

## Original Article

# Puerarin protects against myocardial ischemia/reperfusion injury via the AMPK/Akt/GSK-3 $\beta$ /Nrf2 signaling pathway

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**Abstract:** Puerarin is an active isoflavones derivatives used for the treatment of ischemic diseases. The main purpose of the present study was to investigate the protective effect of Puerarin on ischemia/reperfusion (I/R) through AMPK/Akt/GSK-3 $\beta$ /Nrf2 pathway and explore its underlying mechanism. The index of myocardial injury, inflammatory biomarkers were measured, respectively. Proteins levels were investigated by Western blotting. The results demonstrated that puerarin can decline lactate dehydrogenase (LDH), serum creatinine kinase (CK) levels, attenuated serum interleukin-6 (IL-6), interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor (TNF- $\alpha$ ) production. Moreover, puerarin markedly enhanced the activities of superoxide dismutase (SOD) and reduced the amounts of malondialdehyde (MDA) in I/R rats. The expression of Nrf2, AMPK, AKT and GSK-3 $\beta$  were significantly increased by puerarin. It was assumed that Puerarin might be a new therapeutic candidate for the treatment of I/R possibly through the inhibition of the AMPK/Akt/GSK-3 $\beta$ /Nrf2 pathway.

**Keywords:** Puerarin, ischemia/reperfusion, AMPK/Akt/GSK-3 $\beta$ /Nrf2 signaling

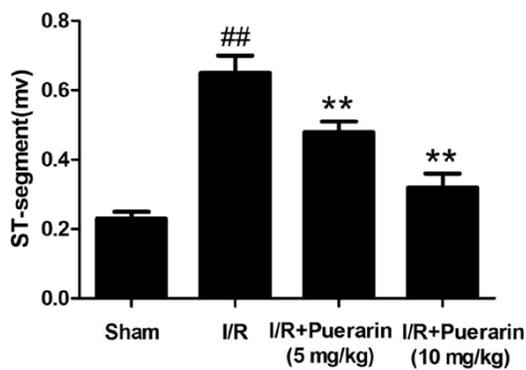
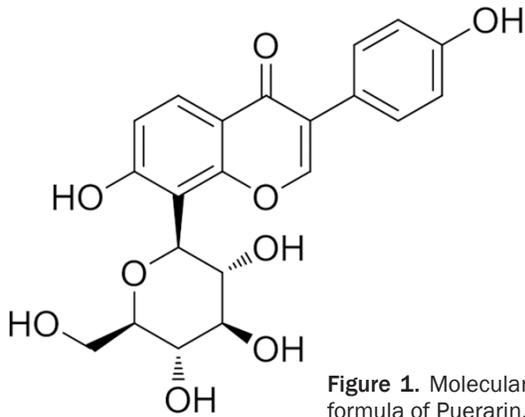
## Introduction

Ischemia heart disease continues to be the leading cause of human disability and mortality worldwide [1]. Early coronary reperfusion is the most effective strategy to alleviate the ischemic injury; however, reperfusion itself may lead to additional myocardial injury, a phenomenon known as ischemia/reperfusion (I/R) injury [2]. The pathophysiology of myocardial I/R injury have been reviewed everywhere [2, 3], and involve intracellular Ca<sup>2+</sup> overload and oxidative stress, which in turn initiate myocardial cell apoptosis and necrosis. However, to date no clinically approved therapy exists [3], highlighting the need to identify the new effective targets.

5'-AMP-activated kinase (AMPK) which was known to play a key role in regulating both glucose and fatty acid homeostasis and controlling whole body energy metabolism, has become one of the strategic cellular targets for the treatment of cardiovascular disease [4, 5].

Moreover, metabolism during myocardial ischemia appear to be affected by impairment of AMPK phosphorylation in the heart [6]. It has been proposed that pharmacological targeting of the Akt pathway may potentially diminish IR injury. The NF-E2-related factor (Nrf2) pathway is regarded as the most important factors associated with the cellular response to oxidative stress [7]. Nrf2 is a nuclear transcription factor that binds to antioxidant-response element (ARE) and regulates expression and coordinated induction of a battery of chemoprotective genes in response to antioxidants, oxidants, and radiations. Nrf2 is free from Keap1 and translocates into the nucleus where it up-regulates the expression of numerous cytoprotective phase II detoxifying enzymes and antioxidant genes [8]. Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine/threonine kinase that has versatile biological functions in cells, including regulation of metabolism, cell growth/death, and gene transcription [9]. Although both GSK-3 $\alpha$  and GSK-3 $\beta$

## Protective effects of puerarin against myocardial ischemia/reperfusion injury



**Figure 2.** Effects of Puerarin on ST-segment elevation. Values are expressed as means  $\pm$  SDs. Compared with Sham: \* $P < 0.05$ , \*\* $P < 0.01$ ; Compared with I/R: \* $P < 0.05$ , \*\* $P < 0.01$ .

appear to be equally important in certain aspects, GSK-3 $\alpha$  has a more critical role in regulating hepatic glucose metabolism and insulin sensitivity, and GSK-3 $\beta$  is the predominant regulator of glycogen synthase, Wnt signaling and sensitization to apoptosis [10]. Contrary to most signaling kinases, GSK-3 $\beta$  is active in unstimulated cells and sensitizes cells to death-promoting insults. In heart, GSK-3 has several important roles. Recently, inhibition of GSK-3 $\beta$  during ischemia and reperfusion (I/R) has been implicated as a cardioprotective mechanism [11]. However, the underlying mechanisms of cardioprotection afforded by GSK-3 $\beta$  in puerarin remain largely unknown.

Interestingly, various traditional Chinese medicine materials have been shown to safely suppress the pro-inflammatory and pro-fibrotic pathway and control myocardial fibrosis in several studies [12, 13]. Puerarin, [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one-8-( $\beta$ -D-

gluco-pyranoside)] (as shown in **Figure 1**), the major bioactive ingredient derived from the puerarialobata, has been widely used for thousands of years in traditional Chinese medicine. Recently, several reporters demonstrated it had healing and anti-fibrotic effects on cardiac remodeling through its anti-inflammatory, antioxidant effects [14, 15]. Since little is known about the effects of puerarin on GSK-3 $\beta$  and the relationship between GSK-3 $\beta$  and Nrf2. We performed this study to determine whether puerarin protected against myocardial ischemia/reperfusion injury via the AMPK/Akt/GSK-3 $\beta$ /Nrf2 signaling pathway.

### Materials and methods

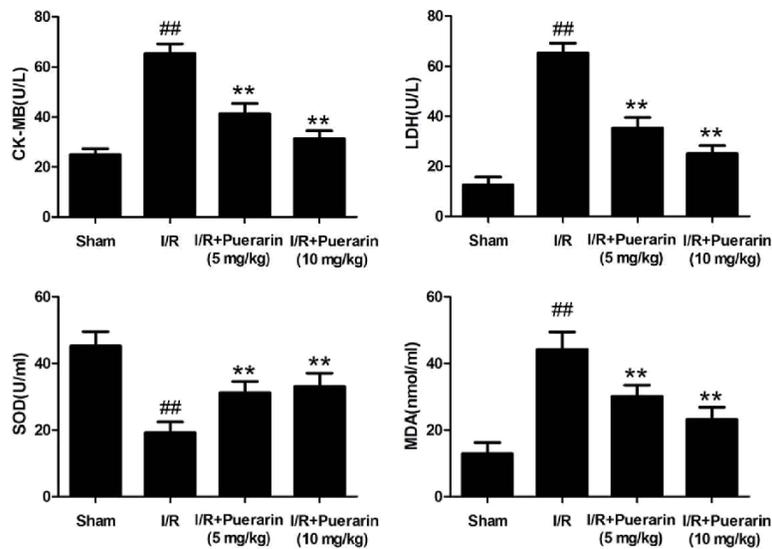
#### Materials

Puerarin were obtained from National Institutes for Food and Drug Control (Beijing, China). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6, IL-1 $\beta$  and TNF- $\alpha$  were produced by Nanjing Key GEN Biotech. CO., LTD. (Nanjing, China). CK, LDH, MDA and SOD kits were provided by Jiancheng Bioengineering Institute (Nanjing, China). All antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). All the studies were performed using male Sprague Dawley rats weighing 200-220 g. The animals were fed with a normal rodent chow diet and had free access to tap water ad libitum. Besides, rats were housed at a constant temperature and relative humidity under a regular 12 h light/12 h dark schedule.

#### Experimental protocol

Rats were randomly assigned to four groups: sham group, I/R group, I/R + Puerarin (5 mg/kg) group and I/R + Puerarin (10 mg/kg) group. The surgical procedures were performed as described previously by Bhindi *et al.* [16]. The successful establish of the myocardial ischemia/reperfusion model was verified by regional cyanosis of the myocardium and ST-segment elevation of electrocardiogram (ECG). To examine cardiac function, reperfusion was prolonged to 24 h. At 4 h of reperfusion, Puerarin was administered in one more doses. The experiments were conduct on non-diseased hearts without abnormal ECG. Except accidental deaths due to anesthesia or failed surgery, the number of rat in each group was as follows:

## Protective effects of puerarin against myocardial ischemia/reperfusion injury



**Figure 3.** Effects of Puerarin on CK-MB, LDH, SOD and MDA in serum. Values are expressed as means  $\pm$  SDs. Compared with Sham:  $^{\#}P < 0.05$ ,  $^{\#\#}P < 0.01$ ; Compared with I/R:  $^*P < 0.05$ ,  $^{**}P < 0.01$ .

sham group (control,  $n = 8$ ), I/R group ( $n = 8$ ), I/R + Puerarin (5 mg/kg) group ( $n = 7$ ), I/R + Puerarin (10 mg/kg) group ( $n = 7$ ).

### Electrocardiographic (ECG)

The ECGs was recorded using the BL-420S Biologic Function Experiment system (Chengdu, China).

### Detections of LDH and CK-MB in serum

Myocardial cellular damage was evaluated by measuring serum LDH and CK-MB levels. 3 h after reperfusion, serum CK-MB and LDH activities were measured spectrophotometrically according to the manufacturer's instructions.

### Detections of SOD and MDA in serum

SOD and MDA levels were measured by a rate assay using an RT-9600 Semi-automatic Biochemical Analyzer (ShenZhen LeiDu life Science, LLC). All the experimental procedures were performed according to the manufacturers' instructions.

### Detections of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ in serum

The levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in serum were measured using ELISA kits according to the manufacturer's instructions. The concentra-

tions of the cytokines were quantified by referring to standard curves.

### Histological examination of myocardium

Immediately after the sacrifice of rats, hearts were removed and fixed in 10% formalin solution. The heart tissue was processed for sectioning and staining by standard histological methods. Sections from the left ventricle were stained with hematoxylin and eosin (H&E) and examined by light microscopy at 400 magnification.

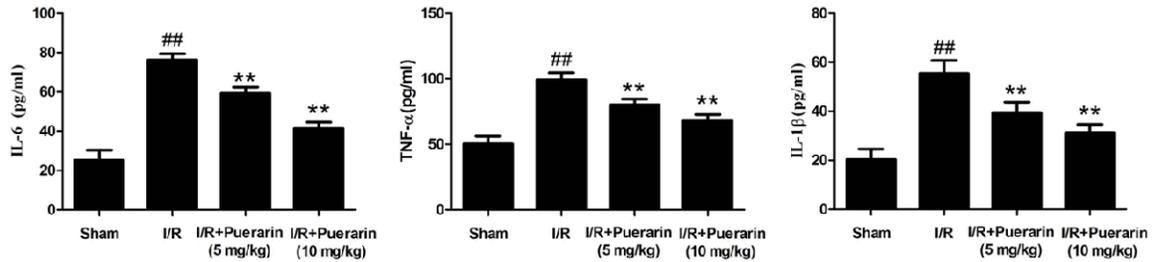
### Western blotting

For western blot analysis, reperfusion was stopped at 6 h and total proteins were extracted from area at risk zones of the heart. The myocardial tissues were placed in RIPA (Radio-Immunoprecipitation Assay) lysis buffer containing 1% PMSF (phenylmethanesulfonyl fluoride) and 1% protease inhibitor cocktail (Roche Applied Science), homogenized and then centrifuged. The cells were harvested and lysed. Cell lysates were centrifuged at 12,500 g for 20 min at 4°C, and the supernatant was collected and stored at -80°C. The protein concentration of each sample (cell or tissue) was determined using a BCA protein assay kit (Nanjing Jiancheng). Equal amounts of protein (50  $\mu$ g) were separated by SDS-PAGE on 10% gels and electrophoretically transferred to a polyvinylidene fluoride membrane (PVDF). Nonspecific binding was blocked with 5% BSA for 1 h at room temperature. The membranes were incubated with primary antibodies diluted 1:500 overnight at 4°C and then incubated with the secondary antibody at room temperature for 1 h. The immunoreactive bands were detected using the ECL method. Optical densities of the bands were scanned and quantified image analysis systems.  $\beta$ -actin served as an internal control.

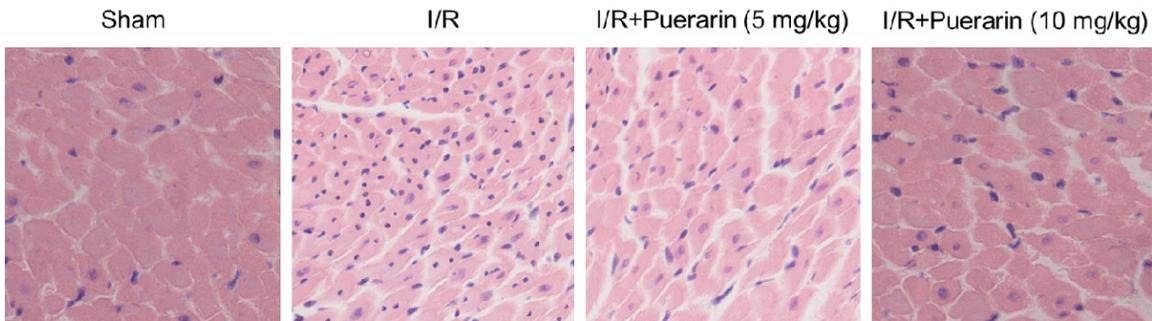
### Statistical analysis

Each experiment was repeated at least three times. All values are shown as the mean  $\pm$  stan-

## Protective effects of puerarin against myocardial ischemia/reperfusion injury



**Figure 4.** Effects of Puerarin on serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Values are expressed as means  $\pm$  SDs. Compared with Sham: #P < 0.05, ##P < 0.01; Compared with I/R: \*P < 0.05, \*\*P < 0.01.



**Figure 5.** Effects of Puerarin myocardial on histology ( $\times 200$ ).

standard deviation, and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using SPSS version 13.0 software, a  $P$ -value of less than 0.05 was considered significant and  $P < 0.01$  was considered to be statistically very significant.

### Results

#### *Puerarin reduced ST-segment elevation*

The ST-segment elevation was observed in the I/R group, which represented that the I/R damage model had been established. Puerarin (5, 10 mg/kg) significantly decreased ST-segment elevation compared with the sham group (Figure 2).

#### *Puerarin decreased CK-MB and LDH serum levels*

As marker enzymes of myocardial injury, the levels of serum CK and LDH were detected. CK-MB and LDH in serum dramatically increased in the I/R model rats compared with those in the sham group. Puerarin decreased CK-MB and LDH levels in comparison with rats in the I/R model group (Figure 3).

#### *Puerarin decreased SOD and MDA serum levels*

Significant increases of MDA and decreases of SOD in serum were observed in the I/R model group compared with those in the control group. Puerarin down-regulated MDA and up-regulated SOD compared with rats in the I/R model group (Figure 3). The obtained data suggested that Puerarin evidently enhanced the activities of enzymatic antioxidant defense system.

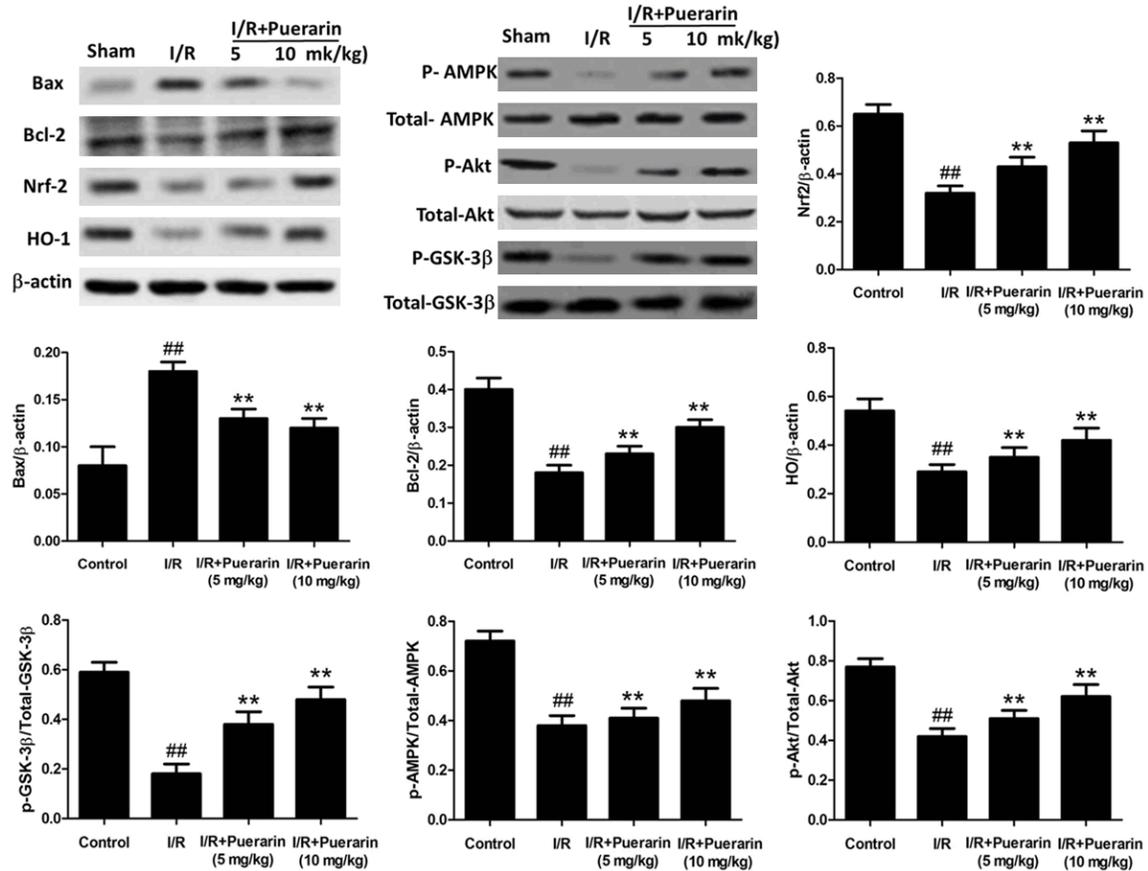
#### *Puerarin decreased TNF- $\alpha$ , IL-6 and IL-1 $\beta$ serum levels*

Compared with the sham group, serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels increased markedly in the I/R model group. Puerarin decreased serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6 levels compared with the I/R group (Figure 4).

#### *Puerarin improved myocardial structure turbulence induced by I/R Injury*

Light microscopy of tissue sections from myocardium of sham rats exhibited a normal myofibrillar structure with striations, branched

## Protective effects of puerarin against myocardial ischemia/reperfusion injury



**Figure 6.** Effects of Puerarin on AMPK/Akt/GSK-3 $\beta$ /Nrf2 pathway protein expressions. Values were expressed as means  $\pm$  SDs. # $P < 0.05$ , ## $P < 0.01$  vs. Sham group. \* $P < 0.05$ , \*\* $P < 0.01$  vs. I/R.

appearance and continuity with adjacent myofibrils. Tissues from the I/R rats showed obvious myocardial cell swelling, degeneration, loss of transverse striations and large numbers of infiltrating inflammatory cells. Tissues from rats treated with Puerarin showed normal, well preserved of cardiac muscle cell histology (Figure 5). The results displayed that Puerarin could attenuate the histopathological condition in myocardial tissue.

*Puerarin decreased Bax-2 levels, and increased Nrf, HO-1, AMPK, Akt, GSK-3 $\beta$  and Bcl-2 levels*

Bax/Bcl-2 are two very specific indicators of cardiomyocyte apoptosis. Bax/Bcl-2 ratio were significantly increased in the I/R group (Figure 6 and Supplementary Figure 1). And these indicators substantially were reduced in Puerarin group compared to those in I/R group, with statistical significance ( $P < 0.01$ ). Nrf2 expression was found to be significantly decreased after

I/R and Puerarin treatment resulted in a dose-dependent increase of it, as well as the expression levels of HO-1. To investigate whether AMPK, Akt and GSK-3 $\beta$  might be responsible for the activation of Nrf2 and protective effect of Puerarin, protein kinase activities in heart were assessed by determining the phosphorylated forms of AMPK, Akt and GSK-3 $\beta$ . Pretreatment with Puerarin significantly increased the phosphorylation of GSK-3 $\beta$ . Moreover, Puerarin treatment increased the phosphorylation of Akt and AMPK. These results indicated that treated with Puerarin facilitated the expression of Nrf2, and the possible mechanism was through phosphorylation of AMPK/Akt/GSK-3 $\beta$  pathway.

### Discussion

In the present study, Puerarin has been demonstrated to reduce the cardiac ischemic/reperfusion damage in rats. The I/R model was established and confirmed by loss of integrity of

## Protective effects of puerarin against myocardial ischemia/reperfusion injury

myocardial membranes on histological examination, ST segment elevation and serum CK-MB and LDH enzymes increased. Puerarin treatment alleviated myocardial histological injury and ST-segment elevation and decreased CK-MB and LDH enzyme in dose-dependent manners. Rats treated with Puerarin also exhibited a smaller infarct size compared with the I/R rats. Myocardial functions were also improved due to the administration of Puerarin. Also Puerarin decreased Bax-2 levels, and increased Nrf2, HO-1, AMPK, Akt, GSK-3 $\beta$  and Bcl-2 levels.

Anti-oxidant enzymes are involved in intracellular mechanisms against inflammatory stresses. One of the major causes of I/R injury is an imbalance between oxidants and antioxidant defense. The activity of SOD and MDA are among the main patho-physiological parameters in assessing free radical metabolisms. The capability of cellular scavenging/quenching free radicals is reflected by SOD activity. However, MDA, the end product of lipid peroxidation, is often used as an indicator of oxidative stress. Increased serum MDA indicates serious oxidative stress. In the current study, SOD activity was significantly decreased, but MDA level was markedly increased in serum of I/R rats, which demonstrated that severe oxidative stress was induced during I/R injury. Puerarin administration dramatically increased SOD activity and decreased MDA level in the myocardium. Thus, the anti-oxidative property might be one of the mechanisms by which Puerarin protected the heart against I/R injury. The proinflammatory cytokines such as IL-6, IL-1 $\beta$  and TNF- $\alpha$  are small secreted proteins that mediate and regulate inflammation. In our study, Puerarin treatment significantly decreased the levels of serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6, which suggested that its cardioprotective effects were possibly related to anti-inflammatory properties.

Our investigations in the underlying mechanisms of the cardioprotective effects of Puerarin manifested the activation of the Nrf2 and AMPK/Akt/GSK-3 $\beta$  pathways. The Nrf2 pathway was regarded as the most important in the cell to protect against oxidative stress [17]. Nrf2 binds to the antioxidant response element (ARE) and regulates ARE-mediated antioxidant enzyme genes expression, including NQO1,

HO-1 and other antioxidants [18]. Therefore, it is of particular interest to determine whether Puerarin can activate Nrf2 in association with HO-1 up-regulation. In this study, Puerarin significantly upregulated Nrf2 activation, and such was correlated with a significant upregulation of HO-1 expression in heart tissues. This result demonstrated that Puerarin mediated its cardioprotective effects against oxidative insults through activation of the Nrf2 pathway. Glycogen synthase kinase 3 beta (GSK-3 $\beta$ ), a multifunctional serine/threonine kinase, controls switching off of Nrf2 activation of gene expression. Our data displayed that GSK-3 $\beta$  phosphorylation was induced by Puerarin treatment. The modulation of AMPK activity in the heart may improve cardiac function and overcome the increased susceptibility of the heart to I/R injury. In this study, the cytoprotective effect of Puerarin against I/R injury depended on AMPK activation. The serine survival kinase Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K). The PI3K/Akt pathway plays a critical role in promoting cell survival in the heart, and there is increasingly evidence to indicate cross talk between the Nrf2 and PI3K/Akt pathways in response to oxidative insults [19, 20]. In the present study, I/R decreased Akt phosphorylation and Puerarin pretreatment augmented Akt phosphorylation significantly. Puerarin not only induced phosphorylation of AMPK but also caused the phosphorylation of Akt and GSK-3 $\beta$  in an AMPK-dependent manner. These results indicated that the AMPK/Akt/GSK-3 $\beta$  pathway is responsible for Puerarin's pharmacological action.

In summary, this study demonstrated that Puerarin possesses cardioprotective properties against oxidative injury and myocardium I/R. The underlying mechanisms of Puerarin mediated cardioprotection may be attributable to activation and cross-talk between the AMPK/Akt and GSK-3 $\beta$ /Nrf2 signaling pathways.

### Disclosure of conflict of interest

None.

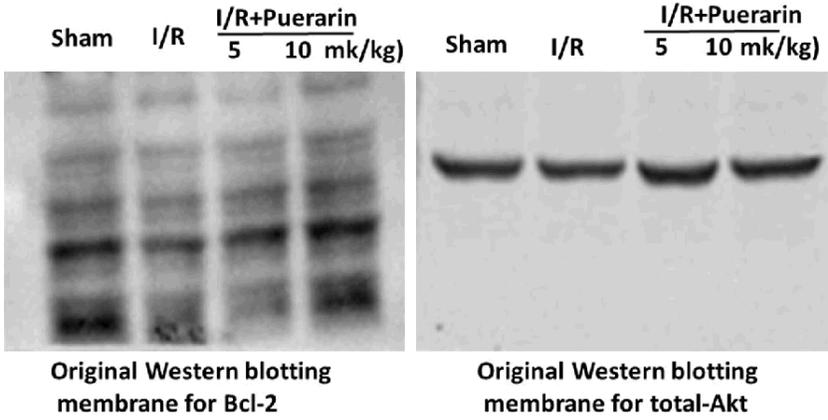
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## Protective effects of puerarin against myocardial ischemia/reperfusion injury

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Protective effects of puerarin against myocardial ischemia/reperfusion injury



Supplementary Figure 1. Original Western blotting membrane for Bcl-2 and total-Akt.