it exerts estrogen-like activity[29].

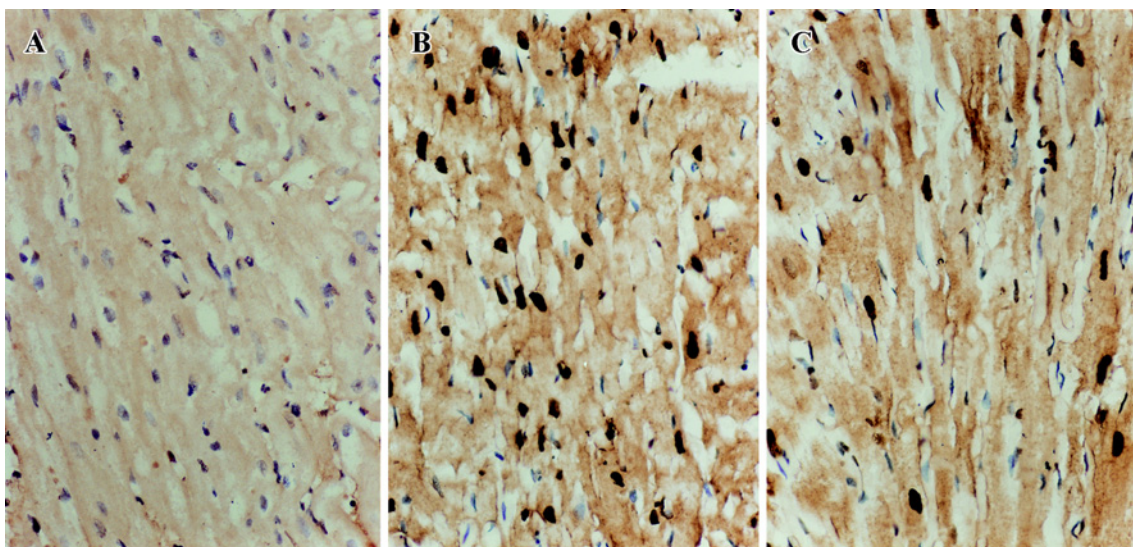Increasing evidence suggests that lethal reperfusion injury possibly consists of two forms of cell death, ne-

Fig 3. Staining of ventricular myocardium by TUNEL in rabbit (× 400). A) non-ischemic myocardium; B) ischemic myocardium in vehicle +I/R group (numerous apoptotic cells); C) ischemic myocardium in GST+I/R group (sparse apoptosis cells).
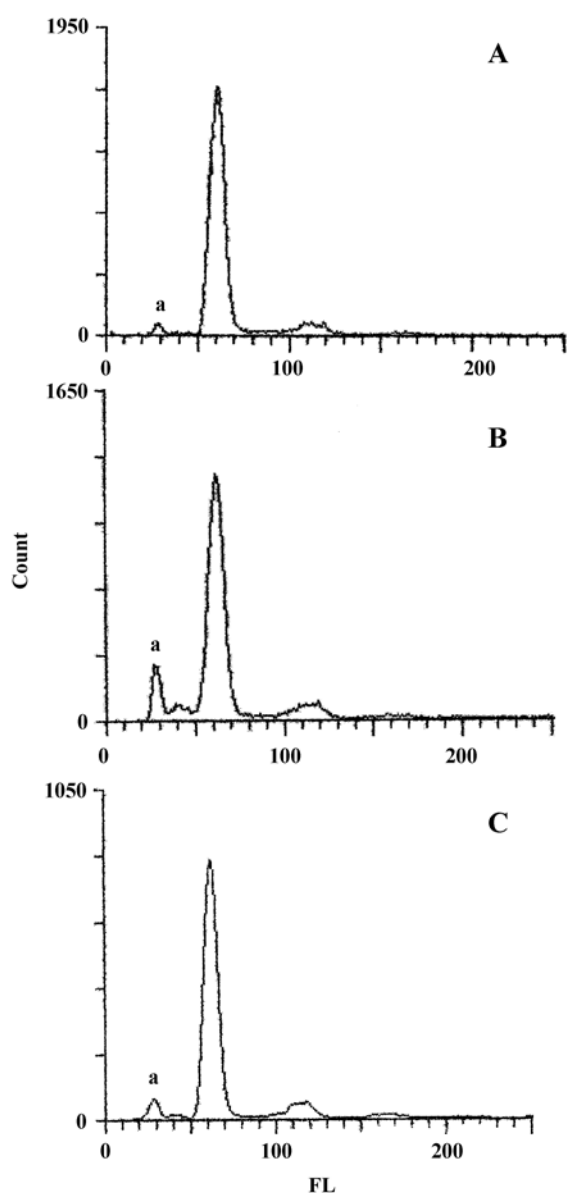
Fig 4. Measurement of cardiomyocytic apoptosis rate by flow cytometry. A) non-ischemic myocardium; B) ischemic myocardium in vehicle +I/R group with a large apoptosis peak; C) ischemic myocardium in GST+I/R group with a small apoptosis peak. a: apoptosis peak.

crosis and apoptosis (programmed cell death). The apoptotic process is initiated shortly after the onset of ischemia, and becomes markedly enhanced during reperfusion. Inhibition of the apoptotic process should then attenuate the irreversible injury in connection with reperfusion. Fas ligand, a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas and induced Fas protein overexpression, thus inducing apoptosis[30]. Yue *et al* reported that I/R induced myocardial apoptosis in rabbit, and accompanied with Fas protein overexpression[31]. Oltvai *et al*

reported[32] that Bax, that has extensive amino acid homology with Bcl-2, overexpression accelerated apoptotic death. Overexpressed Bax also counters the death repressor activity of Bcl-2, while the ratio of Bcl-2 to Bax determines survival or death following on apoptotic stimulus. Our present study showed that Fas and Bax protein expression were increased, while the ratio of Bcl-2 to Bax was decreased in I/R and vehicle+I/R groups. GST reduced the Fas, Bax protein expressions and increased the Bcl–2/Bax ratio in I/R model, implying that GST protected against myocardial I/R injury by inhibiting the apoptosis.

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