

Original Article

Pterostilbene protects against myocardial ischemia-reperfusion injury via activating eNOS in diabetic rats

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Received July 11, 2016; Accepted September 1, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Diabetic patients are more susceptible to myocardial ischemic injury. Pterostilbene (Pte), an analog of resveratrol, exerts a cardioprotective effect. However, the protective effect of Pte against myocardial ischemia-reperfusion (MI/R) injury in diabetes remains unclear. Male SD rats were fed with high-fat diet and injected with streptozotocin once to induce diabetes. Rats were injected intraperitoneally with Pte before subjected to MI/R injury. The left ventricular function, infarct size, serum LDH and CK-MB levels, cell apoptosis and oxidative stress were assessed. Pte treatment improved cardiac function and reduced infarct size. Moreover, Pte reduced malonaldehyde (MDA) content and increased superoxide dismutase (SOD) activity. Additionally, Pte increased eNOS phosphorylation in diabetic rats. In conclusion, Pte reduces diabetes-exacerbated MI/R injury and oxidative stress via activating eNOS in diabetic rats, suggesting that Pte may be a promising therapeutic agent for myocardial ischemia in diabetic patients.

Keywords: Pterostilbene, diabetes, myocardial ischemia-reperfusion injury, eNOS

Introduction

Type 2 diabetes mellitus has been a serious problem to public health with rapidly increasing incidence worldwide [1, 2]. Numerous studies have suggested that cardiovascular diseases are the leading cause of morbidity and mortality among diabetic patients [3, 4]. Diabetic patients are more susceptible to myocardial ischemic injury than non-diabetic patients, with worse clinical prognosis and higher mortality [5, 6]. However, the effective strategy is limited for the treatment of myocardial ischemia in diabetic patients. So, it is urgent to seek new therapeutic targets against ischemic heart diseases under diabetic condition.

Pterostilbene (Pte), a natural dimethylated analog of resveratrol from blueberries, is known to confer diverse pharmacological activities such as anti-cancer, anti-inflammation and anti-oxidation activities [7]. Under most circumstances, Pte is either equally or significantly more potent than resveratrol. Pte may have greater biological activity due to better bioavailability

resulting from the substitution of a hydroxyl group with a methoxyl group, which increases the molecule's lipophilicity [8]. Pte has been shown to be beneficial for some cardiovascular diseases, such as myocardial ischemia and abnormal vascular smooth muscle cells proliferation [9, 10]. And Pte has been suggested to be beneficial against diabetes [11]. However, the effect of Pte on myocardial ischemia-reperfusion (MI/R) in diabetic condition remains elusive. Therefore, this study aims to investigate the effect of Pte treatment on MI/R injury in diabetic rats and further explore the underlying mechanisms.

Materials and methods

Animals

Male Sprague-Dawley rats (250-300 g) were purchased from Southern Medical University Laboratory Animal Center, China. Rats were kept with free access to standard rat chow and water in accordance with the principles of the Animal Management Rule of the Minis-

try of Health, People's Republic of China (Document No. 55, 2001) and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised, 1996). All study protocols were approved by the Southern Medical University Animal Care Committee (Guangzhou, China).

Induction of type 2 diabetes

The high-fat diet-fed and streptozotocin-induced (HFD-STZ) type 2 diabetic rat model was developed according to previous studies [12] by providing HFD (D12451, Research Diets, NJ, USA) containing 45% fat (kcal%), 35% carbohydrate, and 20% protein for 4 weeks and then one-shot injection of streptozotocin (STZ) (40 mg/kg i.p.) (Sigma, St. Louis, MO, USA). HFD was continuously fed after STZ injection, and then hyperglycemic rats (fasting blood glucose ≥ 11.1 mmol/L, from at least three samplings) 1 week after STZ injection were considered to have developed type 2 diabetes and were studied. A normal non-diabetic control group was included and fed with a control diet (10% fat, 20% protein, and 70% carbohydrates, D12450H, Research Diets).

Myocardial ischemia-reperfusion model and experimental protocol

The rats were anesthetized by intraperitoneal (i.p.) administration of 3% pentobarbital sodium. Myocardial ischemia was induced by reopening the chest followed by a slipknot (6-0 silk suture) around the LAD coronary artery about 2-3 mm near its origin. Regional myocardial ischemia was verified by the development of a pale color in the ischemic area and changes of electrocardiogram (ST-segment elevation). The slipknot was loosened after 30 min of ischemia, and the ischemic myocardium was reperfused for 3 h. In sham rats, the silk suture was passed underneath the LAD artery without ligation.

Rats were randomly assigned into five experimental groups: (1) NS-non-diabetic sham rats (n = 10); (2) NIR-non-diabetic rats receiving vehicle were subjected to I/R (n = 10); (3) DS-diabetic sham rats (n = 15); (4) DIR-diabetic rats receiving vehicle were subjected to I/R (n = 15); (5) Pte-diabetic rats receiving Pte were subjected to I/R (n = 15). Pte was dissolved in normal saline with 0.05% dimethyl

sulfoxide (DMSO) and was administered intraperitoneally at a dose of 10 mg/kg body weight 20 min after ischemia induction. The same volume of vehicle was administered at the same time point.

Cardiac function assessment

Cardiac function was monitored continuously during the whole period of I/R. A microcatheter was inserted into the left ventricular cavity under anesthesia through the right common carotid artery to assess the left ventricular developed pressure (LVDP). Hemodynamic data were recorded on a polygraph (RM-6240C; Chengdu Instrument Co., LTD, China). The maximal rate of rise and decline of left ventricular pressure (\pm LV dP/dt_{max}) were derived by digital computer algorithms.

Myocardial infarct size measurement

The slipknot around the LAD coronary artery was retied at the end of the reperfusion, and 1 mL of 1% Evans blue dye was injected into the aortic artery. The heart was then quickly excised and frozen at -80°C . After that, the frozen heart was sliced transversally into 1-mm thick sections and then incubated at 37°C for 30 min in 2% TTC solution. Then, digital images were captured and analyzed. TTC-unstained pale area (infarct zone), TTC-stained red area (ischemic but viable myocardium) and Evans blue-unstained regions (area-at-risk, AAR) were analyzed by using Image Pro Plus 6.0. Myocardial infarct size was calculated as a percent of infarct zone (INF) over total AAR (INF/AAR \times 100%).

Assay of serum creatine kinase-MB and lactate dehydrogenase

After the 3 h reperfusion, blood samples were collected from the carotid artery. Serum creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) levels were determined with the use of commercial kits (Nanjing Jiancheng Bioengineering, China). The activities of these two enzymes were expressed as U/L.

Myocardial apoptosis assay

After the reperfusion, the hearts were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h at room temperature. The fixed tissues were then embedded in paraffin, and TUNEL staining was performed according to the ma-

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Table 1. Animal blood glucose level

Group	NS	NIR	DS	DIR	Pte
Glucose (mmol/L)					
Baseline	4.52±0.08	4.42±0.07	4.53±0.06	4.47±0.05	4.47±0.07
Diabetes induction	4.67±0.11	4.86±0.15	16.24±2.87*	15.73±2.91*	16.27±2.85*

NS: Non-diabetic sham; NIR: Non-diabetic ischemia/reperfusion + vehicle; DS: Diabetic sham; DIR: Diabetic ischemia/reperfusion + vehicle; Pte: Diabetic ischemia/reperfusion group + Pte treatment. Results presented are mean ± SEM. * $P < 0.05$ vs. baseline, $n = 6$.

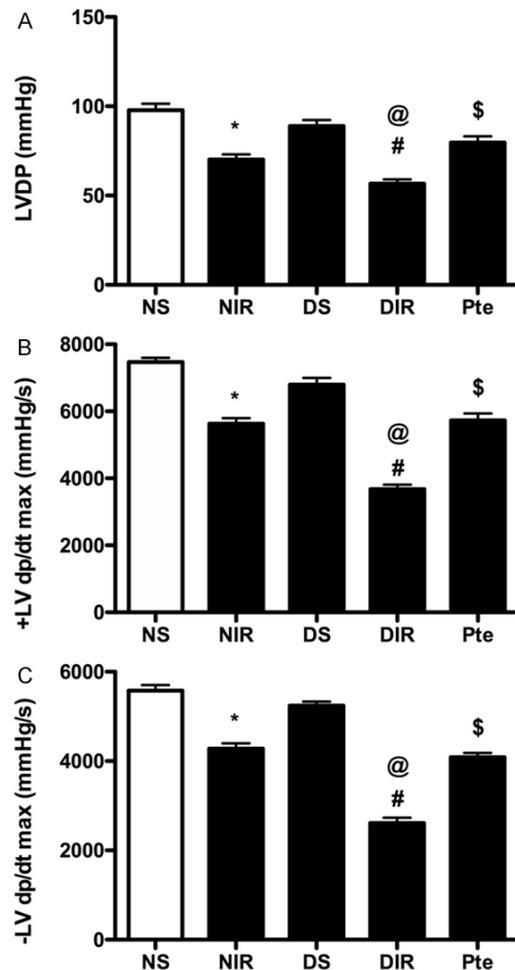


Figure 1. Pterostilbene treatment improved cardiac function after MI/R in diabetic rats. A. LVDP, left ventricular developed pressure. B. +LV dp/dt_{max}. C. -LV dp/dt_{max}. NS, non-diabetic sham rats; NIR, non-diabetic I/R rats receiving vehicle; DS, diabetic sham rats; DIR, diabetic I/R rats receiving vehicle; Pte, diabetic I/R rats receiving Pte. Values are mean ± SEM, $n = 6$. * $P < 0.05$ vs. NS; # $P < 0.05$ vs. NIR; @ $P < 0.05$ vs. DS; \$ $P < 0.05$ vs. DIR.

nufacturer's instructions. All nuclei were stained by DAPI. Apoptotic index was calculated as the percentage of stained, apoptotic cells × 100/total number of nucleated cells. Myocardial caspase-3 activity was determined as de-

scribed previously [13] by using a caspase colorimetric assay kit (Chemicon, Temecula, CA, USA) according to manufacturer's protocol.

Oxidative damage measurement

The MDA level and the activity of SOD in the heart homogenates were determined with the application of commercial kits (Nanjing Jiancheng Bioengineering, China). The data was analyzed using a microplate reader (Multiskan Spectrum, Thermo Scientific, USA).

Myocardial eNOS activity

The AAR of rat hearts was homogenized in 0.9% NaCl. The tissue homogenate was centrifuged at 12,000 g for 10 min at 4°C, and the supernatant was collected to determine myocardial eNOS activity using a spectrophotometrical assay kit (Nanjing Jiancheng Bioengineering).

Western blot

The proteins from the rat hearts were extracted in RIPA buffer containing protease inhibitors. The concentration of the protein extracted from each specimen was quantified using the BCA protein assay kit. After separating the proteins by SDS-PAGE, they were transferred to NC membranes (Millipore, MA, USA). The membranes were probed with the primary antibodies against p-eNOS (phosphorylation at Ser 1177), or β-actin (1:1000 in TBST) overnight at 4°C. After washing three times with TBST, the membranes were incubated with the secondary antibody in TBST for 2 h at 37°C, then washed. The positive protein bands were developed using a chemiluminescence system, and the bands were scanned and quantified by densitometric analysis using an image analyzer Quantity One System (Bio-Rad, Richmond, CA, USA).

Statistical analysis

All values are presented as mean ± SEM. Statistical tests were performed using GraphPad

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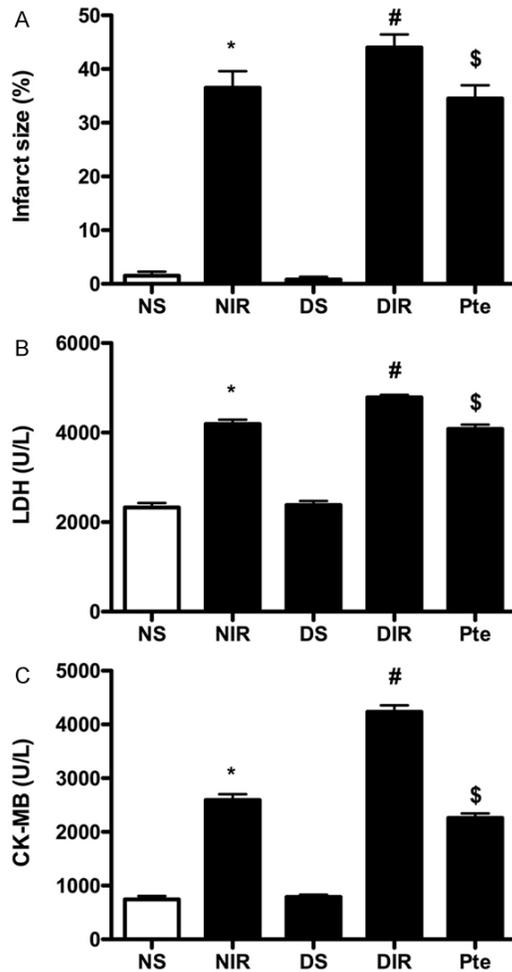


Figure 2. Pterostilbene treatment attenuated MI/R injury in diabetic rats. A. Myocardial infarct size expressed as a percent of infarct area (INF) over total area at risk (AAR). B. Serum lactate dehydrogenase (LDH) level. C. Serum creatine kinase-MB (CK-MB) level. NS, non-diabetic sham rats; NIR, non-diabetic I/R rats receiving vehicle; DS, diabetic sham rats; DIR, diabetic I/R rats receiving vehicle; Pte, diabetic I/R rats receiving Pte. Values are mean \pm SEM, $n = 6$. * $P < 0.05$ vs. NS; # $P < 0.05$ vs. NIR; \$ $P < 0.05$ vs. DIR.

Prism software version 5.0 (GraphPad Software, Inc., San Diego, CA, USA). Differences among comparisons were evaluated with one-way ANOVA followed by Bonferroni corrected t test where appropriate. $P < 0.05$ was taken as statistically significant.

Results

The blood glucose level of animals

As shown in **Table 1**, compared with the non-diabetic rats, diabetic animals manifested sig-

nificantly increased blood glucose, indicating that type 2 diabetic model was created in this study. Pte treatment did not show any significant effect on blood glucose level.

Pte treatment improved cardiac function in diabetic MI/R rats

As shown in **Figure 1**, there were no significant differences in LVDP and \pm LV dP/dt_{max} between NS and DS rats during I/R period. However, after MI/R, DIR rats showed aggravated myocardial functional impairment compared with NIR as evidenced by decreased LVDP and \pm LV dP/dt_{max} during I/R period ($n = 6$, $P < 0.05$). Pte treatment significantly elicited a significant recovery in LVDP and \pm LV dP/dt_{max} in diabetic MI/R rats compared with the DIR rats ($n = 6$, $P < 0.05$).

Pte treatment attenuated MI/R injury in diabetic rats

As shown in **Figure 2**, compared with NS rats, there were obvious myocardial infarction and increased serum CK-MB and LDH levels in NIR rats ($n = 6$, $P < 0.05$). Larger infarct size and further increased serum CK-MB and LDH levels were observed in DIR rats ($n = 6$, $P < 0.05$), indicating that diabetes aggravated MI/R injury. Pte treatment significantly reduced infarct size and serum CK-MB and LDH levels in diabetic rats compared with the DIR rats ($n = 6$, $P < 0.05$).

Pte treatment attenuated myocardial apoptosis in diabetic MI/R rats

As shown in **Figure 3**, the percentage of TUNEL-positive cells and myocardial caspase-3 activity were significantly increased in the NIR group compared with the NS group ($n = 6$, $P < 0.05$). Diabetic MI/R rats showed further increased myocardial apoptotic index and caspase-3 activity compared with NIR group ($n = 6$, $P < 0.05$). Pte treatment significantly reduced myocardial apoptosis in diabetic rats compared with the DIR rats ($n = 6$, $P < 0.05$).

Pte treatment attenuated oxidative stress in diabetic MI/R rats

As shown in **Figure 4**, the activity of SOD was significantly decreased in the NIR group compared with the NS group ($n = 6$, $P < 0.05$). Diabetic MI/R rats showed further decreased SOD

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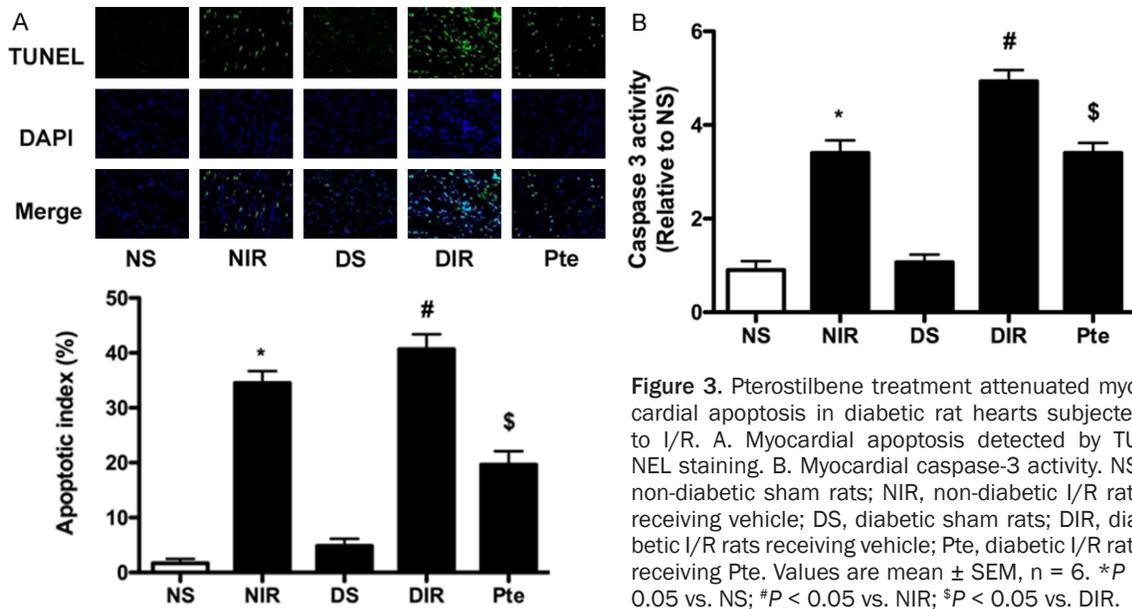


Figure 3. Pterostilbene treatment attenuated myocardial apoptosis in diabetic rat hearts subjected to I/R. A. Myocardial apoptosis detected by TUNEL staining. B. Myocardial caspase-3 activity. NS, non-diabetic sham rats; NIR, non-diabetic I/R rats receiving vehicle; DS, diabetic sham rats; DIR, diabetic I/R rats receiving vehicle; Pte, diabetic I/R rats receiving Pte. Values are mean \pm SEM, n = 6. * P < 0.05 vs. NS; # P < 0.05 vs. NIR; \$ P < 0.05 vs. DIR.

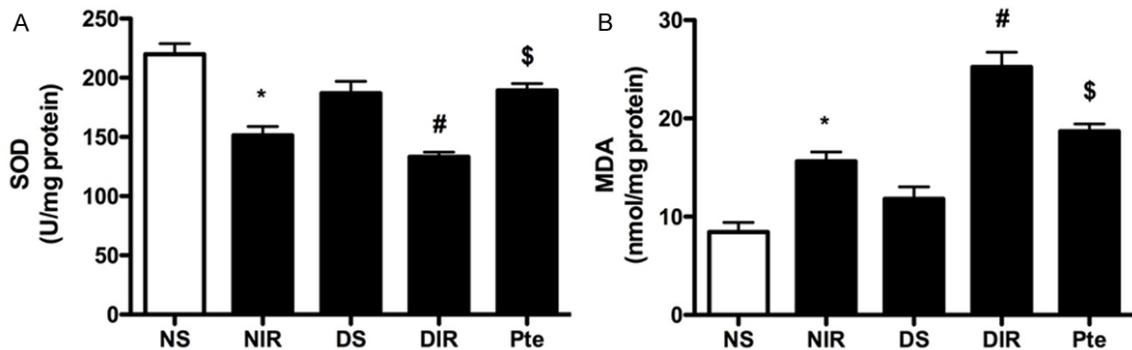


Figure 4. Pterostilbene treatment attenuated oxidative stress in diabetic MI/R rats. A. SOD activity. B. MDA content. NS, non-diabetic sham rats; NIR, non-diabetic I/R rats receiving vehicle; DS, diabetic sham rats; DIR, diabetic I/R rats receiving vehicle; Pte, diabetic I/R rats receiving Pte. Values are mean \pm SEM, n = 6. * P < 0.05 vs. NS; # P < 0.05 vs. NIR; \$ P < 0.05 vs. DIR.

activity (n = 6, P < 0.05). Pte treatment significantly increased SOD activity in diabetic rats compared with the DIR rats (n = 6, P < 0.05). On the contrary, there was a marked increase in MDA content in the NIR group compared with the NS group (n = 6, P < 0.05). Diabetic MI/R rats showed further increased MDA content (n = 6, P < 0.05). Pte treatment significantly decreased MDA content in diabetic rats compared with the DIR rats (n = 6, P < 0.05).

Pte treatment increased eNOS activity and eNOS phosphorylation

As shown in **Figure 5**, the level of p-eNOS was significantly decreased in NIR rats compared

with that in NS rats (n = 6, P < 0.05). Diabetic MI/R rats showed further decreased p-eNOS level (n = 6, P < 0.05). Pte treatment significantly increased p-eNOS level in diabetic rats compared with the DIR rats (n = 6, P < 0.05). Similarly, Pte treatment significantly increased eNOS activity in diabetic rats compared with the DIR rats (n = 6, P < 0.05).

Discussion

In this study, we provide evidence that Pte is protective against MI/R injury in diabetes. First, we found that Pte alleviated MI/R injury and improved cardiac function in diabetic rats. Second, the cardioprotective effects of Pte are

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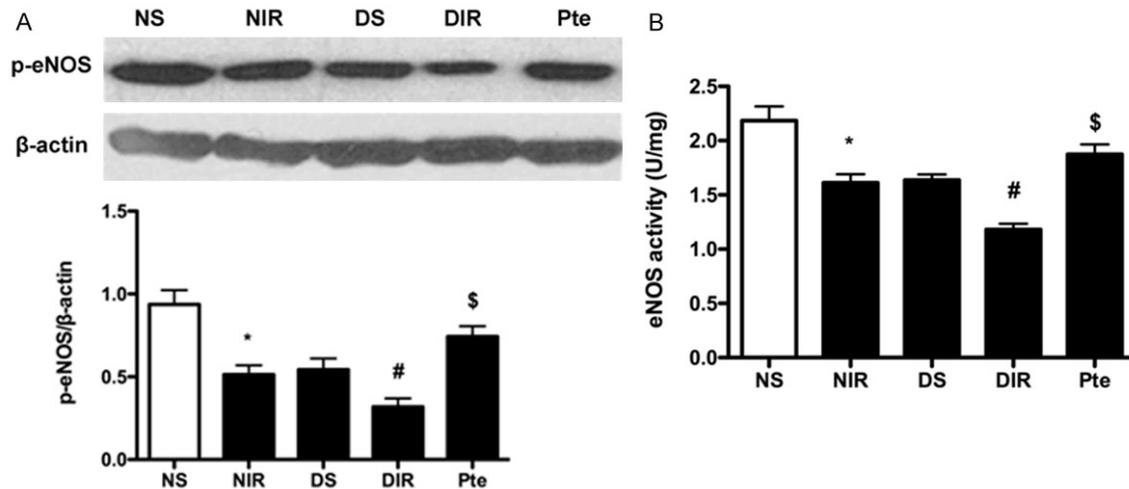


Figure 5. Pterostilbene treatment increased eNOS phosphorylation and eNOS activity. A. The expression of p-eNOS detected by Western blot. B. eNOS activity. NS, non-diabetic sham rats; NIR, non-diabetic I/R rats receiving vehicle; DS, diabetic sham rats; DIR, diabetic I/R rats receiving vehicle; Pte, diabetic I/R rats receiving Pte. Values are mean \pm SEM, n = 6. * P < 0.05 vs. NS; # P < 0.05 vs. NIR; \$ P < 0.05 vs. DIR.

associated with its inhibition of oxidative stress. Third, the mechanism of the cardioprotection is mediated by eNOS activation.

Accumulating researches have demonstrated that the diabetic heart is more sensitive to I/R injury [14, 15]. It has been suggested that diabetes can exacerbate MI/R injury and blunt the protective effect of various therapeutic agents [16, 17]. Therefore, novel strategies are urgently needed to reduce myocardial susceptibility to I/R injury in diabetes. To investigate this issue, high-fat diet-fed and streptozotocin-induced (HFD-STZ) type 2 diabetic animal model was developed in the present study. HFD elicited insulin resistance and STZ administration reduced insulin levels, so the animals were unable to maintain normal glucose levels and develop hyperglycemia. This model has been suggested to be suitable for studying the pathophysiology of type 2 diabetes as well as for testing agents for the treatment of type 2 diabetes in several studies [18, 19].

Pte, a natural dimethylated analog of resveratrol from blueberries, has been suggested to confer protection against diabetes [11]. In this study, we found that Pte alleviated cardiac dysfunction and myocardial injury (as evidenced by increased \pm LV dP/dt_{max} and decreased serum CK-MB and LDH activities, myocardial infarction and cardiomyocyte apoptosis) in diabetic rats, suggesting that Pte treatment reduces

myocardial susceptibility to I/R injury in diabetic rats.

Myocardial oxidative stress contributes to diabetic pathophysiology and hyperglycemia enhances oxidative stress, and reduces antioxidant defenses [20]. MDA is an unsaturated fatty acid in free radical and lipid peroxidation metabolites, which is an indirect marker of cellular damage degree. MDA content reflects the extent of systemic lipid peroxidation. On the contrary, the antioxidant SOD protects cells by reducing free radical-induced injury. SOD level reflects the capacity to scavenge oxygen free radicals. In the present study, myocardial MDA content was increased in the diabetic animal group, which was further increased by MI/R. Myocardial SOD content was attenuated in the diabetic animal group, which was further decreased by MI/R. The results suggest that hyperglycemia-enhanced oxidative stress may exacerbate MI/R injury. However, Pte treatment decreased MDA content and increased SOD activity in the diabetic MI/R rats.

It has been suggested that eNOS activation reduces myocardial infarct size and improves cardiac function in MI/R [21]. In the physiological conditions, eNOS phosphorylation and subsequent NO production exert anti-apoptotic and anti-oxidative effects in I/R hearts [22]. Decreased myocardial eNOS-NO availability has been found in both patients and animals

with diabetes, which leads to the development of diabetic complications [23]. In the present study, we found that p-eNOS level and eNOS activity was reduced in diabetic hearts, and Pte treatment increased p-eNOS level and eNOS activity in the diabetic MI/R rats.

In conclusion, the current study demonstrated that Pte is protective in reducing myocardial susceptibility to I/R injury under diabetic condition. Pte reduces diabetes-exacerbated MI/R injury and oxidative stress via modulating eNOS activity in diabetic rats. The findings suggest Pte may be a promising novel drug for cardiac complications in diabetes patients.

Acknowledgements

This study was supported by Grants from the Shenzhen Municipal Science and Technology Innovation Committee (jcyj20150403101028-192, 20150314161839), Grants from the Health and Family Planning Commission of Shenzhen Municipality (201504001), and Science and Technology Innovation Foundation of Shenzhen (JCYJ20160422151912249).

Disclosure of conflict of interest

None.

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