Original Article
Acacetin protective myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via the PI3K-Akt pathway

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Abstract: Acacetin (5,7-dihydroxy-4'-methoxyflavone) a natural flavonoid compound extracted from natural plants, exhibits a wide range of biological activities, including antioxidant and anti-myocardial ischemia/reperfusion (I/R) injury. However, its mechanism protective of anti-myocardial I/R injury remains unknown. This study aimed to investigate the effect of acacetin protective myocardial I/R injury and potential molecular mechanisms of cardiomyocytes apoptosis. Pre-treatment with acacetin markedly reduced infarct size, increased reactive oxygen species (SOD) activity, and decreased malondialdehyde (MDA) levels in rats with I/R. In addition, histopathology showed that cellular edema and infiltration of inflammatory cells were alleviated in myocardial tissues. Furthermore, acacetin pre-treatment significantly augmented cell viability and decreased levels of creatine kinase-MB (CK-MB). Lactate dehydrogenase (LDH) was also reduced in H9c2 cells subjected to Hypoxia/reoxygenation (H/R). The potential mechanism involved activation of PI3K-Akt signaling in cardiomyocytes. Taken together, acacetin was protective against myocardial ischemia/reperfusion injury and inhibited apoptosis of H9c2 cardiomyocytes. Therefore, acacetin treatment represents a promising strategy for ischemic heart disease.

Keywords: Acacetin, myocardial ischemia/reperfusion injury, anti-oxidative, protective effect, hypoxia/reoxygenation, apoptosis

Introduction

Myocardial ischemia/reperfusion (I/R) injury occurs following myocardial hypoxia/ischemia resulting from the partial or complete cessation of blood circulation through the myocardium [1, 2]. Myocardial I/R injury clinical manifestations included reduced blood perfusion and cardiac oxygen supply, myocardial energy metabolism that does not support the normal functioning of the heart [3, 4]. Currently, rapid restoration of blood flow is the most effective therapeutic strategy for improving clinical outcomes. However, reperfusion itself may aggravate myocardial damage [5-7]. Although mechanisms of I/R have not yet been fully elucidated, oxidative stress and apoptosis play important roles in the pathogenesis of myocardial I/R injury [8-10]. Thus, strategies to inhibit oxidative stress and apoptosis in cardiomyocytes may have a potential application for preventing myocardial damage during myocardial ischemia/reperfusion injury.

Acacetin (5,7-dihydroxy-4'-methoxyflavone) is an natural flavone which is found in many plants [11]. As shown in **Figure 1**, acacetin has been reported to able to exert outstanding anti-peroxidative [12-14]. Currently, its anti-I/R injury myocardial protection mechanism remains unknown. It is only in recent years that efforts have been made to explore methods for against myocardium injury. In this sturdy, the effect of acacetin on myocardial I/R injury and the potential molecular mechanisms cardiomyocytes apoptosis were explored. In the present study, the antioxidative and cardioprotective effects of acacetin on I/R-induced cardiac dysfunction. In addition, the results provide evidence that acacetin protective myocardial I/R injury and inhibits apoptosis of H9c2 cardiomyocytes.
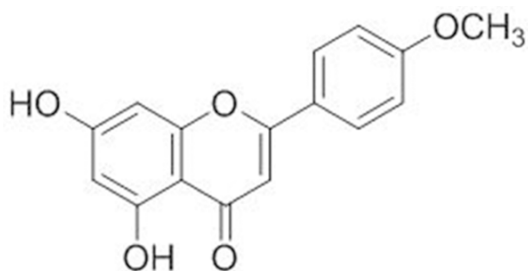
Figure 1. Chemical structure of acacetin.

Materials and methods

Animals and in vivo experimental protocols

Forty-eight male Sprague dawley (SD) rats were randomly divided into the following groups (n=8): sham (control); ischemia/reperfusion (I/R); propranolol hydrochloride + I/R (propranolol + I/R); high-, medium- and low-dose acacetin groups (10, 20, 40 mg/kg, acacetin + I/R). The SD rats were purchased from the Xinjiang Animal Center, China. The rats were housed in the temperature at 23 ± 1°C, relative humidity at 50 ± 5%, UV disinfection sterilization (60 minutes/12 hours) and 12-hour light/dark cycle, with free access to food and water.

I/R injury model

The I/R injury model was established as previously described [15]. SD rats were anesthetized via with 10% Chloral Hydrate (350 mg/kg, i.p) and restrained in the supine position. The animals were tracheostomized and ventilated with a small-animal ventilator (respiration rate 70 time/min, respiration-to-expiration ratio 1:1.5, and tidal volume 50 mL/kg) during the surgical procedures. The chest was opened through the third and fourth intercostal space, and the pericardium was then rived to expose the heart. Myocardial ischemia was induced by placing a 6-0 silk suture with a slipknot around the left anterior descending coronary artery (LAD) during the period of ischemia (30 min). After 30 minutes of ischemia, the slipknot was released and rats received reperfusion for 120 minutes. The model was considered successfully verified when the ST-segment elevated during myocardial ischemia and dropped at least 50% during reperfusion on ECG. At the end of reperfusion, rat abdominal arterial blood was collected, and serum was separated by centrifugation at 3000 rpm for 15 minutes at 4°C. Samples were then stored at -70°C. The mouse hearts were removed following the collection of blood and immediately placed in cold saline. The intracardiac blood was rinsed off, and the left ventricle tissue was line-clipped under ligation and cryopreserved.

Evaluation of myocardial infarct size

After myocardial I/R injury, then abdominal artery blood, hearts were removed and flushed using saline, according to the thickness is 1 mm cut from apex is 5 pieces. All sections to incubate 1% TTC in the dark at 37°C in 15 minutes, then 10% formaldehyde solution fixed. The infarction size percentage was calculated using the following equation: infarct volume % = Infarct volume/total volume of slice × 100.

Measurements of oxidative stress

Serum levels of superoxide dismutase (SOD) and Malondialdehyde (MDA) content activity were evaluated. The indexes were done according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Myocardial histopathology

Each rat heart was fixed in 10% formaldehyde and preserved at room temperature. The heart was observed under an optical microscope after hematoxylin and eosin (HE) coloration. A small piece (2 mm × 1 mm × 1 mm) of subendocardial myocardium from the root of the left ventricular papillary muscle was fixed in a 0.1 mM phosphate buffer (pH 7.2) containing 3% glutaraldehyde and 1.5% paraformaldehyde at 4°C. Afterward, the tissue was dehydrated by alcohol followed by dimethylbenzene and embedded in epoxy resin 618. The tissue was located by semi-thin sectioning and then sliced into ultrathin sections (60 nm). The sections were dyed with uranium acetate and lead citrate and observed under an optical microscope.

Ischemic myocardium ultrastructure

Transmission electron microscopy (TEM, Hitachi, Japan) was used to observe the ultrastructure of the myocardium. The liver tissue (<1 mm³) samples were harvested and then fixed in 2.5% glutaraldehyde and embedded in Epon resin. Then, ultrathin sections were cut and counterstained with uranyl acetate and lead citrate and examined with a JEM-1230 TEM.

Table 1. Primers Sequences

Primers	Sequences
PI3K	Forward: 5'-AGGTTCATGTGCTGGATACT-3'
	Reverse: 5'-TGGGCTCCTTTACTAATCTC-3'
Akt	Forward: 5'-ACGATGAATGAGGTGTCTGT-3'
	Reverse: 5'-TCTGCTACGGTGAAGTTGTT-3'
Caspase-3	Forward: 5'-GAGACAGACAGTGGAACTGACG-3'
	Reverse: 5'-GGCGCAAAGTGACTGGATGA-3'

Cell viability

Cell viability was determined colorimetrically using MTT assay. Cells at the exponential phase were seeded at 1×10^4 cells/well in 96-well plates. After different treatments, 20 ml of 5 mg/ml MTT solution was added to each well (0.5 mg/ml final concentration in medium), and wells were incubated for 4 hours at 37°C. The supernatants were aspirated, the formazan crystals in each well were dissolved in 150 ml DMSO, and optical density at 490 nm was read on a Microplate Reader. The reduction in optical density was considered to be the decrease in cell viability. Neither Wortmannin nor U0126 treatment alone significantly affected cell viability.

Hypoxia/reoxygenation (H/R) model

To induce hypoxia, H9c2 cells were exposed to a hypoxic environment of 95% N_2 and 5% CO_2 for 3 h at 37°C. Afterwards, the medium was replaced with fresh oxygenated culture medium, and the culture vessels were transferred to a normoxic incubator (5% CO_2) at 37°C for 6 hours of reoxygenation. Cells under normoxic conditions served as a control.

Measurement of cellular injury

The levels of lactate creatine kinase-MB (CK-MB) and dehydrogenase (LDH) released were measured to evaluate the presence of necrotic cell death [17]. After the experiment, the levels of CK-MB and LDH in the serum were spectrophotometrically determined by using cytotoxicity detection CK-MB and LDH kits (Nanjing Jiancheng Biological product, Nanjing, China).

TUNEL staining for apoptosis in vitro

Cell apoptosis was analyzed by TUNEL assay using an in situcell death detection kit (KeyGEN, Nanjing, China). The *in situ* TUNEL assay was performed in accordance with the manufact-

urer's protocol for cultured cells after fixing the cells in 4% paraformaldehyde for 30 minutes at 4°C. Individual nuclei were visualized at a magnification of × 400 for quantitative analysis. TUNEL-positive cells contained characters of apoptosis, con-densed chromatin and cellular shrinkage. An average of 300-400 nuclei was analyzed in random fields from each slide. Thepercentage of apoptosis cells was calculated as the ratio of thenumber of TUNEL-positive cells to the total number of cells, which were counted in three different random fields.

Real-time quantitative reverse transcription analysis

Total RNA treated cells isolation were performed in accordance with the Trizol reagent manufacturer's instructions. The RNA quantitate was measured by spectrophotometer (at 260 and 280 nm). Total RNA (1 µg) reverse transcription was performed according to the reverse transcription system (Takara, Japan). Real-time PCR was performed using SYBR-Green Supermix with an iCycler® thermal cycler (Bio-Rad) and Primers Sequences **Table 1**, The $2^{-\Delta\Delta Ct}$ method used to calculate relative changes in gene expression.

Western blot analysis

The Western blot was performed according to previously described [16]. The primary antibodies were PI3K, Caspase-3, Akt (PROTEINTECH GROUP, INC.). Then, the blots were visualized using Chemiluminescence Detection System (GE Healthcare, Sweden).
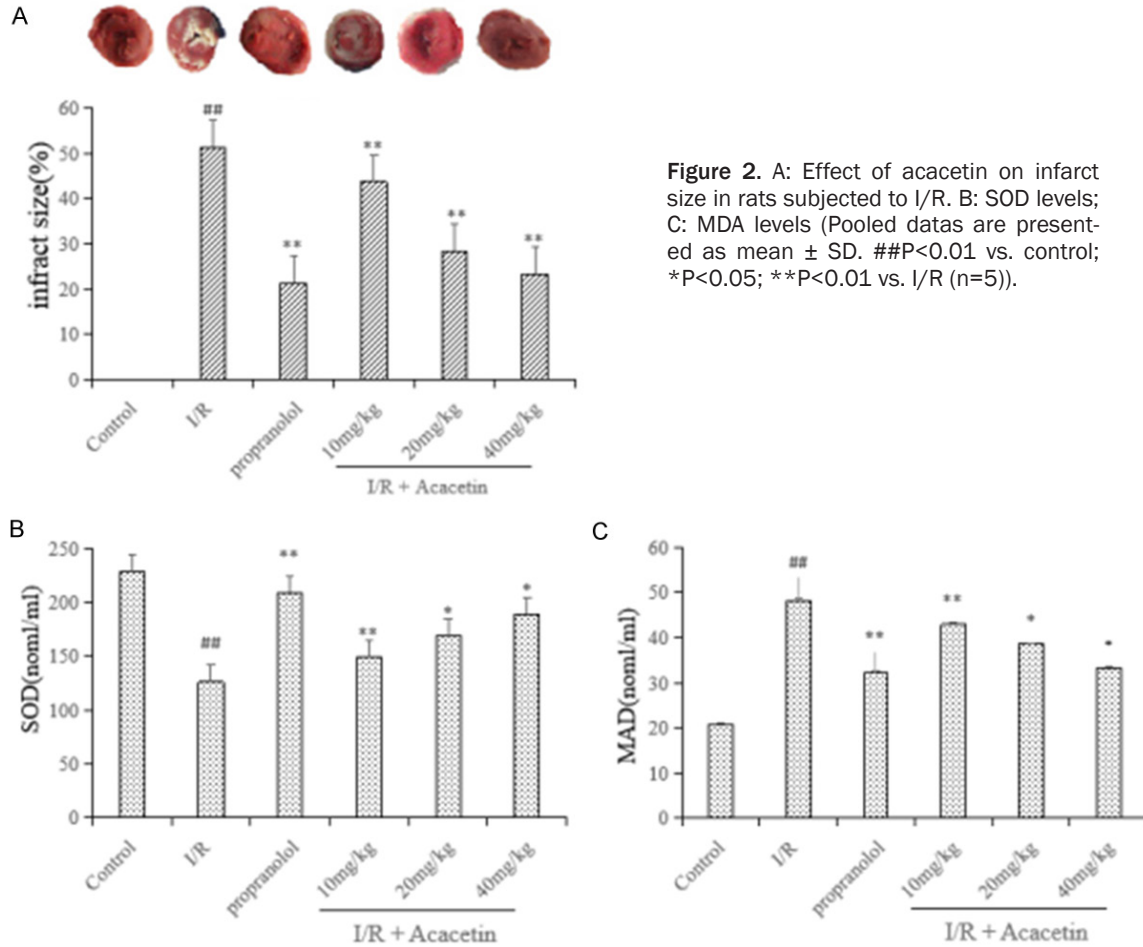
Statistical analysis

Statistical analysis and calculations were performed by using SPSS 20.0 for Windows (Chicago, IL, USA). All values are presented as mean ± SEM. The difference between groups was compared by one-way analysis of variance followed by least significant difference test. $P < 0.05$ was considered statistically significant.

Results

Regulating effect of acacetin on infarct size and serum myocardial injury markers in I/R model rats

As shown in **Figure 2A**, the rats I/R model group showed a significant increase in risk area

Figure 2. A: Effect of acacetin on infarct size in rats subjected to I/R. B: SOD levels; C: MDA levels (Pooled datas are presented as mean ± SD. ##P<0.01 vs. control; *P<0.05; **P<0.01 vs. I/R (n=5)).

infarct. As expected, pretreatment with propranolol and acacetin reduced the infarction area significantly reduced in the I/R rat, respectively. Simultaneously, acacetin alleviated oxidative stress of myocardial tissues induced by I/R injury. The I/R group significantly reduced SOD activity, whereas SOD activity increased significantly after the propranolol and acacetin treatment (**Figure 2B**). Moreover, levels of MDA significantly increased in rats from the I/R group as compared with those in the I/R group (**Figure 2C**).

Acacetin attenuates I/R-induced myocardial morphology changes

The changes in the morphological structures of myocardial tissues were evaluated by HE coloration. As shown in **Figure 3A**, histopathological examination of the myocardium of rats from the control group showed a uniform color and an intact myocardial histology without evidence of myocardial membrane damage. The I/R group showed uneven staining, with an enlarged interstitial region, a small amount of inflammatory cell infiltration around the interstitial region and a small hemorrhage (**Figure 3B**). Pretreatment with acacetin generally improved pathological results compared to the I/R group and showed a trend of decreasing myonecrosis, infiltration of inflammatory cells and extravasation of red blood cells (**Figure 3D-F**). The propranolol group showed similar results as the 40 mg/kg acacetin group (**Figure 3C**).

Acacetin improves I/R-Induced ultrastructural alterations

As shown in **Figure 4**, the representative ultrastructural images of myocardium in the six groups are presented. As expected, the myocardia of the control group revealed clear and

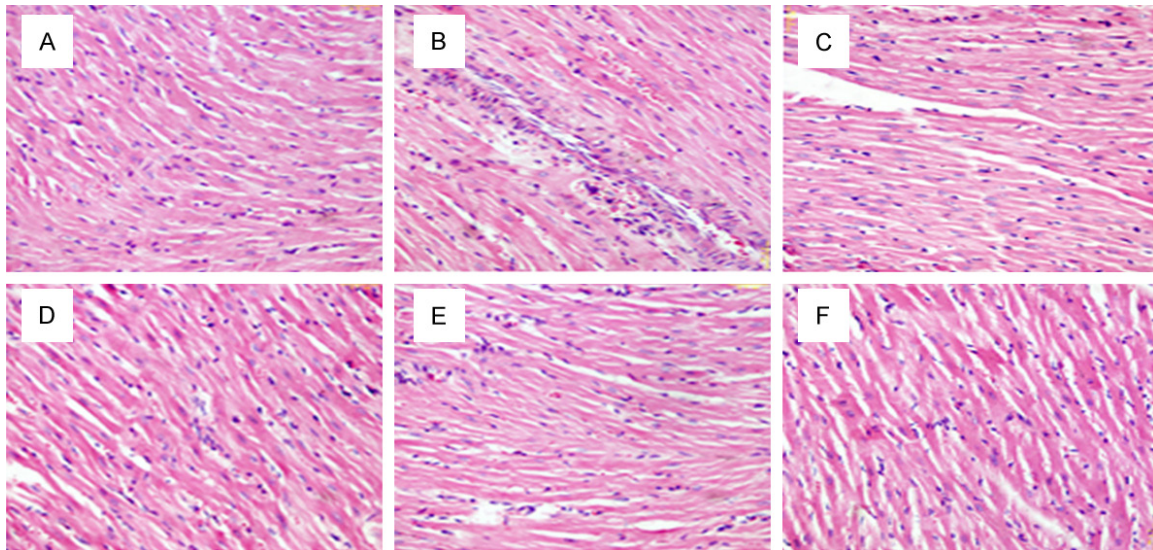**Figure 3.** Effect of acacetin pretreatment on myocardial morphology alterations (× 200), Representative photographs of myocardium stained by HE in different groups. A: Control group, B: I/R group, C: Propranolol + I/R group, D: 10 mg/kg acacetin + I/R group, E: 20 mg/kg acacetin + I/R group, F: 40 mg/kg acacetin + I/R group (n=3).
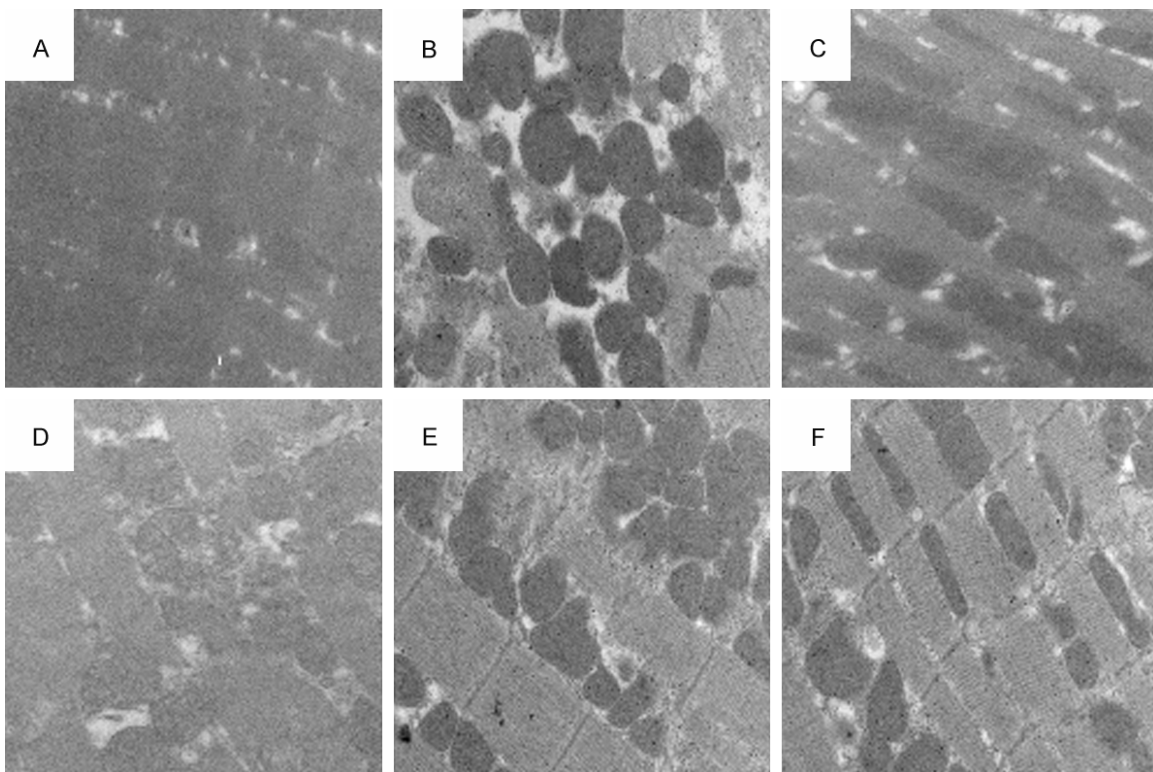

Figure 4. Effect of acacetin pretreatment on ultrastructural alterations (× 20000), Presented are the representative electron micrographs of myocardium from different groups, A: Conntrol group, B: I/R group, C: Propranolol + I/R group, D: 10 mg/kg acacetin + I/R group, E: 20 mg/kg acacetin + I/R group, F: 40 mg/kg acacetin + I/R group (n=3).

integrated mitochondrial structures, and also preserved intercalated discs and myofilaments well (**Figure 4A**). Compared with the control group, apparent alterations were visible in the
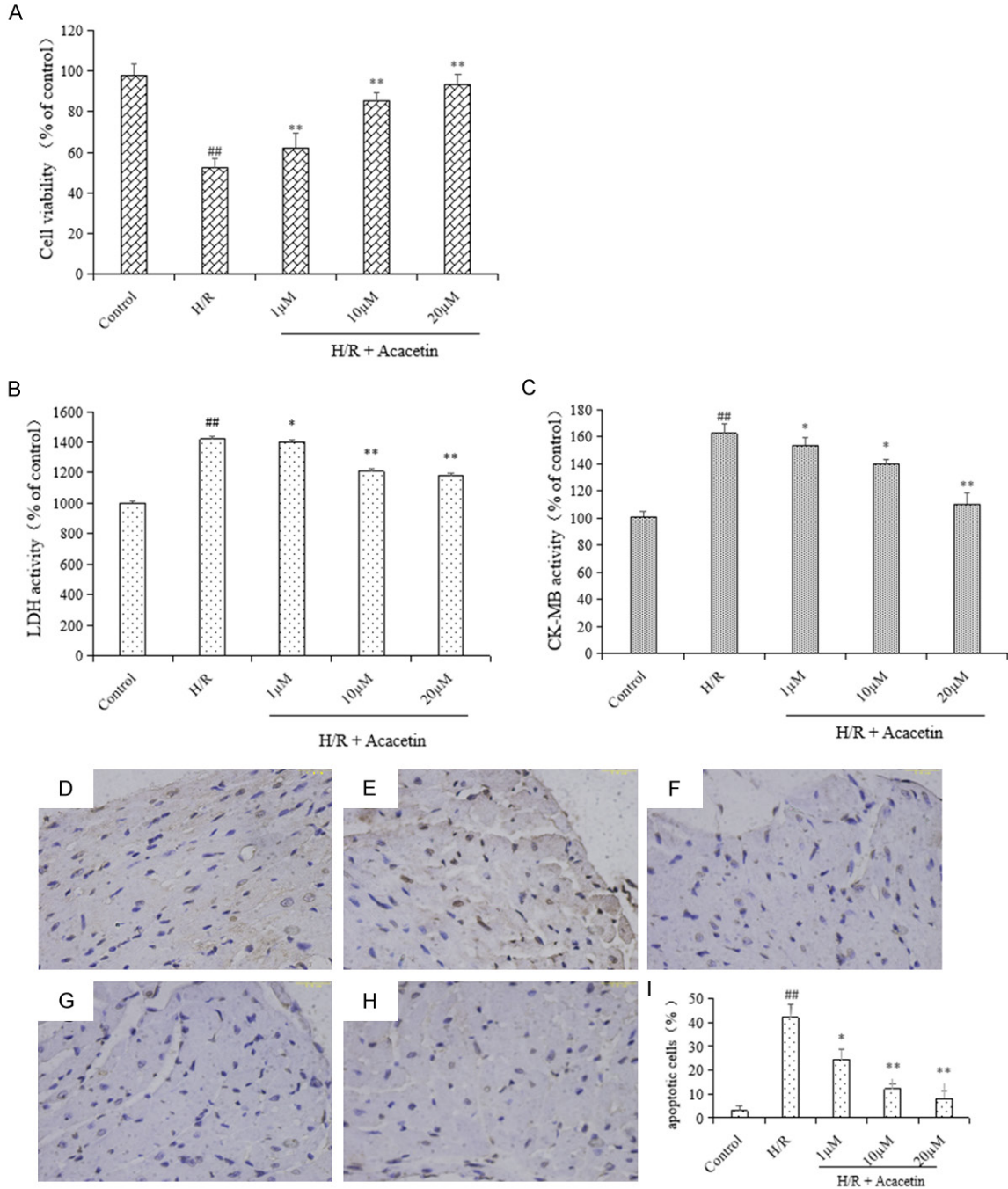
Figure 5. Acacetin prevented against H/R-induced injury of H9c2 cardiomyocytes, (A) Cardiomyocytes were pretreated with acacetin followed by H/R and cell viability was determined by MTT assay. (B) LDH activity. (C) CK-MB activity. Results were expressed as percentages of control and presented as mean ± S.D. for five independent experiments. Control; H/R: Hypoxia/reoxygenation. ##P<0.01 vs. Control; *P<0.05, **P<0.01 vs. H/R. Acacetin attenuated apoptosis in H9c2 cardiomyocytes in response to H/R, (D) Control cells, (E) H/R cells, (F) H/R + acacetin (1 μM), (G) H/R + acacetin (10 μM), and (H) H/R + acacetin (20 μM). Apoptotic cells are characterized by nuclear condensation and stained in dark brown, (I) apoptotic cells. Data were presented as mean ± SD. of five independent experiments. ##P<0.01 vs. Control; *P<0.05, **P<0.01 vs. H/R; P<0.05, vs. H/R + acacetin.

I/R group, including disrupted myofibrils and swelling mitochondria (**Figure 4B**). Pathologic injury in the propranolol group and the acacetin groups were obviously improved (**Figure 4C-F**).
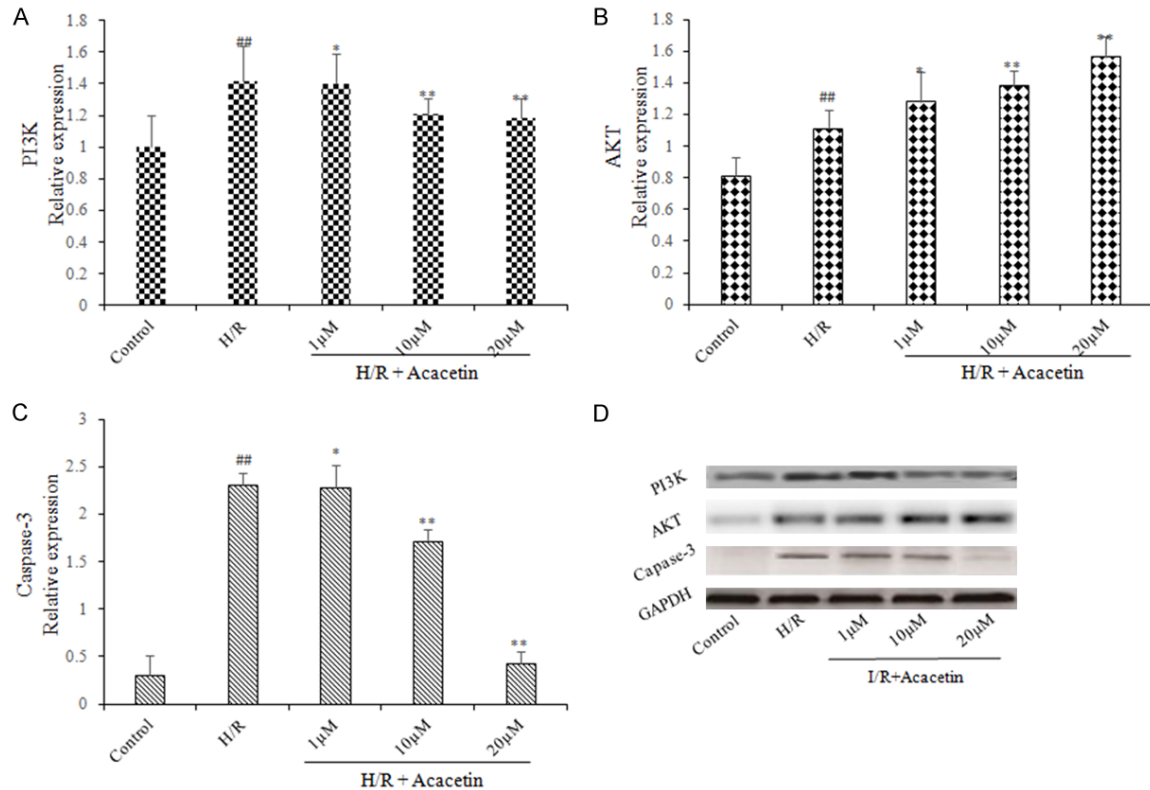
Figure 6. Effects of Acacetin on the expression of PI3K, Akt, and caspase-3. A: The levels of PI3K. B: The levels of Akt. C: The levels of caspase-3. D: The protein levels of PI3K, akt, and caspase-3 were measured by western blot. Data are shown as the mean ± SD. of five independent experiments. ##$P<0.01$ vs. Control, *$P<0.05$, **$P<0.01$ vs. H/R.

Acacetin augmented cell viability and cardiac enzyme in H9c2 cardiomyocytes injury induced by H/R

To analyze the antiapoptotic actions of acacetin at a cellular level, H9c2 cardiomyocytes survival following H/R was assessed by MTT assay. It was found that H/R induced significant decrease in cell viability to 52.20 ± 4.50% as compared to control cells ($P<0.01$), while acacetin pretreatment resulted cell viability significantly increase in H9c2 cardiomyocytes injury induced by H/R (**Figure 5A**). This indicated that cell viability significantly increased by treatment of acacetin.

As CK-MB and LDH release is an acknowledged marker for cell damage, cardiomyocytes injury was also assessed by determining the release of CK-MB and LDH in culture medium at the end of reperfusion. As a consequence of H/R, the serum levels of CK-MB and LDH were significantly higher in the H/R than in the control ($P<0.01$). Compared with the H/R, serum levels of CK-MB and LDH were lower in the pretreated with 1 μM, 10 μM, and 20 μM acacetin ($P<0.05$

or <0.01) (**Figure 5B, 5C**). This indicated that acacetin significantly reduces the levels of CK-MB, LDH after H/R injury.

Acacetin inhibited H9c2 cardiomyocytes from apoptosis induced by H/R

Apoptosis plays a critical role in myocardial H/R injury [15]. In order to determine whether the anti-apoptotic effect is involved in acacetin induced cardioprotection, myocardial apoptosis was further examined following I/R using TUNEL assays. As shown in **Figure 5D-I**, TUNEL staining revealed that the I/R group exhibited severe tissue damage that appeared to markedly increase the number of TUNEL-positive cells, while acacetin pretreatment resulted in a marked reduction in the number of TUNEL-positive cells in H9c2 cardiomyocytes injury induced by H/R.

Acacetin regulated PI3K-Akt pathway activation in H/R stimulated H9c2 cells

To determine whether pretreatment with acacetin would induce the activities of PI3K-Akt sig-

naling pathway in H/R H9c2 cells, additional experiments were performed. **Figure 6A-C** shows that compared with the H/R group, the mRNA expression of PI3K, caspase-3 were significantly decreased and the mRNA expression of Akt were significantly increased in the pretreatment acacetin group. Moreover, the protein activities of PI3K, caspase-3, and Akt were measured using Western blot. As depicted in **Figure 6D**, we found that the protein activities of caspase-3, PI3K were significant decreased in the pertreatment acacetin compared with the H/R group. However, the protein activities of AKT were markedly increased in the pertreatment acacetin.

Discussion

In the present study, acacetin pre-treatment mitigated myocardial I/R injury in rats, as manifested by a reduction of the infarct area, a decrease in the levels of cardiac enzymes and an improvement of cardiac function. In addition, acacetin significantly suppressed oxidative stress and apoptosis in H9c2 cells exposed to H/R treatment. Exploration of the underlying mechanisms of its action indicated that acacetin regulated the activation of PI3K/Akt signaling pathway in H/R-stimulated H9c2 cells.

At present studies have shown that oxidative stress is related to the production of ROS and anti-oxidant capacity, which play a major factor in the etiopathogenesis of I/R injury [16]. Excessive oxygen free radicals are the key factors leading to oxidative damage, which affect antioxidant activation, cardiomyocytes apoptosis, and further cause inflammation mediator in cardiomyocytes [17]. As an endogenous antioxidant enzyme, SOD can protect cardiomyocytes of oxidative stress in I/R injury. In addition, MDA is a major index in lipid peroxidation, which leads to destruction of cellular and proteins structural [18]. These results show that SOD activities were markedly elevated by acacetin, whereas MDA levels were markedly reduced in the I/R rat. Move importantly, myocardial histopathological changes showed that acacetin can significantly attenuated ultrastructure of the myofilaments and mitochondria in the myocardial from I/R. Therefore, one of the cardioprotective mechanisms of acacetin is the suppression of free radicals, peroxide, and increased antioxidant enzyme activity.

When cardiomyocytes are damaged, the cardiac membrane becomes porous or ruptured, causing myocardial enzyme LDH and CK-MB inside the cells to be released into the blood [19]. Pre-treatment with acacetin reduced the contents of CK-MB and LDH enzymes in ischemic heart tissues. Cardiomyocyte apoptosis plays a key factor in the development of myocardial injury and cardiac dysfunction after ischemia [20, 21]. In addition, cardiomyocytes apoptosis plays vital factor pathogenic aspect for myocardial I/R injury [22]. However, suppression of cardiomyocyte apoptosis shall be deemed to successful treatment strategies for the therapy of myocardial I/R injury. In the present study, acacetin significantly decreased myocardial apoptosis, which was also consistent with myocardial enzyme and infarction size analysis.

Although acacetin protective myocardial I/R injury have long drawn scholars' attention, but the studies on molecular mechanisms reports are still insufficient. The PI3K/Akt signaling pathway plays a key role for the intracellular transduction of membrane receptor, it involves every aspect of cell proliferation, metabolism and apoptosis. PI3K is a major apoptotic inhibitory protein, which can be involved in the regulation of apoptotic signal by regulating the integrity of mitochondrial membrane, promoting the survival of cardiac myocytes and inhibiting apoptosis. However, Akt activation can promote the production of anti-apoptotic proteins and inhibit the formation of caspase-3, thereby inhibiting apoptosis. Therefore, this study confirmed further the molecular mechanisms by which acacetin inhibits cardiomyocytes apoptosis via PI3K-Akt pathway.

In conclusion, acacetin exhibited significantly protective effects in I/R-induced myocardial injuries in rats in this study. The protective mechanisms of acacetin protective myocardial I/R injury and inhibits apoptosis of H9c2 cardiomyocytes via PI3K-Akt pathway. This suggests that acacetin is a potentially useful drug for cardioprotection against I/R injury.

Disclosure of conflict of interest

None.

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