

Original Article

Protective effects of quercetin against myocardial ischemia/reperfusion injury in rats

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Abstract: The present study was to investigate the effects of quercetin on myocardial ischemia/reperfusion (MI/R) injury and investigated the underlying mechanisms. The expression of tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxidase dismutase (SOD) was evaluated by enzyme-linked immunosorbent assay (ELISA). Infarct size was measured by Evans blue/TTC double staining. Pathological changes of heart tissues were observed by HE staining. Levels of nuclear factor- κ B (NF- κ B) p65, PI3K, Akt, were determined by Western blotting. Pretreatment with quercetin significantly augmented rat cardiac function, as evidenced by increased left ventricular ejection fraction (LVEF) and \pm dP/dt. Quercetin reduced myocardial infarct size, markedly decreased LDH, CK, CK-MB, AST and MDA levels, and increased SOD, GSH-Px, CAT in the MI/R group. The protein levels of NF- κ B p65 and Bax-2 were significantly decreased, while the protein levels of PI3K, Akt and Bcl-2 were significantly increased in a dose-dependent manner compared to the model group, respectively. Together, these data demonstrate that quercetin might protect myocardial ischemia via regulating PI3K/Akt/NF- κ B p65 signaling.

Keywords: Quercetin, myocardial ischemia/reperfusion, PI3K/Akt/NF- κ B signaling

Introduction

Ischemic heart disease (IHD) is a major health problem and according to the World Health Organization, it will be the leading cause of death in the world [1, 2]. In the past few decades, extensive studies have been performed to explore effective strategies and drugs to ameliorate or prevent ischemic heart injury, such as flavonoids, that possess cardio protective effects. Quercetin (Que; 3,3',4',5,7-pentahydroxyflavone) is one of the major flavonoids found in many vegetables and fruits such as onion and apple. It is nontoxic and has a broad range of pharmacological and biological activities including anticarcinogenic, antioxidative, vasoprotective, antidiabetic, and antiplatelet effects [3]. Several studies indicated that Que protects the myocardium from ischemic heart injury [4]. Recently, in another study, a significant reduction of the myocardial infarct size in both normal and diabetic animals by Que has been reported [5]. However, the molecular

mechanism underlying Que-mediated cardioprotection is still not completely elucidated.

Although the pathogenesis of myocardial ischemia is far from clear, the anatomic changes and the characterized biochemical markers have been well illustrated. The overproduction of reactive oxygen species (ROS) and the activation of inflammatory cascades are major causative factors of cardiomyocyte abnormalities. Activation of the PI3K/Akt pathway has been reported to prevent neuronal apoptosis and protect the brain from cerebral ischemia/reperfusion (I/R) injury [6-8]. Additionally, PI3K/Akt pathway activation attenuates mitochondria-mediated apoptosis [9, 10]. Serine/threonine kinase Akt is a primary mediator of the downstream effects of phosphatidylinositol-3 kinase (PI3K), preserving mitochondrial integrity by phosphorylating molecules such as the Bcl-2 family. Nuclear factor- κ B, a nuclear transcription factor, is a regulator of inflammatory processes. NF- κ B is required for maximal

transcription of numerous cytokines, including TNF- α , IL-1 β , and IL-6 [11], which are detrimental to the myocardial functions [12].

The present study was to investigate the effects of quercetin on myocardial ischemia/reperfusion (MI/R) injury and investigated the underlying mechanisms.

Materials and methods

Materials

Male Sprague-Dawley (SD) rats weighing 190–210 g were provided by Shanghai Slac Laboratory Animal Co.LTD (Shanghai, China). Animal experiment was carried out in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996, Jan 12). All animals were allowed free access to food and water, and were maintained at 22–24°C under a 12 hour: 12 hour light-dark cycle. Quercetin with purity of over 99% were purchased from National Institutes for Food and Drug Control (Beijing, China). Tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), lactate dehydrogenase (LDH), creatine kinase (CK), malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and superoxidase dismutase (SOD) test kits were all purchased from Nanjing Jiancheng Biological Engineering research institute (Nanjing City, China).

Experimental protocol

Adult male Sprague-Dawley rats were fasted overnight, and anesthetized via intraperitoneal (IP) administration of 50 mg/kg pentobarbital sodium. A micro-catheter was inserted into the left ventricle through the right carotid artery to measure the left ventricular pressure. Myocardial ischemia was produced after exteriorizing the heart via a left thoracic incision, and placing a 6-0 silk slipknot suture around the left anterior descending coronary artery approximately 2–3 mm from its origin. Ischemia was monitored and confirmed by ST segment elevation upon electrocardiogram (ECG). After 30 minutes ischemia, the slipknot was released, and myocardial reperfusion for 3 hours. Rats were randomly divided into the following groups: (1) sham group; (2) I/R group; (3) I/R+ propranolol (as a positive control, 25 mg/kg); (4) I/R+ Quercetin (200 mg/kg); (5) I/R+ Quercetin (400 mg/kg). The rats in quercetin-

treated group and propranolol-treated group were pretreated with quercetin or propranolol every 24 h for 10 days by means of intragastric administration.

Determination of cardiac function

MI/R-induced cardiac dysfunction was determined by invasive hemodynamic evaluation methods. A micro-catheter was inserted into the left ventricle via the right carotid artery to measure the left ventricular pressure (LVP). ECG and LVP were simultaneously recorded on a polygraph. Computer algorithms measured left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), first derivative of left ventricular pressure ($\pm dP/dt_{max}$) after 3 hours of reperfusion.

Determination of myocardial infarct size

After reperfusion conclusion, the coronary artery ligation was retied. 4 mL of 2% Evans blue dye (Shanghai Chemical Reagents, Shanghai, China) was injected into the aorta. Dye was circulated and uniformly distributed, except in the cardiac region previously perfused by the occluded coronary artery (defining the ischemic region or area at risk, AAR). Cardiectomy was rapidly performed. Hearts were frozen at -20°C and sliced into 1-mm sections perpendicular to the base-apex. Slices were incubated in 1% TTC in phosphate buffer at 37°C for 10 minutes (pH 7.4). Morpho-metric measurements of AAR and infarct area (INF) were performed by image analysis system. Myocardial infarct size was expressed.

Determination of LDH, CK, CK-MB, TNF- α , IL-6 in the serum

CK, CK-MB and LDH levels were measured by a rate assay using an RT-9600 Semi-automatic Biochemical Analyzer (Shenzhen Lei Du life Science, LLC). TNF- α and IL-6 levels were measured by enzyme linked immunosorbent assay (ELISA, Wuhan Boshide Biological Technology Company, Wuhan, China). All measurements were performed according to the kit manufacturers' instructions.

Determination of SOD and MDA in myocardial tissue

Approximately 0.1 g of myocardial tissue was removed from the apical part of the heart,

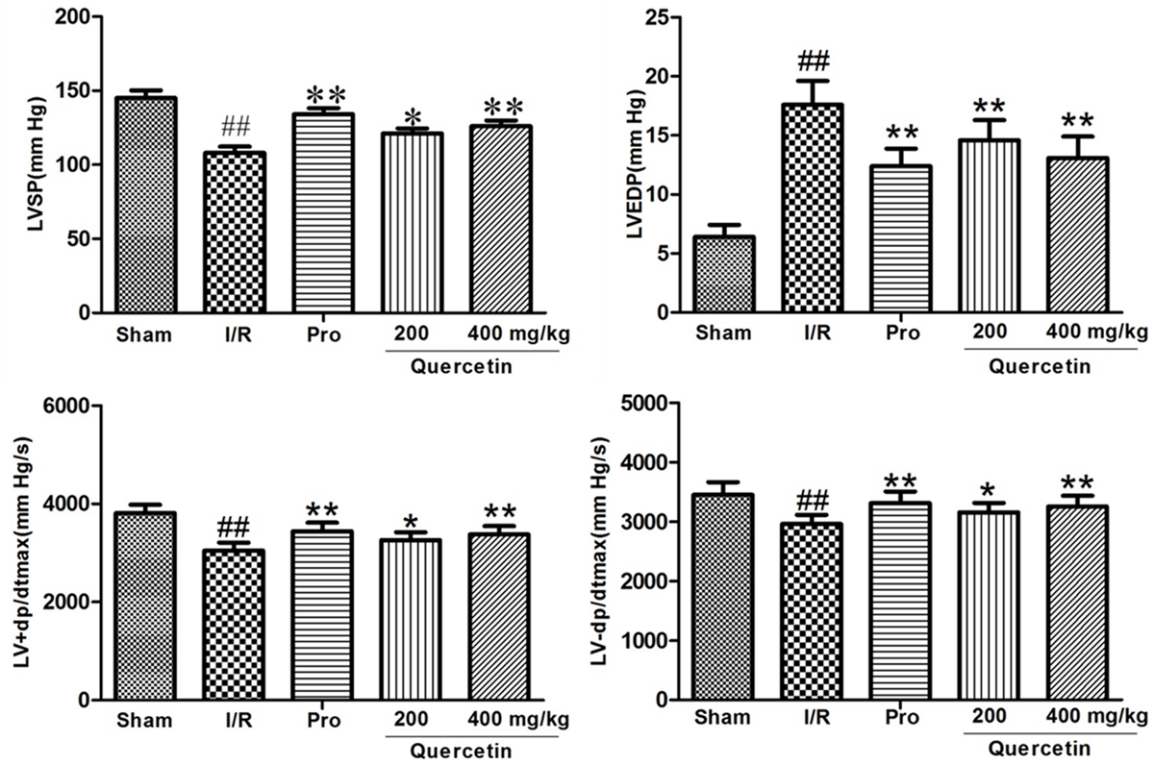


Figure 1. The effect of quercetin on LVSP, LVEDP, LV+dp/dt, LV-dp/dt of rat subjected to I/R injury. All values presented are mean \pm SD. Compared with sham: ^{*} $P < 0.05$, ^{##} $P < 0.01$; Compared with I/R: ^{*} $P < 0.05$, ^{**} $P < 0.01$.

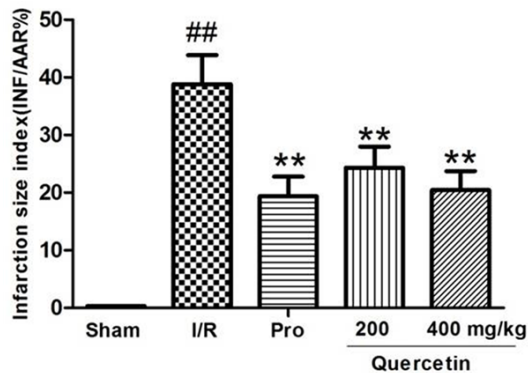


Figure 2. Effects of quercetin on myocardial infarct size in rat subjected to I/R injury. All values presented are mean \pm SD. Compared with sham: [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with I/R: ^{*} $P < 0.05$, ^{**} $P < 0.01$.

immersed in ice-cold saline solution (1:9 W/V) and homogenized. The homogenate was centrifuged at 3000 rpm for 15 min, and the supernatant was used for the biochemical assays. SOD and MDA were determined by ELISA kits according to the instructions recommended by the manufacturers.

Myocardial tissues histopathology

Immediately after the sacrifice of the rats, the hearts were removed and fixed in 10% formalin solution. The heart tissue was processed for sectioning and staining by standard histological methods. Sections (5 mm, Leica RM 2125, Germany) from the left ventricle were stained with hematoxylin and eosin (H&E) and examined by light microscopy (Nikon, Tokyo, Japan) at 200 \times magnification.

Western blotting

Myocardium tissues were homogenized in buffer containing phenyl methanesulfonyl fluoride. Homogenates were mutated at 4 $^{\circ}$ C for 30 min and centrifuged at 13000 \times g for 20 min. Equal amounts of protein were loaded onto each lane and run on SDS-PAGE under reducing conditions. Samples were then electro blotted onto nitrocellulose filter membrane, blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) at room temperature for 2 h, and incubated overnight at

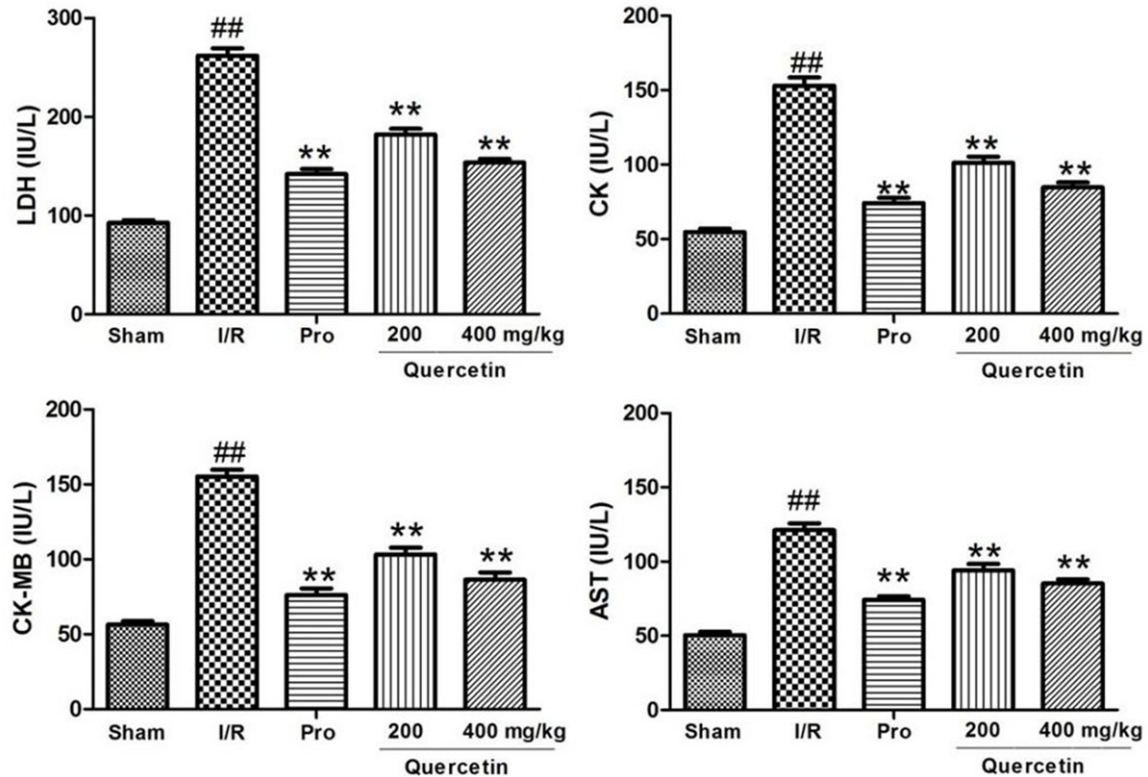


Figure 3. Effects of quercetin on serum levels of LDH, CK, CK-MB and AST in rat subjected to I/R injury. All values presented are mean \pm SD. Compared with sham: $^{\#}P<0.05$, $^{\#\#}P<0.01$; Compared with I/R: $^*P<0.05$, $^{**}P<0.01$.

4°C with different first antibodies. The membrane was washed with TBST for three times per 15 min. The HRP-linked secondary antibody was incubated with the membrane for 1 h at room temperature. Excess antibody was washed off with TBST for three times per 15 min before incubation ECL for 1 min.

Statistical analysis

All values were expressed as the mean \pm S.D. 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

Quercetin improved rat cardiac systolic and diastolic function after MI/R

Quercetin had no effects on blood glucose, blood pressure and cardiac function in the absence of MI/R. Pretreatment with quercetin enhanced \pm LVdP/dt max after 3 hours reperfusion compared to MI/R group (**Figure 1**). Addi-

tionally, quercetin markedly decreased LVEDP compared to MI/R group. Hemodynamic data support quercetin improved rat cardiac systolic and diastolic function after MI/R.

Quercetin decreased infarct size and reduced biochemical marker enzymes

Myocardial infarct size and LDH, CK, CK-MB and AST were measured to assess myocardial injury. Myocardial INF expressed as percentage of AAR (**Figure 2**). No myocardial infarction was observed in sham-group hearts. Quercetin (400 mg/kg) treatment significantly decreased infarct size. Significant increases in the myocardial injury biomarker enzymes, CK, AST, CK-MB and LDH were observed in I/R group compared with the sham-group. Pretreatment with quercetin decreased CK, AST, CK-MB and LDH levels compared with rats in I/R group in a dose-dependent manner (**Figure 3**).

Quercetin increased SOD activity and decreased MDA, TNF- α and IL-6 levels

To determine whether quercetin attenuated MI/R-induced oxidation index, plasma SOD,

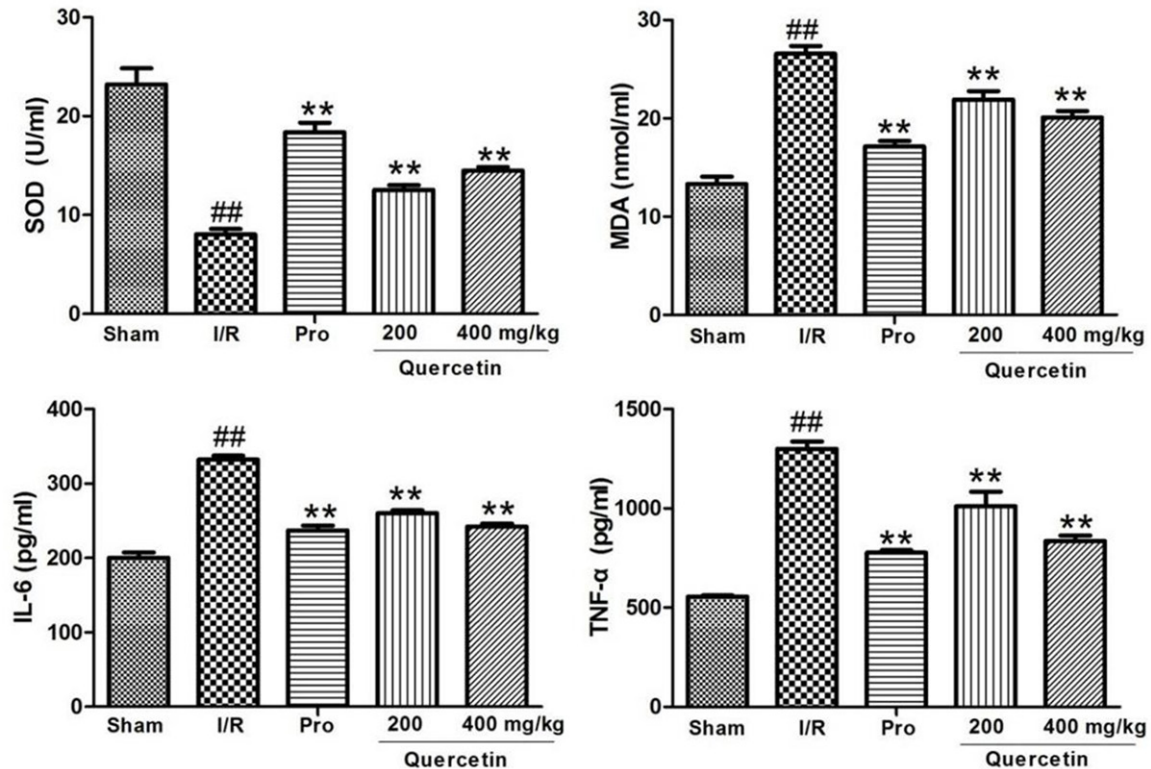


Figure 4. Effects of quercetin on serum levels of SOD, MDA, TNF-α and IL-6 in rat subjected to I/R injury. All values presented are mean ± SD. Compared with sham: [#] $P < 0.05$, ^{##} $P < 0.01$; Compared with I/R: ^{*} $P < 0.05$, ^{**} $P < 0.01$.

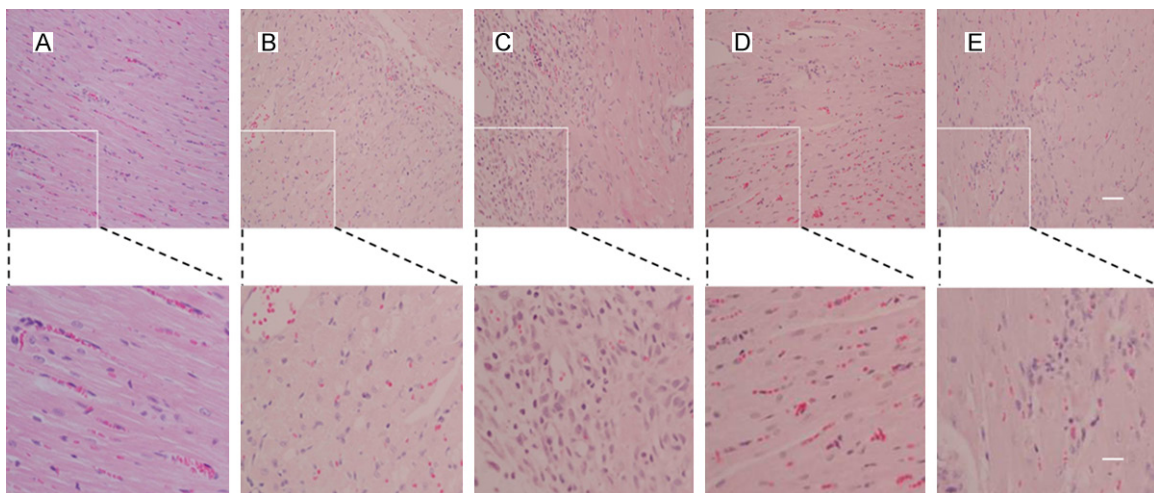


Figure 5. Effects of quercetin on morphological changes of rats' myocardial tissues. A: Sham; B: I/R; C: Propranolol; D: Quercetin (200 mg/kg); E: Quercetin (400 mg/kg). Scar bar: 200 mM (upper panel), Scar bar: 50 mM (lower panel).

MDA, TNF-α and IL-6 levels were measured after reperfusion conclusion. Compared with the sham-group, SOD levels in the I/R group decreased significantly. Pretreatment with quercetin increased SOD activity in a dose-

dependent manner compared with the I/R group. Compared with the sham-group, the MDA, TNF-α and IL-6 levels in the I/R group increased significantly. Pretreatment with quercetin decreased MDA, TNF-α and IL-6 levels in a

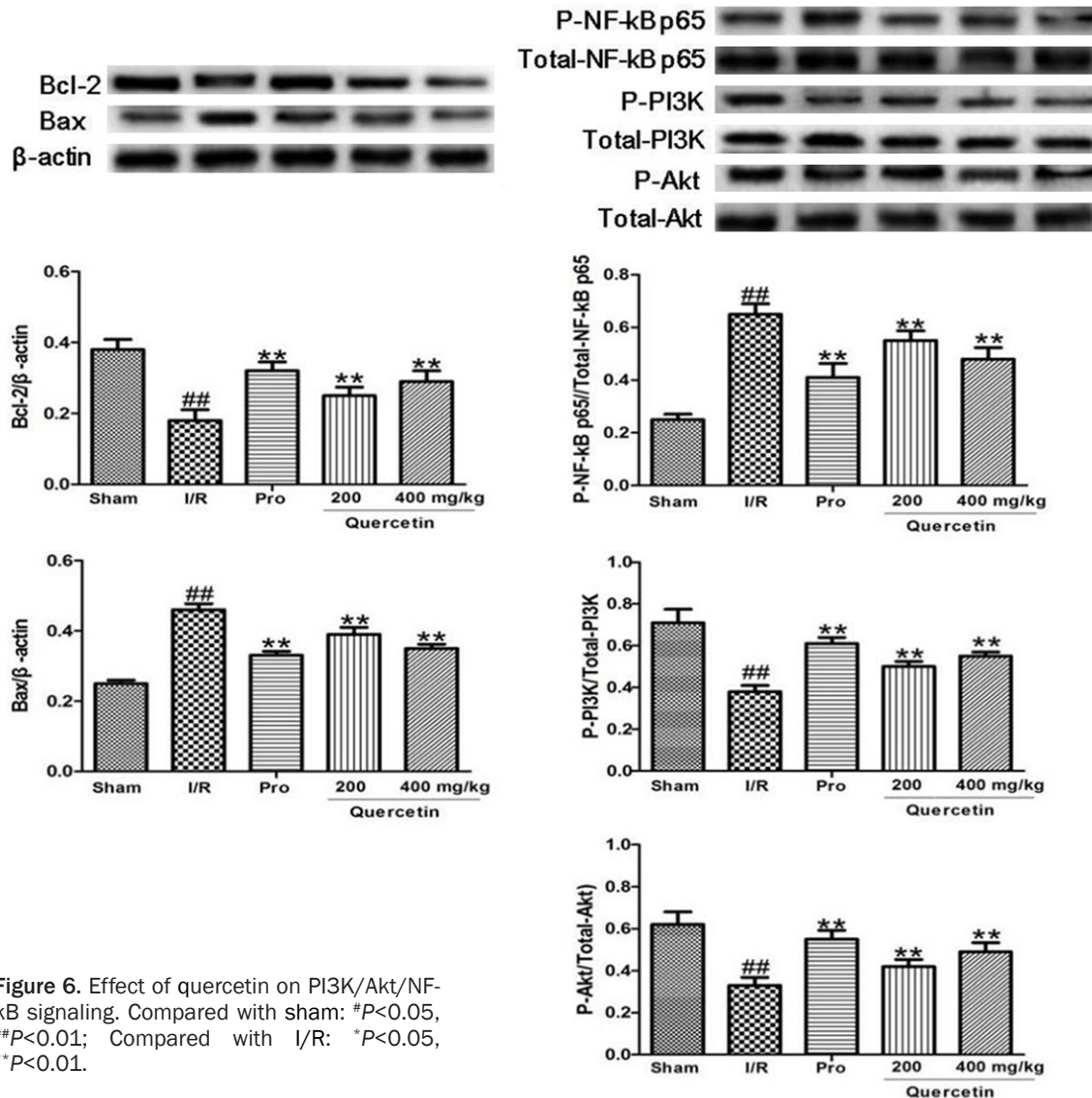


Figure 6. Effect of quercetin on PI3K/Akt/NF-κB signaling. Compared with sham: [#] $P<0.05$, ^{##} $P<0.01$; Compared with I/R: ^{*} $P<0.05$, ^{**} $P<0.01$.

dose-dependent manner compared with the I/R group rats (**Figure 4**).

Quercetin showed normal, well preserved of cardiac muscle cell histology

Histological evaluation of the myocardial tissues (**Figure 5**) by light microscopy demonstrated myocardium cell in I/R group rats arranged in disorder and swell significantly. Obvious myocardial cell swelling, degeneration, loss of transverse striations, and large numbers of infiltrating inflammatory cells also observed in I/R group. Pretreatment with quercetin showed normal, well preserved of cardiac muscle cell histology. The results indicated that quercetin can reduce the degree of pathological changes in myocardial tissues in MI/R.

Quercetin increased PI3K, Akt and Bcl-2 levels and decreased NF-κB p65, Bax-2 levels

The expression of proteins of PI3K, NF-κB p65, Akt, Bcl-2 and Bax-2 were changed in heart. As shown in **Figure 6**, compared with the sham-group, the protein levels of NF-κB p65 and Bax-2 in I/R group were significantly increased. In quercetin treated groups, the protein levels of NF-κB p65 and Bax-2 were significantly decreased in a dose-dependent manner compared to the model group, respectively. Compared with the sham-group, the protein levels of PI3K, Akt and Bcl-2 in I/R group were significantly decreased. In quercetin treated groups, the protein levels of PI3K, Akt and Bcl-2 were significantly increased in a dose-depen-

dent manner compared to the I/R group, respectively.

Discussion

In this study, quercetin improved rat cardiac systolic and diastolic function after MI/R, decreased infarct size and reduced biochemical marker enzymes (CK, AST, CK-MB and LDH). Pretreatment with quercetin also increased SOD activity and decreased MDA, TNF- α and IL-6 levels. And pretreatment with quercetin showed normal, well preserved of cardiac muscle cell histology. These results suggested that quercetin at the doses used in this study had cardioprotective effects in myocardial ischemia/reperfusion that could be attributed to their anti-oxidative and anti-inflammatory properties.

Excessive oxidative stress induced the overproduction of pro-inflammatory cytokines, including IL-6, IL-1 β and TNF- α . Some studies have documented that the elevated levels of inflammatory markers were related to ischemia. Quercetin preconditioning decreased the contents of pro-inflammatory cytokines, which suggested that the cardioprotective effects of quercetin were partially attributed to its anti-inflammatory property. These findings were consistent with the histological evaluation of inflammatory cellular infiltration in myocardial tissues.

SOD (a key antioxidant enzyme) and MDA (a reactive oxygen species) are important indexes of myocardial oxidative damages [13]. It is well known that SOD activity reflects the cellular capability of scavenging/quenching free radicals [14]. Under physiological conditions, low levels of reactive oxygen species play important roles in signal transduction and metabolic pathways [15, 16]. However, under pathologic conditions, excessive reactive oxygen species resulted in the imbalance of antioxidant system. MDA, the end product of lipid peroxidation, was overproduced in the I/R rats. According to the obtained data, the level of MDA was down-regulated with quercetin pretreatment and the activities of SOD was recovered versus those in I/R rats.

The Bcl-2 protein family, comprised of both anti- and pro-apoptotic members, are important mitochondrial regulators during cardiomyocyte

apoptosis. Bcl-2 regulates mPTP opening in opposition to Bax, blocking cytochrome c release, inhibiting caspase activity, and decreasing cell apoptosis. Western blot revealed I/R significantly decreased the Bcl-2 and decreased Bax-2, an effect reversed by quercetin administration, suggesting quercetin-mediated cardioprotection against I/R injury may occur partially via modulating Bcl-2/Bax expression. The serine survival kinase Akt is activated downstream of phosphatidylinositol 3-kinase (PI3K). Activation of PI3K and Akt is cardioprotective against I/R injury, and prevents cardiomyocyte apoptosis. The activation of NF- κ B pathway triggers the overproduction of pro-inflammatory cytokines, such as IL-1 β , IL-6 and TNF- α , which are detrimental to the myocardial functions [17]. As shown in **Figure 6**, The expression of proteins of PI3K, Akt, NF- κ B p65, Bcl-2 and Bax-2 were changed in heart. Compared with the control group, the protein levels of NF- κ B p65 and Bax-2 in I/R group were significantly increased. In quercetin pretreatment group, the protein levels of NF- κ B p65 and Bax-2 were significantly decreased in a dose-dependent manner compared to the I/R group, respectively. Compared with the sham-group, the protein levels of PI3K, Akt and Bcl-2 in I/R group were significantly decreased. In quercetin, the protein levels of PI3K, Akt and Bcl-2 were significantly increased in a dose-dependent manner compared to the I/R group, respectively.

In conclusion, quercetin exerted anti-inflammation and anti-oxidation effects against myocardial ischemia/reperfusion injury in rats, which might be associated with regulating PI3K/Akt/NF- κ B p65 signaling

Disclosure of conflict of interest

None.

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