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Cardioprotective effects of low-dose combination therapy with rosuvastatin and fasudil in the isolated rat heart

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The cardiovascular pleiotropic effects of statins and a Rho-kinase inhibitor (fasudil) could be of interest to prevent myocardial ischemia reperfusion injury (MIRI). In the present study, we investigated whether low-dose rosuvastatin and fasudil, separately not possessing cardioprotection, express cardioprotective effects when combined. The isolated rat hearts underwent 30 min global ischemia and 120 min reperfusion. Rosuvastatin (3 μM) and fasudil (1 μM) were administered 15 min before ischemia. NG-nitro-L-arginine methylester (30 μM) (L-NAME) was given at the onset of reperfusion. Myocardial infarct size, apoptosis, myocardial nitric oxide (NO) content and endothelial nitric oxide synthase (eNOS) expression were evaluated. The combination treatment significantly decreased infarct size and percentage of apoptosis and increased the content of NO and eNOS expression, whereas treatment with rosuvastatin and fasudil alone at the same doses did not lead to cardioprotection. Furthermore, L-NAME reversed the cardioprotective effect of rosuvastatin/fasudil combination treatment. In summary, rosuvastatin combined with fasudil treatment had synergistic protective effects against MIRI, which were mediated by increasing eNOS and NO production. This new concept could be valuable in MIRI prevention.

1. Introduction

Rho-kinase, a serine/threonine protein kinase regulating multiple cellular functions, plays key roles in cardiovascular diseases, including ischemic heart disease, atherosclerosis, myocardial hypertrophy and heart failure (Fukumoto et al. 2007; Wu et al. 2009; Wang et al. 2011; Ho et al. 2012). Furthermore, recent studies have also reported that Rho-kinase is activated in ischemic myocardium, thus inhibition of Rho-kinase activity is a new strategy for preventing myocardial ischemia reperfusion injury (MIRI) (Hamid et al. 2007; Satoh et al. 2011).

The statins, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitors, have been demonstrated to decrease morbidity and mortality in patients who suffered from coronary artery disease through lowering their cholesterol level (Gould et al. 2007). Moreover, several studies have reported that statins can exert beneficial cardiovascular effects independent of cholesterol levels, such as decreasing oxidative stress, inflammation, inhibition of thrombogenic response, and atherosclerotic plaque formation, which is termed pleiotropic effects of statins (Inoue and Node 2007; Mihos and Santana 2011). One of the mechanism behind these beneficial effects has been suggested to be in association with nitric oxide (NO) production and release (Lefer et al. 2001; Kalinowski et al. 2002). Statins exert cardioprotection against MIRI by preventing activation of RhoA, an upstream regulator of Rho-kinase (Bulhak et al. 2007). Therefore, we hypothesize that statins might have synergistic cardioprotective effects for inhibition of Rho-kinase activity in the ischemic heart.

Based on this hypothesis, in this study we investigated the effects of combination therapy with a statin (rosuvastatin) and a Rho-kinase inhibitor (fasudil) using a low-dose of both agents, which might not be effective on MIRI in an isolated rat heart model. Furthermore, we tested the roles of NO and endothelial nitric oxide synthase (eNOS) in the synergistic cardioprotection of rosuvastatin/fasudil combination treatment.

2. Investigations and results

2.1. Effect on the left ventricular function in the isolated rat hearts

As shown in the Table 1, the differences in hemodynamic parameters before ischemia amongst the groups were not statistically significant ($P > 0.05$). The HR, LVDP and ± dp/dt in the combined treatment (RVA + FD) group were significantly increased in comparison to the control group ($P < 0.05$), but The HR, LVDP and ± dp/dt in FD and RVA treated groups were not significantly increased ($P < 0.05$) in comparison to the control group.

2.2. Effect on the myocardial infarction size in the isolated rat hearts

As shown in Fig. 1, the RVA + FD treatment group significantly reduced the size of myocardial infarction compared to the control group (29.0 ± 5.2 % *vs.* 45.8 ± 5.7 %, $P < 0.05$), but the infarction sizes in FD and RVA treatment groups were not significantly reduced compared to the sizes in the control group (43.6 ± 9.6 % and 41.0 ± 10.3 % respectively *vs.* 45.8 ± 5.7%, $P > 0.05$).

Table 1: Hemodynamic parameters (HR, + dp/dt, -dp/dt, and LVDP) before and during reperfusion

TIME	baseline	R-10	R-30	R-120
HR				
Control	254 ± 18	152 ± 10	190 ± 8	151 ± 12
RVA	251 ± 17	149 ± 17	195 ± 12	154 ± 9
FD	253 ± 11	151 ± 9	193 ± 11	155 ± 10
FD + RVA	256 ± 19	169 ± 11*	216 ± 8*	176 ± 7*
FD + RVA + L-NAME	254 ± 15	155 ± 7	188 ± 11	157 ± 9
LVDP(mmHg)				
Control	86 ± 3.9	30 ± 4.1	36 ± 4.0	31 ± 2.4
RVA	85 ± 6.6	31 ± 5.4	33 ± 3.1	32 ± 3.7
FD	85 ± 5.5	34 ± 3.3	40 ± 3.5	36 ± 3.6
FD + RVA	84 ± 8.0	47 ± 4.7*	53 ± 5.5*	48 ± 4.0*
FD + RVA + L-NAME	86 ± 5.4	32 ± 6.2	35 ± 4.5	33 ± 4.2
+ dp/dt(mmHg/s)				
Control	1993 ± 69	1200 ± 34	1341 ± 50	1159 ± 43
RVA	2008 ± 90	1210 ± 49	1335 ± 29	1213 ± 92
FD	1952 ± 77	1212 ± 46	1322 ± 45	1201 ± 51
FD + RVA	1986 ± 90	1311 ± 37*	1491 ± 29*	1308 ± 41*
FD + RVA + L-NAME	1992 ± 86	1189 ± 65	1292 ± 52	1149 ± 78
-dp/dt(mmHg/s)				
Control	1409 ± 36	1004 ± 53	1126 ± 40	980 ± 65
RVA	1424 ± 44	1019 ± 32	1140 ± 69	1002 ± 42
FD	1406 ± 79	1028 ± 43	1155 ± 33	1017 ± 40
FD + RVA	1417 ± 57	1147 ± 39*	1297 ± 47*	1196 ± 31*
FD + RVA + L-NAME	1413 ± 65	1023 ± 51	1148 ± 53	975 ± 58

RVA, rosuvastatin; FD, fasudil; + dp/dt, Positive first order derivative of ventricular pressure; -dp/dt, Negative first order derivative of ventricular pressure; LVDP, left ventricular developed pressure. Values are means ± SD.*$P < 0.05$ *vs*. Control (n = 10).

2.3. Effect on the myocardial apoptosis in rat myocardium

As shown in Fig. 2, the percentage of apoptosis was significantly reduced by RVA combined with FD treatment compared to the control group (28.8 ± 7.1 % vs. 43.9 ± 5.5 %, $P < 0.05$), but treated with RVA or FD alone did not reduce the percentage of apoptosis compared to the control group (39.1 ± 6.4% and 38.0 ± 5.7% respectively *vs*. 43.9 ± 5.5 %, $P > 0.05$).

2.4. Effects on NO content in rat myocardium

As shown in Fig. 3, the content of NO in rat myocardium was significantly increased in the RVA + FD treatment group compared to the control group (6.15 ± 1.60 μmol/g protein *vs*. 2.6 ± 0.78 μmol/g protein, $P < 0.05$), but the content of NO was not increased in the single treatment group (RVA group and FD group) (2.77 ± 1.20 μmol/g protein and 2.65 ± 2.31 μmol/g protein respectively *vs*. 2.6 ± 0.78 μmol/g protein, $P > 0.05$).

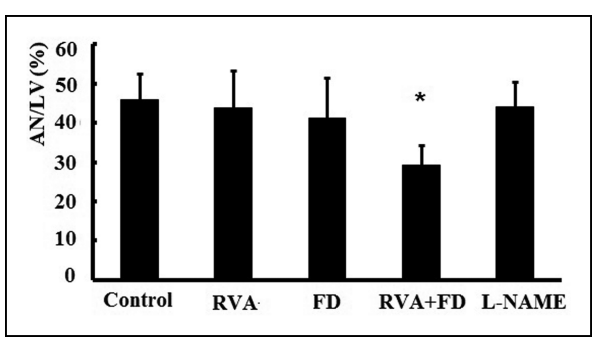

Fig. 1: Area of necrosis (AN) expressed as a percentage of left ventric (LV) area. RVA = 3 μM rosuvastatin was given 15 min before ischemia; FD = 1 μM fasudil was given 15 min before ischemia; L-NAME = 30 μM L-NAME was given at the onset of reperfusion. Data are presented as means ± SD and n = 6 for each. * $P < 0.05$ *vs*. control.

2.5. Effects on eNOS phosphorylation in rat myocardium

As shown in Fig. 4, no significant change in eNOS was observed in all groups. eNOS phosphorylation was also enhanced in RVA + FD group as compared to the control group (1.703 ± 0.162 *vs*. 0.951 ± 0.097, $P < 0.05$), whereas no significant difference in eNOS phosphorylation was shown in RVA group and FD group compared to the control group (0.921 ± 0.072 and 0.943 ± 0.076 respectively *vs*. 0.951 ± 0.097, $P > 0.05$).

2.6. Effects of L-NAME on the cardioprotection of RVA combined with FD treatment

To further assess the role of eNOS in RVA combined with FD treatment, we gave NOS specific inhibitor, L-NAME in RVA combined with FD treatment. The results showed that the cardioprotection of RVA combined with FD treatment was abrogated by treated with L-NAME, as evidenced by no significant differences in hemodynamic parameters, myocardial infarct size, the percentage of cardiomyocyte apoptosis and the content of NO were shown in L-NAME group as compared to the control group ($P > 0.05$).

3. Discussion

Recently, several studies have reported that statins could exert cardioprotection on the ischemic reperfusion myocardium, as evidenced by the fact that statins reduce infarct size in acute myocardial infarction or in an ischemia/reperfusion model (Di Napoli et al. 2001; Ikeda et al. 2003). Furthermore, some reports have demonstrated that statins exert cardioprotection independent of their cholesterol-lowering effects (Bulhak et al. 2005; Adameova et al. 2009). Therefore, in order to observe the direct effects on ischemic reperfusion myocardium, we selected an isolated rat heart model in our study, which is deprived of

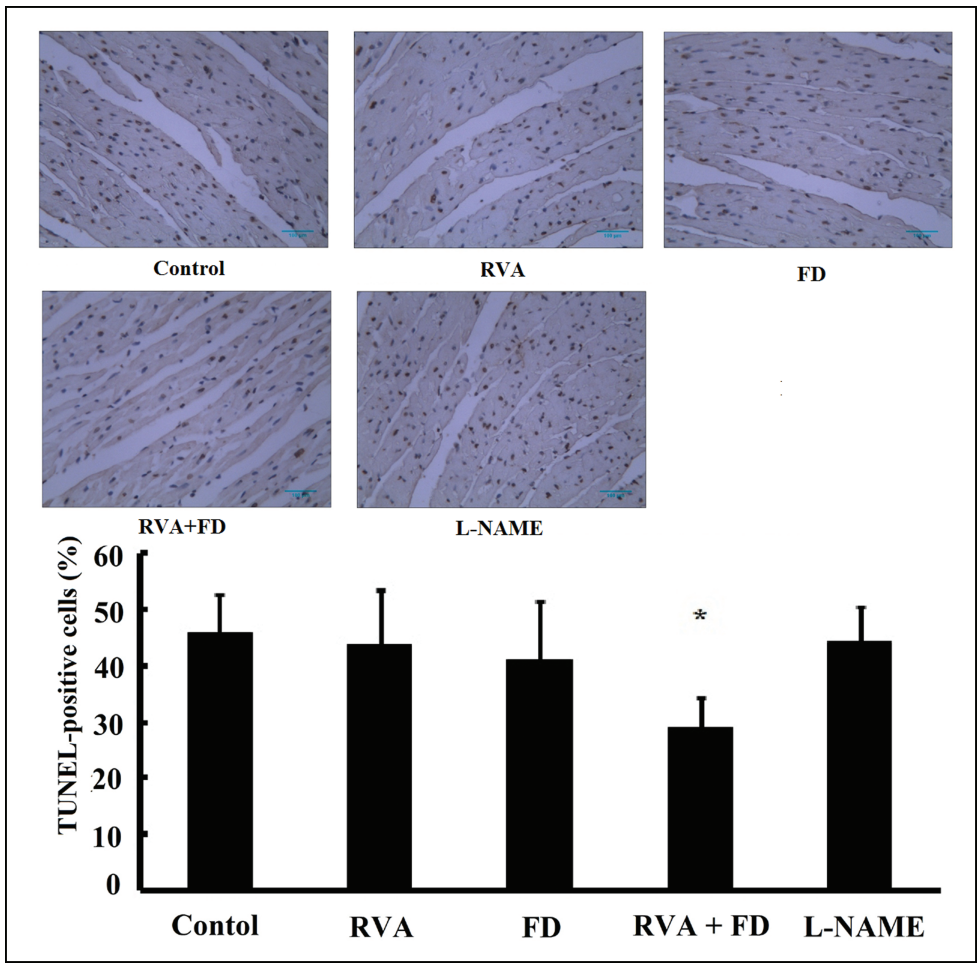

Fig. 2: Apoptosis after myocardial infarction. Apoptotic cardiomyocyte nuclei appear brown stained whereas terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-negative nuclei appear blue. Histogram shows the percentage of TUNEL-positive cells (brown staining). RVA = 3 μM rosuvastatin was given 15 min before ischemia; FD = 1 μM fasudil was given 15 min before ischemia; L-NAME = 30 μM L-NAME was given at the onset of reperfusion. Data are presented as means ± SD and n = 6 for each. * $P < 0.05$ *vs*. control.

neutrophil, humoral hormone or autonomic nervous system effects and is better for us to observe the direct influences on the ischemic reperfusion myocardium. Moreover, some reports have also shown that administration of fasudil can produce beneficial effects on multiple cardiovascular diseases and induce preconditioning or postconditioning against MIRI (Demiryürek et al. 2005; Ichinomiya et al. 2012; Li et al. 2012).

Although the cardioprotection of statin and fasudil on ischemic reperfusion myocardium have been demonstrated in several experiments, the combined effects of statins and fasudil on the cardiovascular system have not been reported yet. In order to explore whether rosuvastatin combined with fasudil has a synergistic cardioprotective effect in myocardial ischemia reperfusion, low doses of rosuvastatin and fasudil were used in our present study. The results of a preliminary study showed that administration of 3 μM rosuvastatin and 1 μM fasudil alone did not show any significant improvement in cardiac function and reduction in infarct size and apoptosis, suggesting that neither 3 μM rosuvastatin nor 1 μM fasudil produced cardioprotective effects on ischemic reperfusion myocardium. In the contrary, in our present study, we firstly showed that pretreatment with 3 μM rosuvastatin plus 1 μM fasudil significantly improved cardiac function and reduced infarct size and apoptosis, although treatment with the same doses of rosuvastatin or fasudil alone did not show such a cardioprotective effect. The result suggested that rosuvastatin combined with fasudil might exist synergistic cardioprotective effects in myocardial ischemia reperfusion.

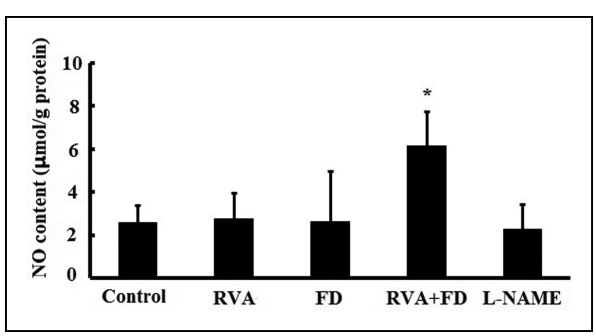

Fig. 3: Content of NO in myocardium. RVA = 3 μM rosuvastatin was given 15 min before ischemia; FD = 1 μM fasudil was given 15 min before ischemia; L-NAME = 30 μM L-NAME was given at the onset of reperfusion. Data are presented as means ± SD and n = 6 for each. * $P < 0.05$ *vs*. control.

NO is an important messenger in cardiovascular regulation and also plays an important role in protecting the myocardium against ischemia reperfusion injury. It was reported that L-arginine, a precursor of NO, improved post-ischemic functional recovery and limited infarct size in the isolated rat heart. Also, L-NAME abolished the effect of L-arginine on MIRI (Izhar et al. 1998; Suematsu et al. 2001). There were reports that both rosuvastatin and fasudil could increase the content of NO production in the myocardium (Di Napoli et al. 2005; Ma et al. 2011). However, in our present study, we found pretreatment with low dose rosuvastatin or fasudil alone confer any increase in myocardial NO production. In the contrary, rosuvastatin combined with fasudil significantly increased myocardial NO production, suggesting that the cardioprotective effects were mediated through increasing NO production.

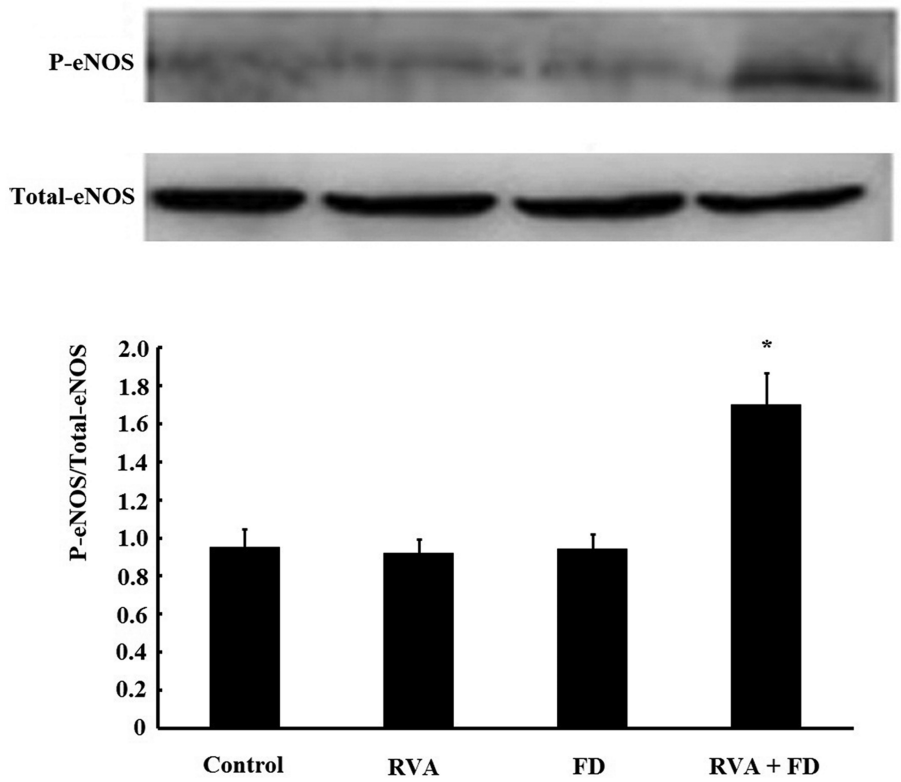

Fig. 4: Myocardial eNOS and P- eNOS expressions. RVA = 3 μM rosuvastatin was given 15 min before ischemia; FD = 1 μM fasudil was given 15 min before ischemia; L-NAME = 30 μM L-NAME was given at the onset of reperfusion. Data are presented as means ± SD and n = 6 for each. * $P < 0.05$ *vs*. control.

The main source of cardiac NO is generated through eNOS expressed by coronary endothelial cells and cardiac myocytes. Recent studies have shown that eNOS plays an important role in preventing MIRI (Bolli 2001; Brunner et al. 2003). Our experiment showed that eNOS phosphorylation expression was increased in the combination treatment (RVA + FD) group but were not increased in the single treatment group. Futhermore, L-NAME reversed the synergistic protective effect of statins combined with fasudil on heart. The results suggested that the increase in NO production resulted from an increase in eNOS activity.

In summary, rosuvastatin combined with fasudil had a synergistic cardioprotective effect against MIRI, which was mediated by an increased eNOS and NO production.

4. Experimental

4.1. Animals

A total of sixty healthy male Wistar rats, weighing 250-300 g, were supplied by the Department of Experimental Animals, The China Medical University. All animals used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH). The study protocol was approved by the institutional ethics committee.

4.2. Drugs

Fasudil and rosuvastatin were purchased from Dalian Meilun Biotech Co., Ltd. 2, 3, 5- triphenyl tetrazoliumchloride (TTC) and the nitric oxide synthase (NOS) inhibitor NG-nitro-L-arginine methylester (L-NAME) were purchased from Sigma (St. Louis, MO, USA).

4.3. Preparation of Langendorff perfusion model of isolated heart

The Wistar rats were anesthetized by intraperitoneal injection of 4 mg/kg 10% chloral hydrate and then heparinized by injecting 3 mg/ml heparin *via* the inferior vena cava. After one minute, the rats were fully heparinized and the heart was dissected and placed in KH solution [127 mmol/l NaCl, 17.7 mmol/l $NaHCO_3$, 5.1 mmol/l KCl, 1.5 mmol/l $CaCl_2$, 1.26 mmol/l $MgCl_2$, 11 mmol/l D-glucose) (pH 7.4)] at 4° C for trimming. The heart was hung on the Langendorff perfusion system. The whole procedure took two minutes and the temperature was controlled at 37° C. The KH solution was saturated with 95% O_2 and 5% CO_2. Further experiments were initiated at 20 min after the solution and heart were equilibrated. Perfusion was maintained at a constant pressure of 75 mmHg. The fluid-filled latex balloon was inserted in the left ventricle *via* the left atrium for pressure measurement. The balloon was connected to a pressure transducer and inflated to an initial LV end-diastolic pressure between 8 and 10 mmHg.

4.4. Experimental protocol

The sixty Wistar rats were randomly divided into five groups: (1) the control group (n = 12): ischemia for 30 min and reperfusion for 120 min without any use of drugs; (2) fasudil treatment group (FD group, n = 12): Perfusion of KH solution containing 1 μM fasudil for 15 min before ischemia, and then ischemia for 30 min and reperfusion for 120 min; (3) rosuvastatin treatment group (RVA group, n = 12): Perfusion of KH solution containing 3 μM rosuvastatin for 15 min before ischemia, and then ischemia for 30 min and reperfusion for 120 min; (4) Rosuvastatin combined with fasudil treatment group (RVA + FD group, n = 12): Perfusion of KH solution containing 3 μM rosuvastatin plus 1 μM fasudil for 15 min before ischemia, and then ischemia for 30 min and reperfusion for 120 min; (5) L-NAME treatment group (L-NAME group, n = 12): 30 μM L-NAME was given at onset of reperfusion, and administration of rosuvastatin and fasudil are the same as RVA + FD group.

4.5. Determination of hemodynamic parameters

The left ventricular cardiac function parameters including heart rate (HR), left ventricular developed pressure (LVDP), positive first order derivative of ventricular pressure (+dp/dt) and negative first order derivative of ventricular pressure (-dp/dt) were recorded using biological signal acquisition system (BIOPAC MP150, USA).

4.6. Determination of the size of myocardial infarction

After two hours' reperfusion, the heart was removed from the perfusion apparatus and placed in the –20 °C freezer for one hour. The frozen left ventricle was cut into six equal sections from the apex to the bottom, along the direction of the atrioventricular groove. The sections were placed in

1% TTC solution (TTC dissolved in pH7.8 Na_2HPO_4/$NaHPO_4$ buffer) for 10 min and then fixed in 10% formaldehyde for another 15 min. Red non-infarcted areas and gray-white infarcted areas were seen in the sections. The sections were scanned and the infarct area was calculated using image J analysis software.

4.7. Determination of myocardial apoptosis

At the end of two hours' reperfusion, the heart was removed as described earlier. Cardiomyocyte apoptosis was detected using an In Situ Cell Death Detection Kit (Roche, USA) according to the manufacturer's instructions. Briefly, the tissue sections were washed in PBS and then fixed in 4 % paraformaldehyde solution before incubation in 20 μg/ml proteinase K for 10 min. After washed in PBS for three times, the tissue sections were incubated with terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37 °C for 60 min for incorporation of biotinylated nucleotides at the 3 -OH DNA ends. The reaction was terminated by transferring the slides to 2 × sodium citrate saline solution. Endogenous peroxidase activity was quenched by incubation in 0.3 % hydrogen peroxide. Finally, streptavidin horseradish peroxidase was bound to the biotinylated nucleotides and peroxidase activity was demonstrated in each section by the application of a stable chromogen diaminobenzidine. In this technique, the apoptotic nuclei are stained dark brown. The sections were counter stained with hematoxylin for total nuclei. Three sections from each myocardial sample were randomly selected and 10 microscopic fields (Olympus BX51 microscope) per section were evaluated by two independent blind observers. In each field, the nuclei were counted and the percentage of TUNEL-positive nuclei was calculated.

4.8. Determination of myocardial NO content

After 30 min of global ischemia followed by 120 min of reperfusion, the hearts were removed quickly from the Langendorff apparatus and homogenized. The content of NO was measured using Nitric Oxide assay kit (Jiancheng, Nanjing, China) according to the manufacturer's instructions.

4.9. Western blotting

After 30 min reperfusion, left ventricles were homogenized in a lysis buffer [(in mmol/l) 10 Tris-HCl, 20 ortho-phosphate, 1 EGTA, 1 EDTA, 2 Na_3VO_4, 1 phenylmethylsulfonyl fluoride; pH 7.4]. After sonication, the lysates were centrifuged, the proteins were separated by electrophoresis on SDS-PAGE and transferred onto a polyvinylidenedifluoride-plus membrane. The membranes were blocked with 5% skim milk followed by incubated overnight at 4 °C with the antibodies: eNOS (1:200; Santa Cruz Biotechnology); phospho-eNOS (at Ser1177, 1:200; Santa Cruz, California, USA). After incubation, the membranes were washed three times with 0.1% Tween-20 for 15 min and incubated with horseradish peroxidase for 2 h. The levels of phosphorylated proteins were normalized to their total protein levels. Relative densitometry was performed using a computerized software package (NIH Image 1.63 software).

4.10. Statistical analysis

Results were presented as means ± SD. SPSS17.0 statistical software was applied in the data analysis and processed. One-way ANOVA was applied in analyzing the difference between the groups. If the difference was statistically significant, SNK method was applied in a further pairwise comparison. $P < 0.05$ was considered statistically significant.

Conflict of Interest: The authors declare that they have no conflict of interests.

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