

# Effect on eNOS/NO Pathway in MIRI rats with preconditioning of GFPC from Dang Gui Si Ni decoction

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## ABSTRACT

**Objective:** In order to discover whether the eNOS/NO (endothelial nitric oxide synthase/nitric oxide) pathway is involved in the protective mechanisms of ischemic myocardium of DGSND (Dang Gui Si Ni Decoction) in MIRI (myocardial ischemia-reperfusion injury) SD rats. **Materials and Methods:** We made I/R (ischemia-reperfusion) model by ligating the left anterior-descending branch of the coronary artery (LAD) for 30 min and releasing the ligature for 120 min. eNOS (nitric oxide synthase) mRNA (message ribonucleic acid) and iNOS (inducible nitric oxide synthase) mRNA were measured by the methods of real-time RT-PCR (Real time Polychainase Chain Reaction), peNOS (phosphorylated eNOS) and iNOS protein were measured by the means of western blot. **Results:** In PPC group, real-time RT-PCR and western-blot analysis showed that eNOS mRNA and peNOS protein increased markedly ( $P < 0.05$ ); iNOS mRNA and protein decreased significantly ( $P < 0.05$ ). **Conclusion:** These results indicate that ischemic preconditioning (IPC) of GFPC from DGSND plays a protective role in I/R heart through regulating the eNOS/NO signal pathway, which could increase the eNOS gene expression and decrease the expression of iNOS mRNA.

**Key words:** Cinnamic acid, eNOS, ferulic acid, glycyrrhizic acid, MIRI, NO, paeoniflorin

## INTRODUCTION

DGSND is a classical decoction which is used to treat blood-deficiency and cold syncope in Shanghanlun. Modern pharmacological studies shown that DGSND have objective effects of anticoagulation, expand blood vessels, analgesia and anti-inflammation,<sup>[1]</sup> which was mainly applied to treat vascular ischemic diseases, such as frostbite, thromboangiitis obliterans, Raynaud's syndrome, coronary heart disease, lower extremity arterial occlusion and venous thrombosis.<sup>[2]</sup> We have extracted four active ingredients: Glycyrrhizic acid, ferulic acid, paeoniflorin and cinnamic acid from DGSND, which all have intervention effects on myocardial ischemia-reperfusion injury (MIRI).

eNOS/NO Pathway plays an important role in the pathogenesis of MIRI.<sup>[3]</sup> Some clinical reports and studies showed that both DGSND and its ingredients have significant effects on vascular ischemic diseases, especially

the MIRI.<sup>[4-11]</sup> However, is the protective mechanism of DGSND for MIRI from the eNOS/NO Pathway or not isn't thoroughly discovered.

NO (nitric oxide) is reported to protect cells from the deleterious effects of some reactive oxygen species such as peroxide.<sup>[12]</sup> Ischemic preconditioning could promote the composition of NO induced by eNOS. NO may contribute to the ischemic preconditioning. In the previous study we selected, we have selected the best combination from DGSND against I/R injury by the means of orthogonal design: Glycyrrhizic acid 50 mg/kg ferulic acid 400 mg/kg, paeoniflorin 100 mg/kg, cinnamic acid 400 mg/kg GFPC<sup>[13]</sup> made an ischemic preconditioning in MIRI rats, and probed whether the eNOS/NO pathway is involved in the cardioprotective effects and mechanisms of DGSND in MIRI rat.

## MATERIALS AND METHODS

### Myocardial I/R

SD rats (250-300g, from Guangdong laboratory animal central) were maintained under conditions of standard lighting (alternating 12-h light/dark cycles),

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temperature ( $22^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) and humidity ( $60\% \pm 10\%$ ) for at least 1 wk before the experiments. The rats were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally). The trachea was cannulated with a PE-90 catheter, and artificial respiration was provided by a respirator with an  $\text{FiO}_2$  (fraction of inspired oxygen) of 0.80, a frequency of 100 strokes/min and a tidal volume of 0.8 to 1.2 mL to maintain normal  $\text{PO}_2$  (partial pressure of oxygen),  $\text{PCO}_2$  (partial pressure of carbon dioxide) and pH. A left lateral thoracotomy was made in the fourth intercostals space; the skin, muscles and ribs were retracted; and the pericardial sac was removed. The left-anterior branch of the descending coronary artery (LAD) was occluded by ligation with a 4-0 silk suture. The LAD ligation was performed by using an easily opened knot set on a PE50 silicon tube lying over the LAD. After 30 min of ischemia, the ligation was loosened and reperfusion occurred. Rats were killed at 180 min of reperfusion. The sham control animals were subjected to the entire surgical procedure and the silk suture was passed beneath the coronary artery, but the LAD was not ligated.<sup>[14]</sup>

The reliability and stability of the model was observed by ECG changes and myocardial HE staining.

### Experimental groups

32 SD rats (body weight 250-300 g) were randomly divided into a sham group (non-ischemic myocardial microvascular endothelial cell, conditions) an I/R group (heart subject to ischemia reperfusion) a PPC group (heart subjected to ischemia-reperfusion treated with GFPC) a ppc + L group (heart subjected to ischemia-reperfusion treated with GFPC and pre-treated with L-NAME, the eNOS inhibitor, N $\omega$ -Nitro-L-arginine methylester, 15 min before reperfusion, 30 mg/kg), 8 per group.

### Nitrate reduction for NO

Serum NO level was tested by methods of nitrate reduction according to the instruction of the kit.

### Real-time quantitative reverse transcription polymerase chain reaction for NOS

Quantitative multiplex reverse-transcribed polymerase chain reaction (RT-PCR) was used to determine mRNA levels of the constitutive eNOS and the inducible iNOS isoforms in rat ventricular tissue. Myocardial samples ( $n = 8/\text{group}$ ), fixed in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  were homogenized in 800 mL of RNA Fast Solution (Celbio, Milan, Italy). Total RNA was isolated as recommended by the manufacturer. RNA was dissolved in DEPC-treated water and quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding RNA (0.1  $\mu\text{g}$ ) to a mixture containing 1 mM deoxynucleoside-tri-phosphates (d-NTP), 1 U/ $\mu\text{L}$  RNase inhibitor, 2.5 U/ $\mu\text{L}$  Moloney murine leukemia virus

reverse transcriptase, 2.5  $\mu\text{M}$  random hexamers, 5 mM  $\text{MgCl}_2$ , 10  $\times$  PCR buffer in a final volume of 20  $\mu\text{L}$ . Reverse transcription was performed at  $42^{\circ}\text{C}$  for 50 min followed by heat inactivation of reverse transcriptase at  $95^{\circ}\text{C}$  for 5 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified from the same amount of RNA to correct for variation of different samples. The PCR solution contained 10  $\mu\text{L}$  of first-strand cDNA, 4  $\mu\text{L}$  10  $\times$  PCR buffer, 2 mM  $\text{MgCl}_2$ , 0.15 mM of both sense (5'-ACC ACA GTC CAT GCC ATC AC-3') and antisense (5'-TCC ACC ACC CTG TTG CTG TA-3') GAPDH primers, 0.15 mM of both sense (5'-CGA GAT ATC TTC AGT CCC AAG C-3') and antisense (5'-GTG GAT TTG CTG CTC TCT AGG-3') eNOS, or 0.15 mM of both sense (5'-TCT GTG CCT TTG CTG ATG AC-3') and antisense (5'-CAT GGT GAA CAC GTT CTT GG-3') iNOS primers, 2 U Thermophilus Aquaticus (Taq) DNA polymerase (Celbio, Milan, Italy), and water to a final volume of 50  $\mu\text{L}$ . These samples were overlaid with mineral oil and subjected to 35 cycles at  $95^{\circ}\text{C}$  for 60 s,  $60^{\circ}\text{C}$  for 60 s, and to one cycle at  $72^{\circ}\text{C}$  for 7 min. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining. Bands on the gel were scanned and quantified using a computerized densitometric system (Bio Rad Gel Doc 1000, Milan, Italy).<sup>[15]</sup>

### Western blotting

Phosphorylation of eNOS and iNOS protein were measured by the means of western blot. Extracting about 200 g tissues from the left ventricular, equal amounts of protein (50  $\mu\text{g}$ ) were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels in running buffer (25 mmol/L Tris, 0.25mol/L glycine, 0.1% sodium dodecyl sulfate, pH 8.3) at 90 V and then electroblotted to nitrocellulose membranes. eNOS and iNOS were detected by monoclonal antibodies (Santa Cruz Biotech, Inc. Santa Cruz, CA). Membranes were blocked at room temperature with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 and then incubated overnight at  $4^{\circ}\text{C}$  with the following primary antibodies: GAPDH (Sigma company, USA; dilutions, 1:5000). GAPDH was used as internal control to correct for variations of different samples. Then the membranes were washed three times in Tween-20 and incubated with the corresponding secondary antibody (Santa Cruz Biochemicals; dilutions, 1:10000) conjugated to horseradish peroxidase at room temperature. Immunoreactive bands were visualized with the chemoluminescence kit (Santa Cruz Biochemicals) according to the manufacturer's instructions. Band intensities and molecular weight were quantified by using a gel image processing system (Bio-Rad, USA).

### Statistics analysis

All results are reported as mean  $\pm$  S.D. Statistical analysis was performed using analysis of variance of orthogonal

design, 1-way and 2-way analysis of variance (ANOVA) for multiple-group comparisons, Chi-square test for data count. The probability of null hypothesis  $< 0.05$  ( $P < 0.05$ ) was considered statistically significant.

## RESULTS

### Effect of GFPC on eNOS and iNOS

To test the hypothesis that eNOS/NO pathway acts to protect ischemic myocardium in ischemic preconditioning of GFPC, we examined the gene and protein expressions of eNOS and iNOS by real-time PCR and western blot analysis. As shown in Figure 1a and b, compared with the sham group, eNOS mRNA and protein of I/R group significantly decreased ( $P < 0.05$ ); compared with the I/R group, eNOS mRNA and eNOS protein of PPC group significantly increased ( $P < 0.05$ ); compared with the PPC group, eNOS mRNA and peNOS protein of PPC + L group significantly reduced ( $P < 0.05$ ). Compared with the sham group, iNOS mRNA and protein of the I/R group significantly increased ( $P < 0.05$ ); Compared with the I/R group, iNOS mRNA and protein of PPC group decreased significantly ( $P < 0.05$ ); but compared with the PPC group, iNOS mRNA and protein of the PPC + L group changes were not statistically significant ( $P > 0.05$ )

Figure 1a shows real-time RT-PCR analysis of eNOSmRNA in rat hearts of different groups (sham, I/R, PPC, PPC + L). The expression of eNOS mRNA attenuated in the I/R group decreased ( $*P < 0.05$  versus shame group) After administration of GFPC, the amount of eNOSmRNA significantly increased ( $\Delta P < 0.05$  versus the I/R group); and in the PPC + L group, the amount of eNOS mRNA was significantly reduced ( $\#P < 0.05$  versus the PPC group).

Figure 1b shows the gene expression of iNOS in rat hearts of different groups (shame, I/R, PPC, PPC + L) evaluated by real-time quantitative RT-PCR. The expression of iNOS mRNA was markedly increased in the I/R group ( $*P < 0.05$  versus sham group) After administration of GFPC, the amount of iNOSmRNA significantly decreased ( $\Delta P < 0.05$  versus the I/R group); The amount of iNOS mRNA has no obvious difference ( $P > 0.05$ ) between ppc group and ppc + L group.

Figures 2b and 3b show western blot analysis of 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 ( $P < 0.05$  versus the sham group); After administration of GFPC, the amount of peNOS protein was increased in PPC ( $P < 0.05$  versus

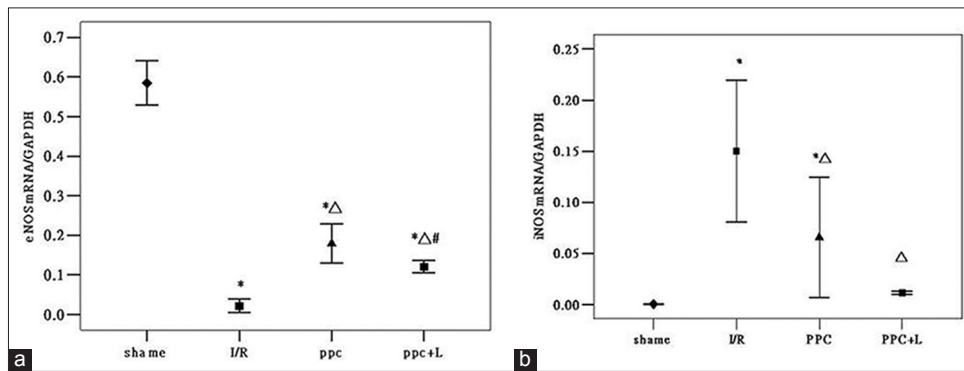


Figure 1: (a) eNOS mRNA statistical analysis (b) iNOS mRNA statistical analysis

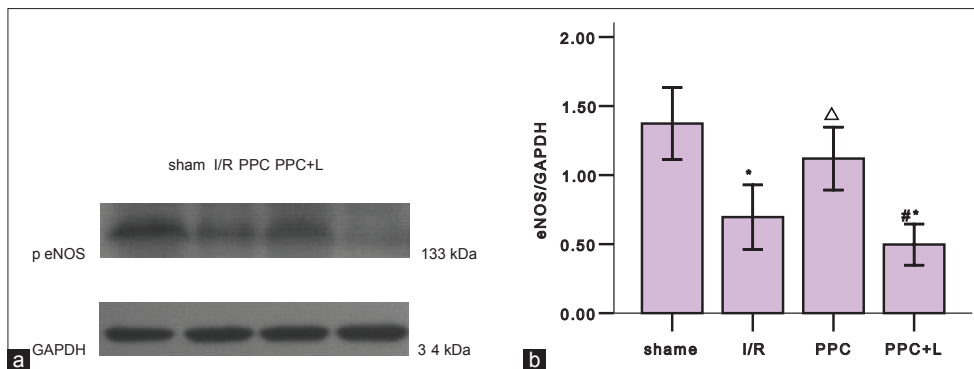
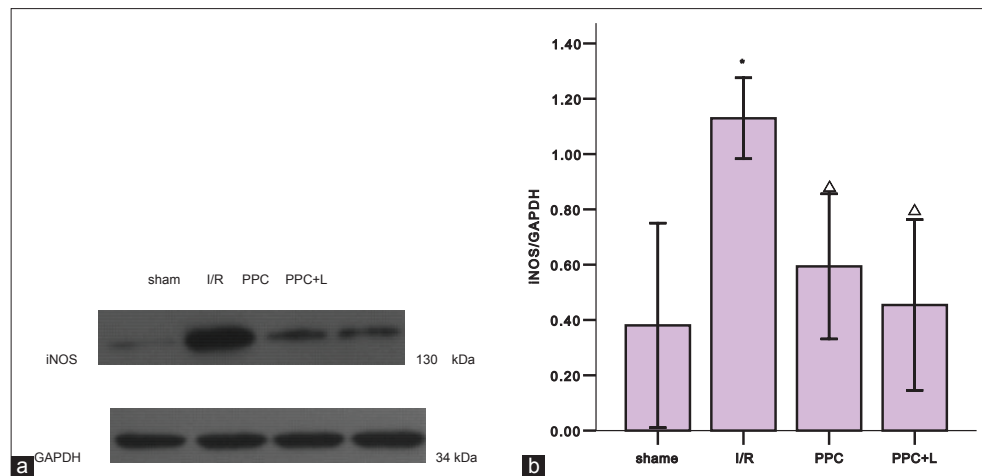


Figure 2: (a) Western blot analysis of peNOS of different groups in rat hearts (b) statistics of western blot



**Figure 3:** (a) Western blot analysis of iNOS of different groups in rat hearts (b) statistics of western blot

the I/R group); and in the PPC + L group, the amount of peNOS protein was reduced significantly ( $P < 0.05$  versus the PPC group).

Figure 1 shows the amount of iNOS protein increased significantly in the I/R group ( $P < 0.05$  versus the sham group); After administration of GFPC, the amount of iNOS protein was decreased in PPC ( $P < 0.05$  versus the I/R group); and between the PPC and PPC + L, the amount of iNOS protein changes was not significant ( $P > 0.05$ ).

## DISCUSSION

In previous studies of L-arginine-NO pathway in I/R injury, NO was found as an endothelium-derived relaxing factor by Palmer in 1987.<sup>[16]</sup> L-arginine-NO pathway contributed to free radical generation, which lead to ischemia-reperfusion injury. NO synthase inhibitors decreased coronary sinus free radical concentration and tissue peroxynitrite formation in an ischemic-reperfusion canine model.<sup>[17]</sup> Various competitive inhibitors of the NOS enzyme have been shown to reduce I/R injury in various settings by reducing myocardial infarct size and improving myocardial contractile function.<sup>[18,19]</sup> L-arginine aggravated myocardial staining through production of peroxynitrite.<sup>[20]</sup> In contrast to these above studies, some studies have shown that an important protective role of NO in the ischemic preconditioning.<sup>[3,21]</sup> NO precursor arginine ameliorated the endothelial dysfunction resulting from global ischemia-reperfusion sequences in an isolated working rat heart model.<sup>[22]</sup> 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 GMP.<sup>[23]</sup>

The results of our study provided the experimental evidence that iNOS expression elevate, while eNOS expression reduce in ischemia-reperfusion myocardium. During reperfusion, the resulting formation of NO decreases. GFPC could increase the expression level of eNOSmRNA and phosphorylation 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. Preconditioning of 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 nitric oxide 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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