Preconditioning with glycyrrhizic, ferulic, paeoniflorin, cinnamic prevents rat hearts from ischemia/reperfusion injury via endothelial nitric oxide pathway

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ABSTRACT

Objective: The objective was to investigate the endothelial nitric oxide synthase (eNOS/NO) pathway is involved or not in the protective effects of glycyrrhizic, ferulic, paeoniflorin, cinnamic (GFPC) in myocardial ischemia-reperfusion injury Sprague-Dawley rats. **Materials and Methods:** Ischemia-reperfusion (I/R) model was made by ligating the left anterior descending branch of the coronary artery for 30 min and releasing for 120 min, then the left ventricular apical was fixed and sliced, morphological changes of myocardial microvascular endothelial cell (MMVEC) was observed by electron microscopy, apoptosis index of MMVEC was observed by means of TUNEL, serum NO was tested by methods of nitrate reduction, lactate dehydrogenase (LDH), creatine kinase MB (CK-MB) was detected by automatic biochemical analyzer; Phosphorylated eNOS (PeNOS) and inducible NOS (iNOS) protein were measured by means of western blot. **Results:** In positive product control group, the serum levels of NO, LDH, CK-MB significantly increased ($P < 0.05$); MMVEC apoptosis was significantly decreased ($P < 0.05$); incidence of area at risk decreased significantly ($P < 0.05$); PeNOS protein increased ($P < 0.05$); iNOS protein decreased significantly ($P < 0.05$). **Conclusion:** Ischemic preconditioning of GFPC from GFPC plays a protective role in I/R heart through regulating the eNOS/NO signal pathway by increasing the PeNOS protein expression and decreasing the expression of iNOS protein.

Key words: Cinnamic, endothelial nitric oxide synthasee, ferulic,glycyrrhizic, inducible nitric oxide synthase, myocardial ischemia- reperfusion injury, myocardial microvascular endothelial cell, paeoniflorin

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INTRODUCTION

DangGuiSiNi Decoction (DGSND) is used to treat blood deficiency and cold syncope in *shanghanlun*, also a Chinese classical decoction. Investigations have shown that DGSND have effects on anticoagulation, dilation of blood vessels, analgesia, and anti-inflammation.[1] Paeoniflorin,[2-5] ferulic acid,[6] licorice acid,[7] cinnamic acid[8] are four components[9] were found have protective effects on ischemia-reperfusion (I/R) injury in rats. Ischemic preconditioning can significantly ease the I/R injury, previously we selected the best combination from DGSND against I/R injury by means of orthogonal design: Glycyrrhizic acid 50 mg/kg, ferulic acid 400 mg/kg, paeoniflorin 100 mg/kg, cinnamic acid 400 mg/kg[10] (GFPC), then to study whether the endothelial nitric oxide synthase (eNOS/NO) pathway is involved in the cardioprotective effects and the mechanisms of GFPC.

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MATERIALS AND METHODS

Myocardial ischemia-reperfusion

The study protocol was approved by the Ethical Committee of Clinical Research in Jinan University. Sprague-Dawley (SD) rats (250–300 g, from Guangdong laboratory animal central) were maintained for at least 1 week before the experiments. The rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally), respiration with a fraction of inspired oxygen of 0.80, left anterior-descending (LAD) was ligated with a 4-0 silk suture. After 30 min of ischemia,

the ligation was loosened. Rats were killed at 180 min of reperfusion.[11]

The reliability and stability of the model were observed by electrocardiogram changes and myocardial hematoxylin and eosin staining.

Experimental groups

Thirty-two SD rats were divided into sham group (animals were subjected to entire surgical procedure and the silk suture was passed beneath the coronary artery, but the LAD was not ligated), I/R group (heart subject to ischemia-reperfusion), positive product control (PPC) group (heart subjected to I/R preconditioning with GFPC), PPC + L group (heart subjected to I/R preconditioning with GFPC and pretreated with L-NG-Nitroarginine methyl ester (L-NAME), the eNOS inhibitor, Nω-Nitro-L-arginine methylester, 15 min before reperfusion, 30 mg/kg), 8/group.

Nitrate reduction for nitric oxide, creatine kinase MB, lactate dehydrogenase

Serum NO level was tested by methods of nitrate reduction according to the instruction of the kit. Serum lactate dehydrogenase (LDH), creatine kinase MB levels were detected by automatic biochemical analyzer.

Apoptosis

Left ventricular tissue (4 mm × 4 mm × 4 mm, close to the apex cordis) was fixed in 4% paraformaldehyde (1:10), embedded in paraffin and cut into sections 4 μm in thickness for observation of apoptosis (TUNEL), and calculating the apoptotic index (each slice count positive apoptotic nuclei of five high-power field/total nuclei, the mean value for the apoptosis index).

Electron microscopy

The sample of tissue (1 mm × 1 mm × 1 mm) was collected from the apex, placed on ice, and then fixed in 2.5% glutaraldehyde at 4°C for 24 h. The myocardium was then washed in phosphate-buffered saline, embedded in epoxy resin, and immersed in Epon812. Longitudinal ultrathin sections collected with an LKB-V microtome (Bromma, Sweden) were stained with uranium acetate, folic acid lead, and captured with a transmission electron microscope (H-600, Hitachi, Japan).

Western blotting

Phosphorylation of eNOS and inducible NOS (iNOS) protein were measured by the means of western blotting. Extracting about 200 mg tissues from the left ventricular, protein were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels in running buffer at 90 V and then electroblotted to nitrocellulose membranes. Membranes were blocked then incubated overnight at 4°C with the following primary antibodies (Santa Cruz Biotech, CA). Then membranes were washed three times in Tween-20 and incubated with the corresponding secondary antibody (Santa Cruz Biochemicals). Immunoreactive bands were visualized with the chemiluminescence kit (Santa Cruz Biochemicals) according to the instructions.

Statistics analysis

All values are presented as the mean ± standard error of the mean Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 15.0, Analysis of Variance (ANOVA) of orthogonal design, one-way and two-way ANOVA for multiple group comparisons, Chi-square test for data count. $P < 0.05$ was considered significant.

RESULTS

Effect of glycyrrhizic, ferulic, paeoniflorin, cinnamic on serum nitric oxide, lactate dehydrogenase, creatine kinase MB levels

As shown in Figure 1a-c, Compared with sham group, serum NO level of I/R group significantly decreased ($P < 0.05$); compared with the I/R group, serum NO level of PPC group significantly increased ($P < 0.05$); compared with PPC group, serum NO level of PPC + L group significantly decreased ($P < 0.05$). Rats in I/R group, the serum level of LDH, CK were significantly higher than that in the sham group ($P < 0.05$), serum LDH, CK levels in PPC group were significantly lower than I/R group ($P < 0.05$), serum LDH, CK levels in PPC + L group were significantly higher than PPC group ($P < 0.05$).

Effect of glycyrrhizic, ferulic, paeoniflorin, cinnamic on myocardial apoptosis

Compared with sham group seen in Figures 2 and 3, I/R group myocardial microvascular endothelial cell (MMVEC) apoptosis rate was significantly higher ($P < 0.05$); compared with the I/R group, PPC group MMVEC apoptosis was significantly decreased ($P < 0.05$); compared with the PPC group, PPC + L group MMVEC apoptosis rate was increased ($P < 0.05$).

Morphological changes of myocardial microvascular endothelial cell by electron microscope

Electron microscope: In sham group, mitochondrial membrane integrity of MMVEC, crest particle exists, within the folds close, no bubble, regular nuclear membrane, chromatin uniform, no concentration phenomenon, nucleolus exists [Figure 4a]; mitochondrial of I/R group swelling, membrane Irregular [Figure 4b], loose vacuoles

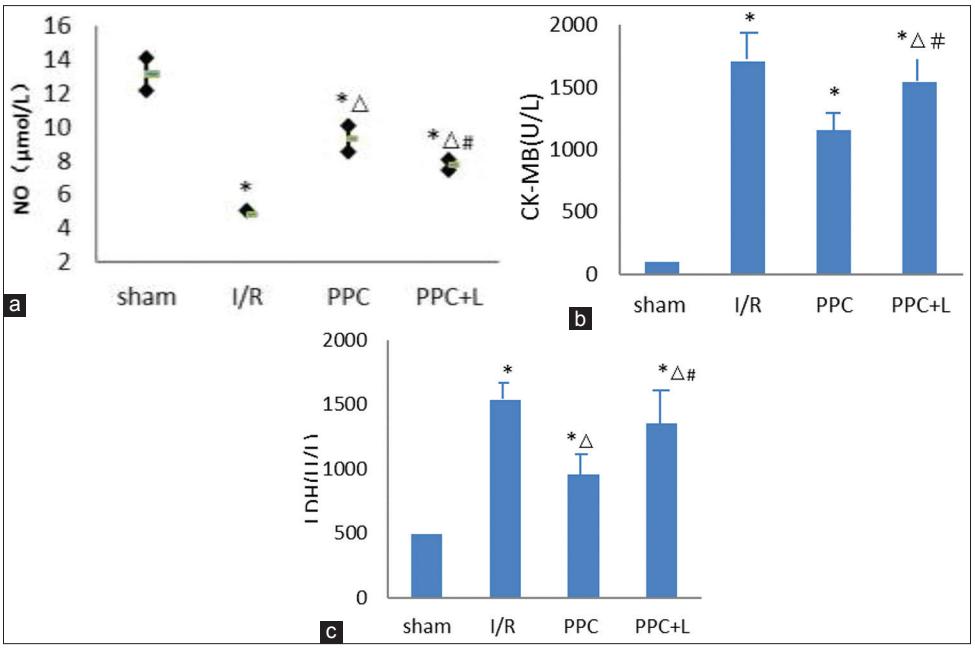

Figure 1: (a) Serum nitric oxide level statistical analysis. (b) Serum creatine kinase MB level statistical analysis. (c) Serum lactate dehydrogenase level statistical analysis

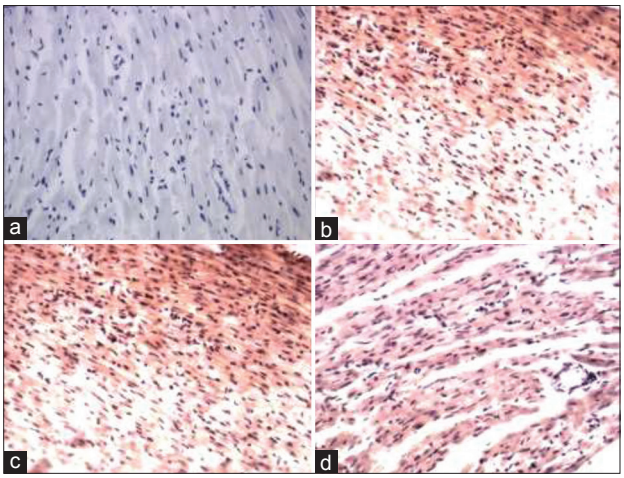

Figure 2: (a) Sham group myocardial microvascular endothelial cell (×200). (b) Ischemia-reperfusion group myocardial microvascular endothelial cell (×200). (c) Positive product control group myocardial microvascular endothelial cell (×200). (d) Positive product control group myocardial microvascular endothelial cell (×200)

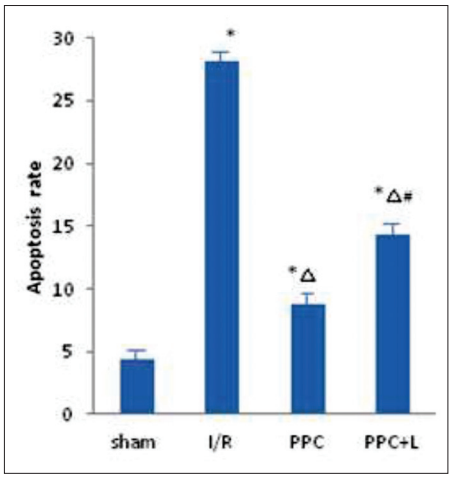

Figure 3: Each group of left ventricular apex myocardial microvascular endothelial cell apoptosis rate (%) comparison. *versus sham group $P < 0.05$, *versus I/R group $P < 0.05$, #versus positive product control group $P < 0.05$

within the wrinkle, ridge particles disappeared, and irregular nuclear membrane [Figure 4c], chromatin condensation and margination, nucleolar disappearance, or even apoptotic bodies [Figure 4d]; compared with I/R group, PPC group significantly improved in symptoms; PPC + L group worse than PPC group.

The incidence of reperfusion arrhythmias

Compared with the sham group, incidence of area at risk (RA) of I/R group significantly increased ($P < 0.05$), Compared with the I/R group, incidence of RA of PPC group decreased significantly ($P < 0.05$); but compared with the PPC group, the incidence of RA of the PPC + L group changes were not statistically significant ($P > 0.05$). GFPC can significantly reduce the incidence of RA in rat myocardial ischemia-reperfusion injury (MIRI) [Figure 5].

Effect of glycyrrhizic, ferulic, paeoniflorin, cinnamic on endothelial nitric oxide synthase and inducible nitric oxide synthase

In Figures 6 and 7, PeNOS and iNOS protein of different groups in rat hearts. A. significant reduction in amount of PeNOS protein was observed in the I/R group ($P < 0.05$ vs. the sham group); After administration

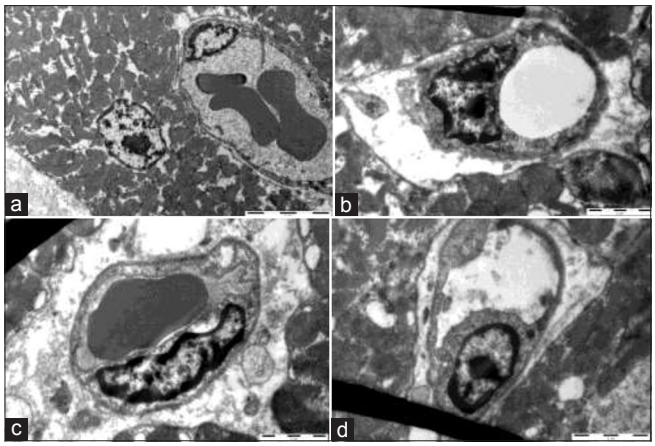

Figure 4: (a) Sham group myocardial microvascular endothelial cell figure. (b) Ischemia-reperfusion group myocardial microvascular endothelial cell. (c) Positive product control group myocardial microvascular endothelial cell. (d) Positive product control group myocardial microvascular endothelial cell

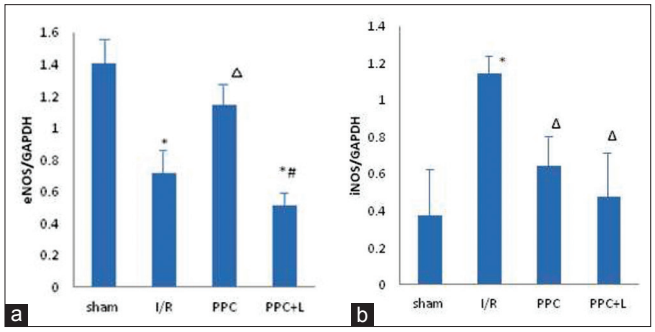

Figure 6: (a) Protein endothelial nitric oxide synthase protein expression (b) Inducible nitric oxide synthase protein expression. *versus sham group $P < 0.05$, versus I/R group $P < 0.05$, #versus positive product control group $P < 0.05$

of GFPC, the amount of PeNOS protein was increased in PPC ($P < 0.05$ vs. the I/R group); and in the PPC + L group, the amount of PeNOS protein was reduced significantly ($P < 0.05$ vs. the PPC group); the amount of iNOS protein increased significantly in the I/R group ($P < 0.05$ vs. the sham group); After administration of GFPC, the amount of iNOS protein was decreased in PPC ($P < 0.05$ vs. the I/R group); and between the PPC and PPC + L, the amount of iNOS protein changes was not significant ($P > 0.05$).

DISCUSSION

Nitric oxide was found as a vascular relaxing factor.[12] L-arginine-NO pathway contributed to free radical generation, which lead to I/R injury. NO synthase inhibitors decreased coronary sinus free radical concentration and tissue peroxynitrite formation in an ischemic-reperfusion canine model.[13] Various competitive inhibitors of the NOS enzyme have been shown to reduce I/R injury in various

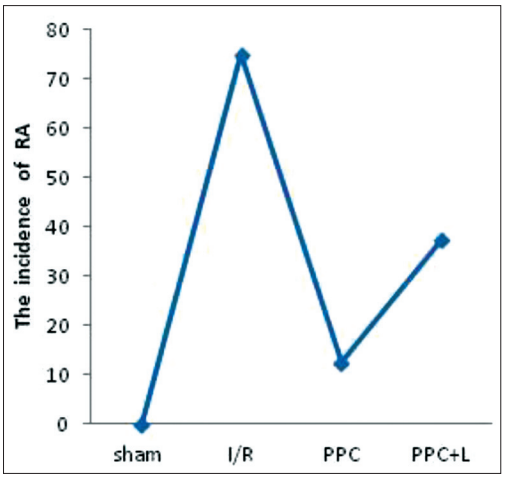

Figure 5: Multiple comparisons among groups of sample rate. *versus sham group $P < 0.05$, Δversus I/R group $P < 0.05$

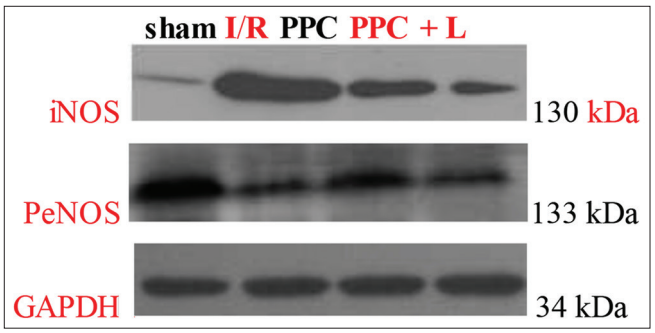

Figure 7: Protein endothelial nitric oxide synthase and inducible nitric oxide synthase of different groups

settings by reducing myocardial infarct size and improving myocardial contractile function.[14,15] L-arginine aggravated myocardial staining through production of peroxynitrite.[16] Some studies have shown that an important protective role of NO in the ischemic preconditioning.[17,18] NO precursor arginine ameliorated the endothelial dysfunction resulting from global I/R sequences in an isolated working rat heart model.[19] In the basic state, NO released from vascular endothelial cells plays an important role in maintaining the cardiovascular system at a relaxation state, regulating blood pressure, ameliorating coronary artery vascular tone and I/R injury. The generation of nitric oxide contributes to the marked antiarrhythmic effects of preconditioning in the canine myocardium, probably through elevation of cyclic guanosine monophosphate.[20]

Apoptosis is considered to be an important part of MIRI, and *in vitro* studies have shown that I/R can lead to apoptosis in VEC,[21,22] slow down the process of apoptosis may improve the prognosis of MIRI, so find the way to block apoptosis signal transduction pathway is very important.[23] The combination use of GFPC playing a multi-target inhibition role of anti-apoptosis

of MMVEC.[24] The results of our study provided the experimental evidence that iNOS expression elevate, while eNOS expression reduce in I/R myocardium. During reperfusion, the resulting formation of NO decreases. GFPC could increase the expression level of eNOS protein, and then promote the L-arginine, NO precursor synthesize nitric oxide. At the same time, GFPC decreases the expression level of iNOS and protein. A large number of iNOS will produce the toxicity NO and inflammatory factors, which will aggravate ischemia-reperfusion injury. Preconditioning with GFPC plays a protective role on ischemic myocardium by increasing eNOS and inhibiting iNOS. But after administration of L-NAME, eNOS inhibitor, phosphorylation of eNOS protein was inhibited. Hence, the generation of NO reduced. It is concluded from these results that the generation of NO is based on the changes of NOS isozyme. Preconditioning of GFPC plays a major protective role on myocardial ischemia in MIRI through the eNOS/NO pathway.

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