Puerarin reduces ischemia/reperfusion-induced myocardial injury in diabetic rats via upregulation of vascular endothelial growth factor A/angiotensin-1 and suppression of apoptosis

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Abstract. Puerarin is an active ingredient of pueraria, which has been developed for puerarin injections, used in the treatment of cardiovascular diseases including arrhythmia, myocardial ischemia and hypertension. However, the molecular mechanisms of puerarin on ischemia/reperfusion (I/R)-induced myocardial apoptosis in diabetic rats are not fully understood. The present study aimed to investigate whether puerarin can attenuate I/R-induced myocardial apoptosis in diabetic rats, and to investigate the underlying mechanism. A hemodynamic analyzing system was employed to analyze the left ventricular developed pressure (LVDP), the left ventricular end-systolic interior dimension (LVIDs) and the left ventricular end diastolic interior dimension (LVIDd). ELISA kits were used to analyze malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels, NO production and caspase-3 activity. Nuclear factor (NF)-κB, ascular endothelial growth factor A (VEGFA), angiotensin (Ang)-I, phosphorylated (p)-endothelial nitric oxide synthase protein expression was analyzed using western blot analysis. Puerarin significantly reduced the myocardial infarct area, and increased left ventricular developed pressure in diabetic rats with myocardial I/R. Oxidative stress, inflammation and nuclear factor-κB protein expression were significantly reduced by puerarin. Furthermore, puerarin activated the protein expression levels of VEGFA and Ang-I, and increased nitric oxide production, phosphorylated-endothelial nitric oxide synthase protein expression and caspase-3 activity. These results demonstrated that the myocardial protective effect of puerarin serves to reduce myocardial I/R injury, via upregulation of VEGFA/Ang-1 and suppression of apoptosis, in diabetic rats with myocardial I/R.

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Introduction

Diabetes mellitus (DM) is a chronic disease that exhibits genetic susceptibility (1). DM is caused by decreased levels or an absolute lack of insulin, resulting from the interaction of various internal and external factors, and is characterized by high blood glucose and lipid metabolism of sugar (1). Diabetic patients are disposed to complications in early and late stages, including nephropathy, eye disease, foot pathologies and cardiomyopathy; diabetic cardiomyopathy belongs to the cardiovascular complications of diabetes and is the predominant cause of mortality in patients with diabetes (2).

Angiotensin-converting enzyme (ACE)2 is the only homologue of ACE that has been identified to date; it exhibits 42% homologous sequences with ACE (3). ACE2 is an inner membrane carboxypeptidase that can hydrolyze angiotensin 1 (Ang-1) into Ang-(1-9), and this may be further hydrolyzed by ACE into Ang-(1-7) (4). ACE2 can also remove the terminal tryptophan of Ang-II, thus directly hydrolyzing Ang-II to Ang-(1-7). Furthermore, *in vitro* studies have demonstrated that the enzymatic activity of ACE2 on Ang-II is 400 times of that on Ang-I (4). The binding of Ang-(1-7) with its specific G-protein coupled receptor Mas promotes the release of bradykinin, nitric oxide (NO) and prostaglandins, which serve roles in vessel expansion, decreasing blood pressure, improving insulin resistance, resisting inflammation, inhibiting cell proliferation, anticoagulation and protecting the vascular endothelium (5). ACE2 hydrolyzes vasopressin, neurotensin and dynorphin A-(1-13), which are involved in cardiovascular

regulation. Cardiovascular protection is enabled by maintaining homeostasis of the renin-angiotensin system, by modulating the antagonizing effect of the ACE2/Ang-(l-7)/Mas receptor axis on the ACE/Ang-1/Ang-II-type 1 (ATI) receptor axis (6).

The vascular endothelial growth factor (VEGF) family serves a major role in the regulation of the angiogenesis network. VEGF has direct effects on vascular endothelial cells by promoting their proliferation and migration; VEGF is also a critical regulator of angiogenesis in the formation human tumors (7). Furthermore, VEGF may be involved in regulating the interaction between endothelial cells and between endothelial cells and the basement membrane, by interacting with receptors on vascular endothelial cells (8).

Diabetic cardiomyopathy (DCM) is a chronic and complex pathological process that can alter cell metabolism, resulting in damage to the endoplasmic reticulum, mitochondria and other organelles, and ultimately result in myocardial hypertrophy and increased apoptosis (9). Furthermore, apoptosis resulting from altered energy metabolism of the cell serves an additional role in the pathology of DCM; large scale apoptosis can aggravate myocardial remodeling and cause further tissue dysfunction, thus creating a destructive pathogenic cycle (10,11).

The active ingredients of pueraria constitute isoflavones, including daidzin, daidzein and puerarin (12). Puerarin has wide pharmacological effects (12); aside from its application in the treatment of cardiovascular and cerebrovascular diseases, with broad prospects for development and clinical application (13). Puerarin inhibits lipid peroxidation and aldose reductase activity, removes superoxide ion radicals, and has a protective effect on endothelial cells (14). Furthermore, puerarin may significantly slow the glucosamine metabolism of endothelial cells, reduce endothelin and platelet surface activity, inhibit platelet aggregation and adhesion, lower blood lipids, cholesterol, blood viscosity and prevent thrombosis (14). Therefore, puerarin may be useful in the protection of vascular endothelial cells, the promotion of angiomalacia and the inhibition of atherosclerosis (15). The present study investigated whether the myocardial protective effects of puerarin can attenuate ischemia/reperfusion (I/R)-induced myocardial apoptosis in diabetic rats, via upregulation of VEGFA/Ang-1 and suppression of apoptosis in rats, and thus exert cardioprotective effects in diabetics.

Materials and methods

Animals and experimental groups. Male Sprague-Dawley rats (8-10 weeks old, n=40) weighing 200-220 g were housed at 22-23˚C, 55-60% humidity, 12 h light/dark cycle and access free to food and water, and randomly assigned into five groups: Sham (n=6); ischemia/reperfusion (I/R; n=10); I/R+L (lower dosage, n=8, 25 mg/kg puerarin); I/R+M (medium dosage, n=8, 50 mg/kg puerarin), I/R+H (higher dosage, n=8, 100 mg/kg puerarin). Rats were injected intraperitoneally with streptozotocin (30 mg/kg) twice, with a rest day between each injection. The following week, the myocardial I/R model was induced with 35 mg/kg pentobarbital sodium; the heart was exteriorized with a left thoracic incision, and a slipknot made using 4-0 silk was placed around the left anterior descending coronary artery. Ischemia was performed for 30 min, the slipknot was released and reperfusion was allowed to occur for 3 h. Rats of the sham group were anesthetized with 35 mg/kg pentobarbital sodium and underwent a sham surgery in which the heart was exteriorized without myocardial I/R. The following day, myocardial I/R rats were gavaged with 25, 50 or 100 mg/kg/puerarin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) every 2 days over 4 weeks. The present study was approved by the ethics committee of Tianjin First Central Hospital (Tianjin, China).

Assessments of myocardial function. After treatment with puerarin for 4 weeks, A hemodynamic analyzing system (Chengdu Xinjin Shifeng Medical Apparatus & Instrument Co., Ltd., Chengdu, China) was employed to analyze the left ventricular developed pressure (LVDP), the left ventricular end-systolic interior dimension (LVIDs) and the left ventricular end diastolic interior dimension (LVIDd) as described in a recent study (16). Under anesthesia (35 mg/kg of pentobarbital sodium), the coronary artery was ligated; after 4 h, the left ventricle was stored at -80˚C for 30 min. The left ventricle was subsequently sliced into 4-mm thick sections to assess the size of the infarct. The heart area assessed was the ischemic heart muscles. The infarct size area was assessed by volume and weight as a percentage of the left ventricle.

Determination of malondialdehyde (MDA), superoxide dismutase (SOD), tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels, NO production and caspase-3 activity. Rats were anesthetized and blood was collected from the eye socket of each rat. Plasma was centrifuged at 8,000 x g for 10 min at 4˚C. ELISA kits obtained from Wuhan Elabscience Biotechnology Co., Ltd., (Wuhan, China) were employed to analyze the levels of MDA (E-EL-0060c), SOD (E-EL-R1424c), TNF-α (E-EL-R0019c) and IL-6 (E-EL-R0015c). Total NO production was measured using an NO analyzer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Caspase-3 activity was measured using a Caspase 3 Activity Assay kit (Beyotime Institute of Biotechnology, Haimen, China). Absorbance values were measured using a Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Western blot analysis. Heart tissue samples were lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) on ice for 30 min, and the supernatant was centrifuged at 8,000 x g for 10 min at 4˚C. Following quantification of the protein concentration using a Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), proteins (50 μg) were separated by 10% SDS-PAGE and transferred onto Immobilon-FL transfer membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4˚C with anti-nuclear factor (NF)-κB (sc-71675, 1:1,000), anti-VEGFA (sc-7269, 1:1,000), anti-Ang-1 (cat. no. sc-6320, 1:1,000), anti-phosphorylated (p)-endothelial nitric oxide synthase (eNOS; sc-293032, 1:1,000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-GAPDH (BM3876, 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China). Blots were subsequently washed 3 times with TBS with

0.1% Tween-20 (TBST), and incubated with goat anti-mouse immunoglobulin G-horseradish peroxidase secondary antibodies (sc-2005, 1:5,000; Santa Cruz Biotechnology) in TBST solution for 1 h at 37˚C. Bands were visualized using BeyoECL Star (P0018A; Beyotime Institute of Biotechnology) quantified using the Quantity image analyzer 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are expressed as the mean ± standard error (n=3) using SPSS 17.0 (SPSS, Inc. Chicago, IL, USA). Between-group differences were determined using one-way analysis of variance followed by Tukey's post-hoc analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Puerarin reduces the myocardial infarct area in diabetic myocardial I/R rats. The chemical structure of puerarin is presented in Fig. 1. The myocardial infarct area was markedly increased in the diabetic I/R model rats, compared with the sham control group. Treatment with puerarin for 4 weeks markedly reduced the myocardial infarct area, compared with untreated I/R diabetic rats (Fig. 2).

Puerarin increases the LVDP in diabetic myocardial I/R rats. A significantly attenuated LVDP in the diabetic I/R rat model group was observed, compared with the sham control group. Puerarin treatment increased the LVDP in diabetic myocardial I/R rats, compared with the diabetic I/R rat model group (Fig. 3A). Notably, a significant increase was observed in LVIDs and LVIDd in the diabetic I/R rat model group, compared with the control group (Fig. 3B and C). However, puerarin treatment markedly decreased LVIDs and LVIDd in diabetic I/R rats, compared with rats in the diabetic I/R model group.

Puerarin inhibits oxidative stress in diabetic myocardial I/R rats. MDA and SOD activity were measured to investigate the protective effect of puerarin on myocardial I/R in diabetic rats. Increased MDA activity (Fig. 4A) and decreased SOD activity (Fig. 4B) were observed in the diabetic myocardial I/R rat model, compared with the sham control group. Puerarin treatment reversed these alterations to MDA and SOD activity, compared with rats in the diabetic myocardial I/R model group (Fig. 4).

Puerarin inhibits inflammation in diabetic myocardial I/R rats. The protective effects of puerarin on myocardial I/R in diabetic rats were further examined by investigating the plasma levels of TNF-α and IL-6. TNF-α and IL-6 levels were significantly increased in the plasma of rats in the diabetic myocardial I/R model, compared with the sham control group (Fig. 5A and B, respectively). However, treatment with puerarin significantly reduced TNF-α and IL-6 levels, compared with rats in the diabetic myocardial I/R model group.

Puerarin inhibits NF-κB and VEGFA protein expression in diabetic myocardial I/R rats. NF-κB and VEGFA protein expression levels were measured to examined the

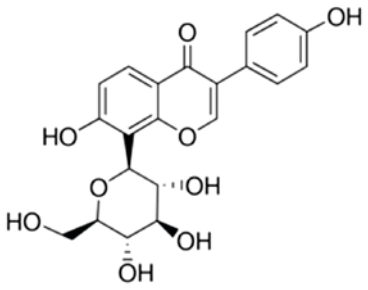

Figure 1. Chemical structure of puerarin.

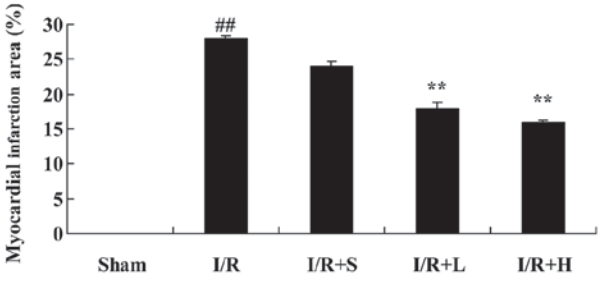

Figure 2. Puerarin reduces the myocardial infarct area in diabetic rats. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

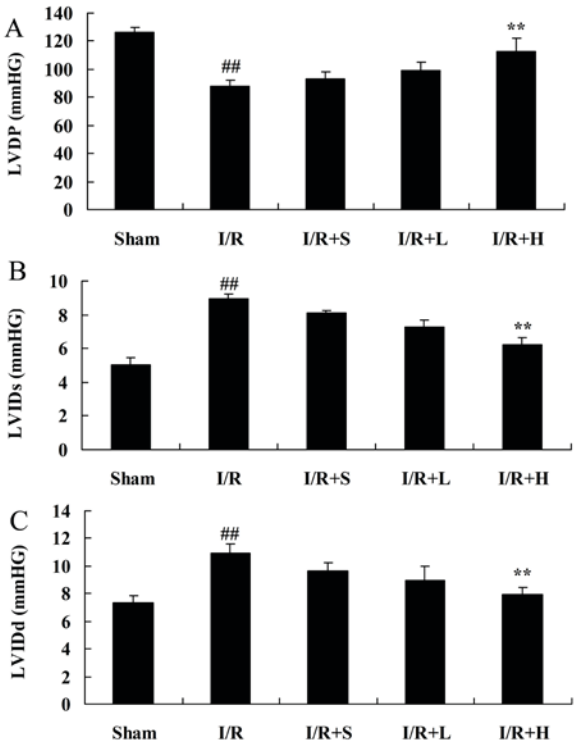

Figure 3. Puerarin increases the LVDP in diabetic myocardial I/R rats. Puerarin increases (A) LVDP and decreases (B) LVIDs and (C) LVIDd. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

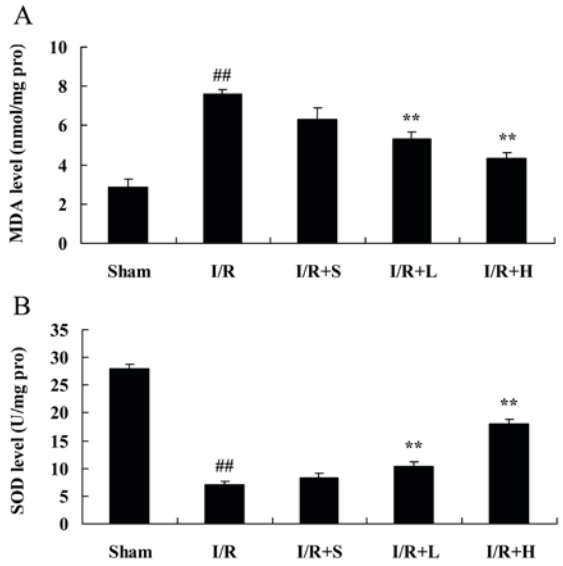

Figure 4. Puerarin inhibits oxidative stress in diabetic rats. Puerarin inhibits (A) MDA and (B) SOD activity in diabetic myocardial I/R rats. Data are expressed as the mean ± standard error. $^{\#\#}$P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

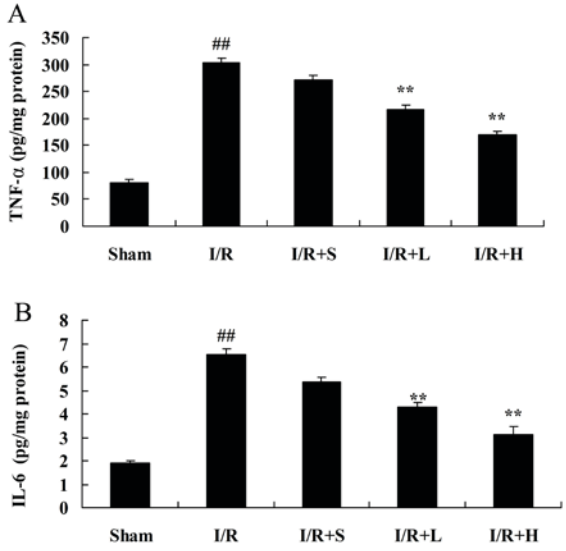

Figure 5. Puerarin reduces inflammation in diabetic myocardial I/R rats. Puerarin inhibits (A) TNF-α and (B) IL-6 levels. Data are expressed as the mean ± standard error. $^{\#\#}$P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

anti-inflammatory effect of puerarin on diabetic myocardial I/R in rats. NF-κB protein expression was significantly induced and VEGFA protein expression was significantly suppressed in diabetic myocardial I/R model rats, compared with the sham control group (Fig. 6). However, puerarin treatment significantly suppressed NF-κB and elevated VEGFA protein expression in puerarin-treated diabetic I/R rats, compared with the untreated diabetic I/R model rats.

Puerarin increases Ang-1 and p-eNOS protein expression and NO production in diabetic myocardial I/R rats. The role of Ang-1 and p-eNOS in puerarin-induced diabetic myocardial I/R protection was investigated. Inhibition of Ang-1 and p-eNOS protein expression and NO production was observed in diabetic myocardial I/R rats, compared with the sham control group (Fig. 7). Puerarin treatment significantly alleviated the I/R-induced inhibition of Ang-1 and p-eNOS protein expression and NO production, compared with the untreated diabetic myocardial I/R rats.

Puerarin decreases caspase-3 activity in diabetic myocardial I/R rats. To investigate whether puerarin protects against apoptosis, caspase-3 activity was assessed (Fig. 8). Increased activation of caspase-3 was observed in diabetic myocardial I/R rats, compared with the sham control group. Treatment with puerarin significantly decreased caspase-3 activity in untreated diabetic myocardial I/R rats.

Discussion

DCM is a chronic cardiac complication directly caused by diabetes, and is independent of coronary heart disease, hypertension and valvular heart disease (17,18). Early stage DCM predominantly manifests with left ventricular hypertrophy and diastolic dysfunction (19). The left ventricular ejection score at this stage may be normal or even elevated; however, systolic dysfunction and a decline in the ejection fraction value are presented as the disease progresses, and these may ultimately result in heart failure (20). DCM pathology also includes cardiac hypertrophy and apoptosis, heart wall thickening, capillary basement membrane thickening, capillary endothelial lesions and microvascular lesions (10). The pathogenesis of DCM is complex; high blood sugar has been recognized as a leading risk factor, accompanied by lipid toxicity (triglycerides in the blood), oxidative stress, inflammation, autonomic neuropathy, microvascular disease and activation of renin-angiotensin-aldosterone system (20). In addition, mitochondrial dysfunction and epigenetic changes have been demonstrated to participate in the occurrence of DCM (21). The results of the present study indicated that puerarin may reduce the myocardial infarct area, increase LVDP and decrease LVIDs and LVIDd in diabetic myocardial I/R rats.

High blood sugar and abnormal glucose metabolism induce excessive reactive oxygen species (ROS) production by the mitochondrial electron transportation chain. ROS and oxidative stress can cause DNA damage, meanwhile activating a DNA repair enzyme, poly ADP-ribose polymerase (22). Advanced glycation endproducts can activate nuclear factor kB (NF-κB) via binding to the galectin-3 receptor (23). The NF-κB pathway can regulate the expression of inflammation-related genes to increase the synthesis of TNF-α and interleukin, amongst others, resulting in myocardial damage (23). The present study discovered that puerarin may reduce oxidative stress and the expression of inflammatory cytokines and NF-κB, in diabetic myocardial I/R rats. Furthermore, Li *et al* (12) demonstrated that puerarin can reduce diabetic aorta injury via the suppression of NADPH oxidase-induced oxidative stress and NF-κB p65 in diabetic rats.

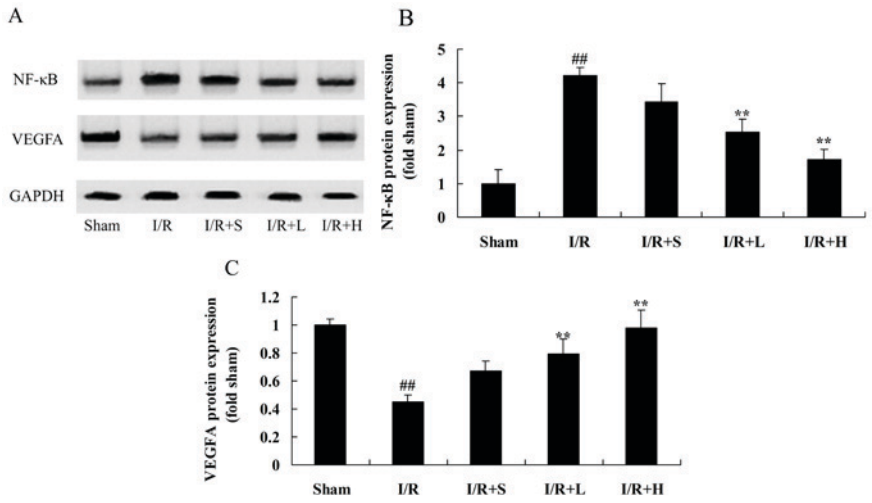

Figure 6. Puerarin inhibits NF-κB and increases VEGFA protein expression in diabetic myocardial I/R rats. (A) Western blot analysis indicated that puerarin inhibits NF-κB and increases VEGFA protein expression. Quantification of (B) NF-κB and (C) VEGFA protein expression. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

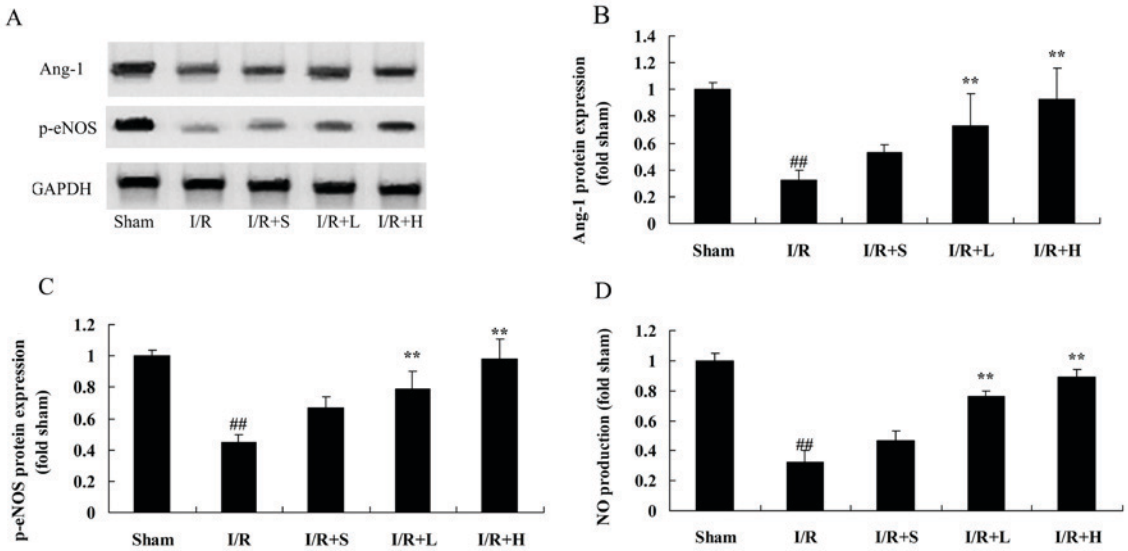

Figure 7. Puerarin increases Ang-1 and p-eNOS protein expression in diabetic myocardial I/R rats. (A) Western blot analysis indicated that puerarin inhibits Ang-1 and p-eNOS protein expression. Quantification of (B) Ang-1 and (C) p-eNOS protein expression. (D) Analysis of NO production. ##P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

Ang-(1-7) predominantly associates with the Mas receptor; however, a low amount of Ang-(1-7) also binds with the Ang-II type 2 (AT2) receptor (6). Association of Ang-(1-7) with the AT2 receptor can antagonize the ATI receptor; activation of eNOS promotes the release of NO, prostacyclin and other vasodilators; this increases the activity of bradykinin and thus antagonizes the effect of Ang-II (5). Furthermore, association of Ang-(1-7) with the Mas receptor can counteract the induction of vasoconstriction induced by Ang-II binding to the ATI receptor (24). In addition, VEGFA is a potent wound healing cytokine; the main functions of which include inducing angiogenesis, promoting endothelial cell proliferation and enhancing microvascular permeability, which results in widespread leakage of plasma proteins (25).

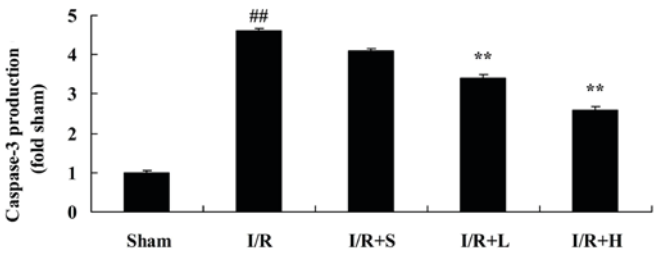

Figure 8. Puerarin reduces caspase-3 activity in diabetic myocardial I/R rats. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham group, **P<0.01 vs. myocardial I/R group. I/R, ischemia/reperfusion injury; Sham, sham group; I/R, myocardial I/R group; I/R+L, myocardial I/R + 25 mg/kg puerarin group; I/R+M, myocardial I/R + 50 mg/kg puerarin group; I/R+H, myocardial I/R + 100 mg/kg puerarin group. I/R, ischemia/reperfusion injury.

These proteins directly or indirectly alter the extracellular matrix components to form a temporary new matrix; this matrix supports the migration of endothelial cells and fibroblasts, which is conducive to wound repair. The present study demonstrated that puerarin significantly increases VEGFA, Ang-1 and p-eNOS protein expression, and activates NO production in diabetic myocardial I/R rats. Ai *et al* (15) demonstrated that puerarin accelerates cardiac angiogenesis and improves cardiac function via upregulation of VEGFA, Ang-1 and Ang-2.

Proliferation and apoptosis occur in the early stages of DCM cardiomyocyte hypertrophy; however, normal heart function can be maintained (26). As the disease develops, the myocardial intracellular environment becomes disordered; myocardial cells lose normal regulation and the rate of apoptosis exceeds the speed of cell proliferation; the apoptotic area increases, and the resulting loss of large numbers of cells eventually leads to a significant reduction in cardiac function (27,28). The regulation of apoptosis involves a series of complex cascades, the most important of which are the caspase-related apoptosis signal transduction pathways, which include the death receptor-mediated apoptosis signaling pathway, the mitochondrial/cytochrome *c*-mediated apoptosis pathway and the endoplasmic reticulum stress-mediated apoptosis pathway (29). In the present study, treatment with puerarin significantly decreased caspase-3 activity in diabetic rats. Notably, Iribarren *et al* (25) demonstrated that puerarin may protect against oxidative stress injury via the downregulation of caspase-3 in neural cells.

In conclusion, the results of the present study indicated that puerarin markedly reduces the myocardial infarct area, increases LVDP and decreases LVIDs and LVIDd in diabetic myocardial I/R rats. Furthermore, puerarin may reduce oxidative stress and the expression of inflammatory cytokines via the inhibition of NF-κB, upregulation of the VEGFA/Ang-1 signaling pathway and the suppression of apoptosis in diabetic rats. The results of the present study indicated that puerarin may be useful as a myocardial protective treatment to reduce cardiomyopathy in diabetic patients.

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Competing interests

The authors declare that they have no competing interests.

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