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Research Paper

Protective effect of polydatin against ischemia/reperfusion injury in rat heart

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Abstract: The aim of the present study was to investigate the protective effect of polydatin against myocardial ischemia/reperfusion injury in rats and the underlying mechanism. In anesthetized rats, ischemia and reperfusion arrhythmia produced by ligating and loosing the coronary artery was recorded and myocardial infarct size was measured. In Langendorff isolated rat heart, cardiac function was recorded before and after 30 min of global ischemia followed by 60 min of reperfusion. The parameters of cardiac function include left ventricular developed pressure (LVDP), maximal differentials of LVDP ($\pm LVdp/dt_{max}$) and coronary flow (CF) were measured. Myocardial superoxide dismutase (SOD) activity, the contents of myocardial malondialdehyde (MDA) and nitric oxide (NO) as well as the activity of nitric oxide synthase (NOS) were measured in isolated heart. The results showed: (1) Arrhythmia score and myocardial infarct size were significantly lower in polydatin group than that in the control group ($P<0.05$, $P<0.01$); (2) The recovery of LVDP, $\pm LVdp/dt_{max}$ and CF during reperfusion in polydatin group were significantly better than that in the control rats ($P<0.05$, $P<0.01$); (3) SOD activity in polydatin group was significantly higher than that in the control group, but MDA content was lower in polydatin group than that in the control group ($P<0.05$); (4) NO content and NOS activity, especially constitutive nitric oxide synthase (cNOS) activity in polydatin group were higher than that in the control group ($P<0.05$); (5) *L*-NAME, the NOS inhibitor, reversed the protective effect of polydatin against ischemia/reperfusion injury. The results suggest that polydatin has a protective effect against ischemia/reperfusion injury in rat heart. The cardioprotection of polydatin is mainly mediated by cNOS which leading to an increase in NO production.

Key words: polydatin; heart; ischemia/reperfusion; protection; rat

白藜芦醇甙对大鼠心脏缺血/再灌注损伤的保护作用

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摘 要：本文利用冠脉结扎/放松方法和Langendorff灌注技术，建立在体和离体大鼠心脏缺血/再灌注(ischemia/reperfusion, I/R)损伤模型，探讨白藜芦醇甙(polydatin)对大鼠I/R心肌损伤的保护作用及其机制。观察白藜芦醇甙对缺血和再灌注心律失常、心肌梗死面积、心脏收缩功能、心肌超氧化物歧化酶(superoxide dismutase, SOD)活性、丙二醛(malondialdehyde, MDA)含量、NO含量以及一氧化氮合酶(nitric oxide synthase, NOS)活性的影响。结果显示：与对照组相比，白藜芦醇甙组大鼠缺血和再灌注心律失常明显降低($P<0.05$, $P<0.01$)；心肌梗死面积显著减少($P<0.01$)；I/R心脏左心室发展压(left ventricular developed pressure, LVDP)、左心室压力上升和下降最大变化速率($\pm LVdp/dt_{max}$)、冠脉流量(coronary flow, CF)明显改善($P<0.05$, $P<0.01$)；心肌SOD活性升高，MDA含量降低($P<0.05$)；NO含量和NOS及cNOS活性也明显升高($P<0.05$)；此外，NOS抑制剂*L*-NAME拮抗白藜芦醇甙对I/R心肌的保护作用。结果提示：白藜芦醇甙具有明显的抗心肌I/R损伤作用，此作用主要由cNOS产生的NO增加所介导。

关键词：白藜芦醇甙；心脏；缺血/再灌注；保护作用；大鼠
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Polydatin, 3,4',5-trihydroxystibene-3-β-mono-*D*-glucoside, also named piceid, is a monocrystalline isolated from a traditional Chinese herbal medicine, *Polygonum cuspidatum* Sieb. et Zucc. Previous studies showed that polydatin could inhibit platelet aggregation, reduce neutrophil-endothelial adhesion, improve microcirculation, antagonize shock, and has antioxidative activity[1-4]. It was reported that polydatin protected cardiomyocytes against the injury induced by deprivation of oxygen and glucose[5]. Besides, it was proved that polydatin had protective effect against ischemia/reperfusion (I/R) injury in brain, intestine and remote organs[6-8].

Polydatin is a combination of resveratrol and glucose. Both polydatin and resveratrol belong to stilbene compound and share some similar pharmacological effects, such as antioxidative activity and inhibition of platelet aggregation[1,4,9]. But there are some different effects between the two compounds. Resveratrol reduces, but polydatin increases intracellular free calcium concentration ($[Ca^{2+}]_i$) in myocardial cells[10,11]. It was reported that resveratrol had protective effect on hearts against I/R injury[12]. However, it is not known whether polydatin has the same effect. The aim of the present study was to confirm the hypothesis that polydatin protects heart against I/R injury, and explore the underlying mechanism.

1 MATERIALS AND METHODS

1.1 *Experimental animals and biochemicals*

Fifty-two male Sprague-Dawley (SD) rats weighting 280-320 g (grade II, Certificate No. 04036) were provided by the Experimental Animal Center of Hebei Province. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Polydatin was provided by Neptunus Pharmaceutical Co. Ltd. (Shenzhen, China). Superoxide dismutase (SOD), malondialdehyde (MDA), NO and nitric oxide synthase (NOS) assay kits were purchased from Nanjing Jiancheng Biotechnology Company (Nanjing, China). *L*-NAME, Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma (St Louis, Missouri, USA).

1.2 *I/R injury of in vivo heart*

To study the effect of polydatin on *in vivo* hearts, rats were randomly divided into the control and polydatin groups. Rats were anesthetized with sodium pentobarbital (30 mg/kg body weight, i.p.). Body temperature was maintained at (37.0±0.5) ºC. Animals were ventilated with a rodent ventilator (HX-300S, Chengdu TME Technology Co. Ltd., China) at 60 to 70 breaths per minute with tide volume of about 1.5 mL/100 g. Electrocardiogram (ECG) in lead II together with the blood pressure of carotid artery, was continuously monitored and recorded using a data acquisition system (PowerLab/8 s, AD Instrument, Australia). Heart rate (HR) was calculated from the R-R interval in ECG. Catheter in the left femoral vein was used for administration of biochemicals. The left thoracotomy was performed in the 3rd or 4th intercostal space, and pericardium was opened to expose the heart. A 5/0 silk suture was passed through the left descending coronary artery (LDA). After the state of cardiac function was kept steady for 15 min, myocardial ischemia was produced by ligating LDA and reperfusion was produced by loosing the ligation[8]. Successful ischemic sign following coronary arterial occlusion was indicated by a significant ST-segment elevation in ECG immediately after ligation, together with a slight blood pressure reduction.

1.3 *Measurement of arrhythmia*

In this part of experiment, rats were treated with 10 min of ischemia followed by 60 min of reperfusion. In polydatin group (n=6), polydatin (0.1% polydatin solution, 0.2 mL/100 g body weight) was injected via the femoral vein at 10 min before ischemia. In the control group (n=6), saline (0.9%) of the same volume as that in polydatin group was injected. In this study, the dose of polydatin was determined according to the published reports on polydatin[2,7,9,11] and our preliminary experiments. Ventricular arrhythmias included ventricular premature beat, ventricular tachycardia and ventricular fibrillation. Arrhythmias were defined in accordance with the guideline of the Lambeth Conventions for analysis of experimental arrhythmias, and quantified with arrhythmia score according to Johnston standard[13].

1.4 *Determination of myocardial infarct size*

In this part of experiment, rats were treated with 30 min of ischemia followed by 60 min of reperfusion. Polydatin (0.1% polydatin solution, 0.2 mL/100 g body weight) in polydatin group (n=6) and saline (0.9%) of the same volume in the control group (n=6) were given via the femoral vein at 10 min before ischemia, respectively. At the end of reperfusion, after the LAD was ligated completely, the aorta was ligated also and 2% Evans blue (1 mL) was injected to the heart via the left free ventricular wall, then, the heart was removed quickly and frozen. The frozen heart was cut into 5 slices that were incubated in 1% TTC (phosphate buffer, pH 7.4) to be stained for 15 min.

Normal myocardium that stained by Evans blue and TTC looked blue, ischemic myocardium that stained by TTC looked red, and infarct myocardium that was not stained by either Evans blue or TTC looked pale. The photos were taken with a digital camera, and input into the computer and analyzed by image processing system (JIE DA-108, Jiangsu). The extent of ischemic myocardium (area at risk) was expressed as the percentage of ischemic size to the left ventricular size. The extent of infarct myocardium was expressed as the percentage of infarct size to ischemic size.

1.5 *Ischemia and reperfusion of isolated heart in vitro*

Rats were anesthetized with sodium pentobarbital (30 mg/kg body weight, i.p.) and hearts were quickly excised and mounted on a Langendorff apparatus via aorta for retrograde perfusion with Krebs-Henseleit (K-H) buffer at constant pressure (100 mmH_2O). K-H solution (in mmol/L) was composed of: NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, and glucose 11.0. The medium was continuously gassed with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 37 ºC. A water-filled latex balloon connected to a pressure transducer (Gould P23Db) was introduced into the left ventricle through atria to record isovolumic left ventricular pressure. The balloon volume was adjusted to achieve a stable left ventricular end-diastolic pressure (LVEDP) of 5 to 10 mmHg during initial equilibration. The left ventricular developed pressure (LVDP), LVEDP, maximal differentials of LVDP ($\pm LVdp/dt_{max}$), HR and coronary flow (CF) were monitored with PowerLab system (AD Instrument Ltd., Australia). In this part of experiments, rats were randomly divided into the control, polydatin, *L*-NAME and *L*-NAME + polydatin groups. The control hearts, after stabilization for 20 min with K-H buffer solution, were subjected to 30 min of no-flow global ischemia followed by 60 min of reperfusion. The hearts in polydatin group were treated with 0.05 mmol/L polydatin for 15 min before ischemia and reperfusion. In *L*-NAME group, the hearts were treated with 0.1 mmol/L *L*-NAME for 20 min before ischemia and reperfusion. In *L*-NAME + polydatin group, the hearts were perfused for 5 min with 0.1 mmol/L *L*-NAME firstly, then were treated with 0.05 mmol/L polydatin and 0.1 mmol/L *L*-NAME together for 15 min before ischemia and reperfusion.

1.6 *Measurements of SOD, MDA, NO and NOS*

After 30 min of global ischemia followed by 60 min of reperfusion, the hearts were removed quickly from the Langendorff apparatus and homogenized. The activity of SOD was measured by xanthine oxidase method and MDA content was measured by thibabituric acid chromatometry. The content of NO was measured by nitrate reductase method. NOS and inducible NOS (iNOS) activities were measured directly by catalyzing *L*-arginine method[14] and constitutive NOS (cNOS) activity was calculated from NOS and iNOS.

1.7 *Statistical analysis*

All data were presented as mean±SD. The paired *t*-test was used to compare the data within groups and ANOVA was used to compare the data between groups. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Effects of polydatin on blood pressure and HR in anesthetized rats during I/R

During I/R, blood pressure and HR in anesthetized rats decreased in both control and polydatin groups, but the decrease in blood pressure and HR in polydatin group was smaller than that in the control group ($P<0.05$, $P<0.01$) (Table 1).

2.2 Effect of polydatin on arrhythmia in anesthetized rats during I/R

As shown in Fig.1, arrhythmia scores of ischemic and reperfusion arrhythmia in polydatin group were 1.0±1.5 and 2.0±1.8, respectively, and were lower than those in

Table 1. Changes in mean arterial pressure (MAP) and heart rate (HR) during myocardial ischemia/reperfusion in anesthetized rats

	MAP (mmHg)		Heart rate (beats/min)	
	Control	Polydatin	Control	Polydatin
Pre-ischemia	88.9±13.4	84.4±13.4	381.5±33.8	374.3±24.4
Ischemia	72.6±23.2*	78.6±16.1	352.3±35.5*	349.8±25.3*
30 min of perfusion	60.4±10.5**	76.9±10.8$^{*\#}$	326.5±22.0**	348.3±32.3*
60 min of perfusion	53.4±10.1**	73.8±9.1$^{**\#\#}$	293.8±40.4**	347.1±32.1$^{*\#}$

n=6. mean±SD. $^{*}P<0.05$, $^{**}P<0.01$ *vs* corresponding pre-ischemia group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* corresponding control group.

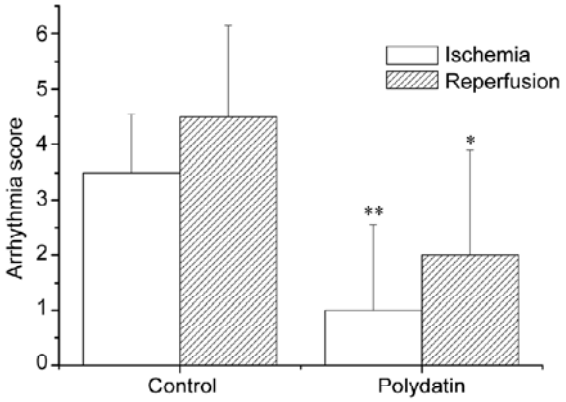

Fig. 1. Effect of polydatin on arrhythmia induced by ischemia and reperfusion in anesthetized rats. n=6. mean±SD. $^{*}P$<0.05, $^{**}P$<0.01 *vs* control group.

the control group (3.5±1.0 and 4.5±1.6, P<0.05, P< 0.01), suggesting that polydatin had an antiarrhythmic effect on I/R-injured hearts.

2.3 Effect of polydatin on infarct size induced by I/R injury in anesthetized rats

The infarct size in polydatin group at 60 min after reperfusion was (14.6±3.7)%, much smaller than (46.7±7.6)% in control group (P<0.01). There was no significant difference in area at risk between the two groups (Fig.2). The results suggest that polydatin reduced myocardial infarct size induced by I/R injury.

2.4 Effect of polydatin on ventricular function in isolated rat hearts during I/R

There were no significant differences in the functional parameters between the control and polydatin groups before ischemia (Table 2). The functional parameters, including LVDP, +LV$dp/dt_{max}$, -LV$dp/dt_{max}$ and CF decreased significantly in both control and polydatin groups during I/R (P<0.01)(Table 2, Fig.3), showing the damage effect of I/R on left ventricular function. The rats in polydatin group displayed a better recovery during reperfusion after ischemia than the control rats. After 60 min of reperfusion, the recovery percent of LVDP, +LV$dp/dt_{max}$, and -LV$dp/dt_{max}$ were 43.3%, 42.9%, and 42.4%, respectively, in polydatin group, much higher than those in the control group (22.5%, 23.1%, and 24.1%, respectively) (P<0.05, P<0.01). Thus polydatin significantly promoted recovery of post-ischemic myocardial function, evidenced by significantly higher CF after 60 min of reperfusion. No significant differences in HR were observed in all rat hearts (Table 2). All the results suggest that polydatin increased the tolerance of hearts to I/R injury.

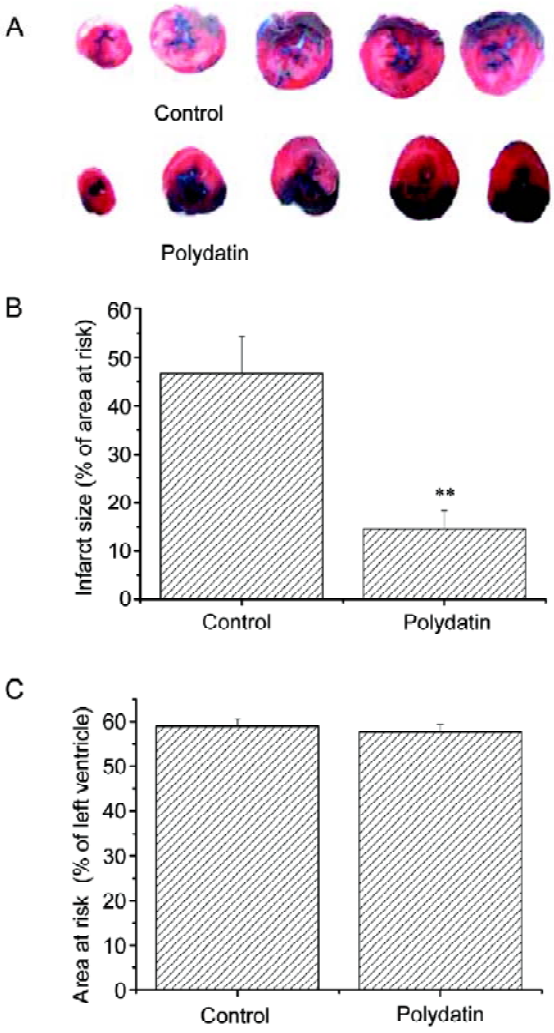

Fig. 2. Effect of polydatin on myocardial infarct size induced by ischemia/reperfusion. *A*: Representative TTC staining of control and polydatin-treated hearts after ischemia/reperfusion. *B*: Necrotic myocardium (infarct size). *C*: Ischemic myocardium (area at risk). n=6. mean±SD. $^{**}P$<0.01 *vs* control group

2.5 Effects of polydatin on SOD activity and MDA content in rat myocardium during I/R

As shown in Table 3, the activity of SOD [(965±77) U/g protein] was higher in polydatin group than that in the control group [(788±137) U/g protein]. The content of MDA was lower in polydatin group than that in the control group (P<0.05).

2.6 Effects of polydatin on NO content and NOS activity in rat myocardium during I/R

The content of NO was higher in polydatin group than that in the control group (P<0.05). Besides, NOS activity, especially cNOS activity, in polydatin group, was higher than that in the control group (P<0.05)(Table 4).

Table 2. Hemodynamic parameters in isolated rat hearts before ischemia and 60 min after reperfusion

		Control	Polydatin
Pre-ischemia	LVDP (mmHg)	110.1±25.3	112.5±13.6
	LVEDP (mmHg)	10.2±4.9	10.8±5.7
	+LV$dp/dt_{max}$ (mmHg/s)	4 422±976	4 327±575
	-LV$dp/dt_{max}$ (mmHg/s)	-2 580±606	-2 420±320
	HR (beats/min)	257±31	250±18
	CF (mL/min)	12.5±2.7	12±2.1
Reperfusion	LVDP (mmHg)	24.6±12.1**	49.1±13.1$^{**\#\#}$
	LVEDP (mmHg)	63.3±13.2**	50.3±16.0$^{**\#}$
	+LV$dp/dt_{max}$ (mmHg/s)	1 056±851**	1 916±858$^{**\#}$
	-LV$dp/dt_{max}$ (mmHg/s)	-636±340**	-1 075±504$^{**\#}$
	HR (beats/min)	226±56	222±53
	CF (mL/min)	3.5±1.5**	5.1±1.0$^{**\#}$

n=7. LVDP: left ventricular developed pressure; LVEDP: left ventricular end-diastolic pressure; ±LV$dp/dt_{max}$: maximal differentials of LVDP; HR: heart rate; CF: coronary flow. $^{**}P$<0.01 *vs* corresponding pre-ischemia group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs* corresponding control group.

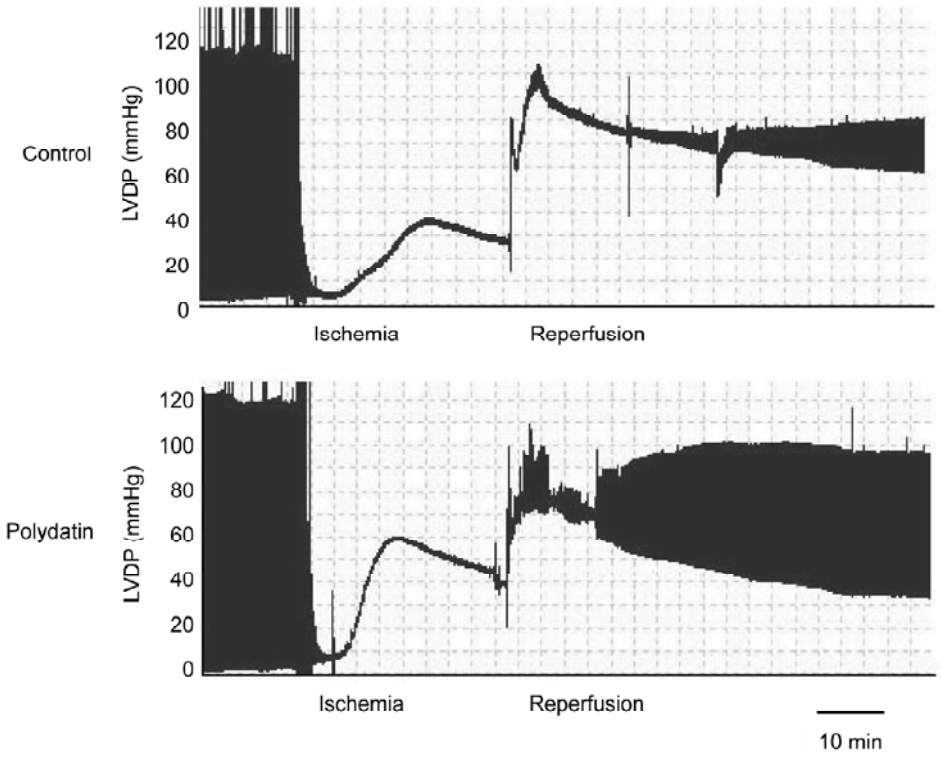

Fig. 3. Original recordings of left ventricular function during ischemia/reperfusion in isolated rat hearts.

2.7 Influence of *L*-NAME on the protective effect of polydatin against myocardial I/R injury in isolated rat hearts

The recovery percent of LVDP, +LV$dp/dt_{max}$, -LV$dp/dt_{max}$, and CF after 60 min of reperfusion in *L*-NAME group was (17.1±6.3)%, (16.1±10.4)%, (18.5±8.5)%, and (23.4±5.6)%, respectively, with no significant differences from that in the control group (Fig.4), suggesting that perfusion of the isolated heart with 0.1 mmol/L *L*-NAME (an inhibitor of all NOS) alone did not affect the functional recovery of left ventricle after reperfusion. But the addition of 0.1 mmol/L *L*-NAME to K-H solution in polydatin-treated hearts

Table 3. Effects of polydatin on SOD activity and MDA content in isolated rat hearts during I/R

Group	SOD (U/g protein)	MDA (μmol/g protein)
Control	788±137	2.79±0.37
Polydatin	965±77*	2.15±0.50*
L-NAME + polydatin	778±62#	2.73±0.19#

*P<0.05 *vs* control group; #P<0.05 *vs* polydatin group. n=7.

Table 4. Effects of polydatin on NO content and NOS activity in isolated rat hearts during I/R

Group	NO (μmol/g protein)	NOS (kU/g protein)	cNOS (kU/g protein)	iNOS (kU/g protein)
Control	2.08±0.71	0.85±0.16	0.38±0.19	0.51±0.10
Polydatin	3.58±1.47*	1.23±0.34*	0.76±0.35*	0.47±0.08
L-NAME + polydatin	2.00±0.41#	0.77±0.11#	0.31±0.17#	0.46±0.10

*P<0.05 *vs* control group; #P<0.05 *vs* polydatin group. n=7.

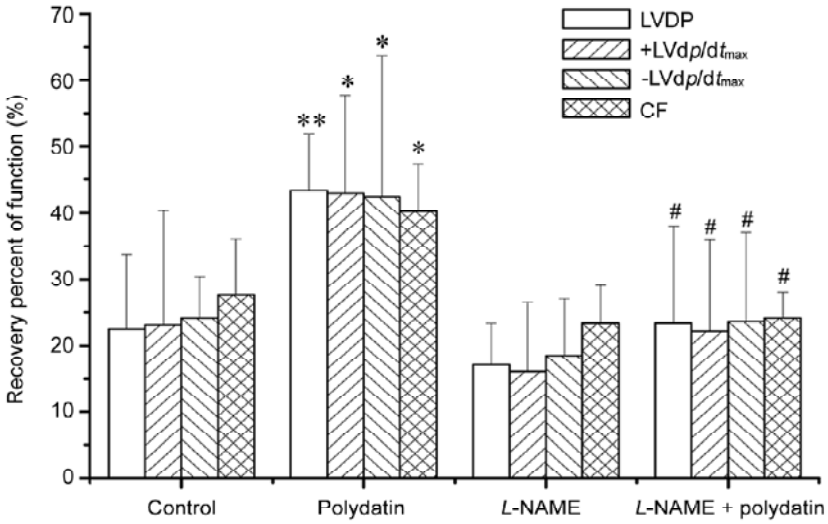

Fig. 4. The influence of *L*-NAME on the cardioprotective effect of polydatin after reperfusion. *P<0.05, **P<0.01 *vs* control group; #P<0.05 *vs* polydatin group. n=7.

reversed the improvement in cardiac function. After pretreatment with 0.1 mmol/L *L*-NAME and 0.05 mmol/L polydatin together, the recovery percent of LVDP, LVEDP, +LV$dp/dt_{max}$, -LV$dp/dt_{max}$, and CF after I/R was (23.4±14.6)%, (22.2±13.8)%, (23.6±13.4)%, and (24.1±3.8)%, respectively (P<0.05 *vs* polydatin group)(Fig.4). The activity of SOD [(778±62) U/g protein] and the content of NO [(2.00±0.41) μmol/g protein] was lower in *L*-NAME + polydatin group than that in polydatin group (P<0.05), and the content of MDA [(2.73±0.19) μmol/g protein] was higher than that in polydatin group (P<0.05). The results suggest that *L*-NAME reversed the protective effect of polydatin against I/R injury.

3 DISCUSSION

Polydatin is a combination of resveratrol and glucose. Both polydatin and resveratrol belong to stilbene compound abstracted from *Poligonum cuspidatum* Sieb. et Zucc, hydroxy chrysophenine compound. Polydatin and resveratrol share some similar pharmacological effects, such as antioxidative activity[4]. Also, polydatin and resveratrol have some different qualities and pharmacological effects. Experimental results showed that resveratrol reduced, but polydatin increased $[Ca^{2+}]_i$ in myocardial cells[10,11]. It was reported that resveratrol had protective effect on hearts against injury[12]. But we don't know whether polydatin

has the same protective effect on hearts. In this study, we observed the effects of polydatin on I/R myocardium using *in vivo* and *in vitro* rat models. The results firstly demonstrated that polydatin had protective effects on hearts against I/R injury, as evidenced by reducing arrhythmia and myocardial infarction, improving myocardial function during I/R. The results also showed that polydatin increased CF and NO production, and enhanced antioxidation in heart, which could be reversed by *L*-NAME, an NOS inhibitor.

The coronary blood circulation has some distinct features in anatomy and function. The blood supply to heart is afforded mainly during diastole because the strong compression of the cardiac muscle around the intramuscular vessels during systole. The heart has a limited potential to increase oxygen supply by enhancing oxygen uptake because oxygen uptake rate is very high under static circumstance. Therefore, the increase in oxygen demand under some circumstance, such as physical exercise or ischemic disorder, can be accomplished mainly through increasing blood flow in coronary circulation[15]. The result of present study showed that CF during reperfusion was significantly higher in polydatin group than that in control group, suggesting that increasing CF might be one of important mechanisms of protective effects of polydatin against myocardial I/R injury.

It is well known that oxygen-free radicals, generated during I/R injury, contributes to the pathogenesis of I/R injury and is also an important factor for the arrhythmia induced by ischemia and reperfusion[16]. Usually, antioxidative ability of heart is evaluated by SOD and MDA activities. SOD is one of important antioxidases that scavenges oxygen-free radicals and MDA is an end-product of free radical chain reaction and lipid peroxidation. There was a report that polydatin protected brain and intestine against the injury induced by oxide through decreasing the content of MDA and increasing the activity of SOD in brain and intestine[8-10]. There was also a report that polydatin protected primarily cultured rat hepatocytes against CCl_4-induced injury by inhibiting the formation of MDA in rat hepatocytes[17]. Consisted with above research results, our present study showed that polydatin enhanced myocardial antioxidative ability, which might be another mechanism of cardiac protection of polydatin against I/R injury.

NO is an important messenger in the cardiovascular regulation and also played an important role in protecting myocardium against I/R 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, the NOS inhibitor *L*-NAME abolished the effect of *L*-arginine on I/R injury[18,19]. The results of present study showed that polydatin increased myocardial NO production and *L*-NAME reversed the protective effect of polydatin on heart, suggesting that NO plays a key role in polydatin-induced cardioprotection. There were reports that NO could maintain coronary vasodilator tone, scavenge free radicals and exert antioxidative activity[20,21]. According to our experimental results, we hypothesize that polydatin protected heart against I/R injury through increasing CF and antioxidative ability as a result of increasing NO production.

NO is formed from *L*-arginine and oxygen by NOS. There are three known isoforms of the enzyme, all of which are expressed in the heart: neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. eNOS and nNOS together are also called cNOS. The main source of cardiac NO is generated through eNOS expressed by coronary endothelial cells and cardiac myocytes. Whereas nNOS expression is low in the heart and iNOS is usually expressed in response to various physiological and pathophysiological stimuli, such as intense exercise and hypoxia. Recent studies have shown that eNOS plays an important role in protecting myocardium against I/R injury[22,23]. In addition, studies have indicated that the cardioprotective effects of late preconditioning observed after 24 h resulted from the upregulation of NOS and, more specifically, of iNOS[24,25]. Our experiment showed that NOS and cNOS activities were increased in polydatin group compared with that in the control group, suggesting that increase in NO production resulted from increase in cNOS activity.

In summary, polydatin had a significant cardioprotection against I/R injury, which was mediated by increase in cNOS and NO production and consequently by increase in CF and antioxidation of myocardium.

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