Apigenin suppresses the apoptosis of H9C2 rat cardiomyocytes subjected to myocardial ischemia-reperfusion injury via upregulation of the PI3K/Akt pathway

ZHENGWEN ZHOU[1], YUE ZHANG[2], LUNING LIN[3] and JIANMEI ZHOU[4]

Departments of [1]Electrocardiogram Diagnosis and [2]Cardiovasology, Zhejiang Hospital;
[3]College of Pharmaceutical Science, Zhejiang University of Traditional Chinese Medicine;
[4]Department of Cardiac Rehabilitation, Zhejiang Hospital, Hangzhou, Zhejiang 310013, P.R. China

Received December 25, 2017; Accepted May 2, 2018

DOI: 10.3892/mmr.2018.9115

Abstract. Apigenin, a flavonoid with multiple physiological and pharmacological activities, is associated with the prevention of cardiovascular diseases. The present study aimed to examine the roles and mechanisms of apigenin in the apoptosis of H9C2 rat cardiomyocytes, which were subjected to myocardial ischemia-reperfusion (MI/R) injury. Cell viability, reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and cellular apoptosis were evaluated using cell counting kit-8 assays and flow cytometry. The content/activity of oxidative stress markers was determined using commercial kits. Western blot analysis and reverse transcription-quantitative polymerase chain reaction assays were used to measure protein and mRNA expression, respectively. The results demonstrated that apigenin had limited cytotoxicity on the viability of H9C2 rat cardiomyocytes. Apigenin reduced the oxidative stress, ROS production and cellular apoptotic capacity of MI/R-induced H9C2 cells. Apigenin additionally increased the MMP level of MI/R-induced H9C2 cells. Furthermore, apigenin modulated apoptosis-associated protein expression and phosphatidylinositol 3'-kinase (PI3K)/RAC-α serine/threonine-protein kinase (Akt) signaling in MI/R-induced H9C2 cells. Treatment with LY294002 reversed the anti-apoptotic effect of apigenin. In conclusion, apigenin suppressed the apoptosis of H9C2 cells that were subjected to MI/R injury by activating the PI3K/Akt pathway. It was suggested that apigenin may be effective as an MI/R therapy.

Correspondence to: Dr Jianmei Zhou, Department of Cardiac Rehabilitation, Zhejiang Hospital, 12 Lingyin Road, Hangzhou, Zhejiang 310013, P.R. China
E-mail: jianmeizhouu@163.com

Key words: apigenin, H9C2, myocardium ischemia, reperfusion injury, reactive oxygen species, mitochondrial membrane potential, phosphatidylinositol 3'-kinase, RAC-α serine/threonine-protein kinase

Introduction

Currently, cardiovascular diseases remain a leading cause of mortality worldwide. Among cardiovascular diseases, coronary heart diseases (CHDs) are frequently occurring cardiovascular diseases (1). It is estimated that 110 million people succumbed to CHD annually in 2015 (2). Approximately half of patients with CHD suffer from acute myocardial infarction and sudden cardiac mortality, of which, the complications bring economic burden to families and society (3,4). Myocardial ischemia-reperfusion (MI/R) injury is a common clinical pathophysiological phenomenon in CHD, which fails to restore normal cardiac function, and aggravates the dysfunction and structural impairments of the heart (5). The clinical manifestations of MI/R are arrhythmia, no reflow phenomenon, myocardial stunning and necrocytosis (6). Currently, there is no effective therapy to prevent MI/R. Thus, the search of novel therapeutic agents and therapeutic strategies for MI/R is important.

RAC-α serine/threonine-protein kinase (Akt), also termed protein kinase B, serves as a critical tumor factor and a downstream effector of phosphatidylinositol 3'-kinase (PI3K) (7). Previous studies have reported that MI/R induces the apoptosis of cardiomyocytes (8,9), and that the upregulation of PI3K/Akt pathway may suppress myocardial apoptosis (10). It was additionally demonstrated that the PI3K/Akt pathway inhibited myocardial apoptosis to protect the heart through numerous mechanisms, including by suppressing caspase-3 activation and DNA damage (11), affecting the metabolism of glucose (12), enhancing the effects of the Bcl-2 family (13) and inhibiting the expression of apoptosis-regulating genes (14). However, to the best of our knowledge, limited research regarding the involvement of the PI3K/Akt pathway in myocardial apoptosis induced by MI/R injury exists.

Apigenin is an important flavonoid that exhibits a variety of physiological and pharmacological activities, and is associated with the prevention of cardiovascular diseases (15). Previous studies have demonstrated that apigenin possess multiple pharmacological activities, including anti-oxidant (16), anti-mutagenesis (17), anti-inflammatory (18) and anti-tumor (19). However, the pharmacological activity and effect

of apigenin on myocardial apoptosis induced by MI/R is unclear.

The present study aimed to investigate whether apigenin acts as a novel therapeutic agent that affects myocardial apoptosis induced by MI/R. Furthermore, the exact roles and mechanisms of apigenin, together with the PI3K/Akt pathway in MI/R injury were investigated.

Materials and methods

Establishment of H9C2 cells subjected to oxygen-glucose deprivation/reoxygenation (OGD/R) injury as a model of MI/R. H9C2 rat cardiomyocytes were obtained from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The H9C2 cells were treated as described in previous literature to establish an *in vitro* model of MI/R (20,21), and the protocols were further developed in the present study. Medium with no serum or glucose served as the ischemic buffer. The ischemic buffer was incubated in an atmosphere with a gas mixture of 95% N_2 and 5% CO_2 for 2 h. The cells were subsequently cultured in glucose-free Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), in an anoxic environment (95% N_2 and 5% CO_2) at 37˚C for 2 h. The H9C2 cells were transferred to fresh medium (DMEM) at 37˚C and the gas mixture of 95% N_2 and 5% CO_2 was replaced with air at a flow rate of velocity of 2 l/min (95% O_2 and 5% CO_2). Following incubation for 1 h, the MI/R cell model was harvested and cells were subsequently observed under an inverted microscope.

Grouping. A total of five treatment groups were prepared in the present study: The control group (H9C2 cells with no treatment); the MI/R group (H9C2 cells treated with OGD/R injury); the 1 μM apigenin + MI/R group (H9C2 cells treated with OGD/R injury, and subsequently treated with 1 μM apigenin); the 6 μM apigenin + MI/R group (H9C2 cells treated with OGD/R injury, and subsequently treated with 6 μM apigenin); and the 25 μM apigenin + MI/R group (H9C2 cells treated with OGD/R injury, and subsequently treated with 25 μM apigenin). Inhibition of PI3K was performed via incubation with LY294002 (25 μM) for 2 h as previously described (22).

Cell viability analysis. A Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) was used to determine the cell viability of the H9C2 cells. H9C2 cells ($6x10^4$ cells/ml) cultured in the logarithmic phase were seeded in 96-well plates, and subsequently maintained in a 5% CO_2 atmosphere at 37˚C for 12 h. Apigenin at different concentrations (1, 3, 6, 12, 25 and 50 μM) was added to the cells. The H9C2 cells were maintained for 12, 24 and 48 h. Subsequently, 10 μl CCK-8 reagent was added to the wells of the 96-well plates and the H9C2 cells were maintained for a further 3 h. A microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to record the absorbance at 450 nm. Cell viability was evaluated as the percentage of cell survival compared with the control.

Enzyme activity detection. H9C2 cells were seeded into 96-well plates ($6x10^3$ cells/well) and treated according to the aforementioned protocol. Cells were subsequently collected and the content/activity of lactate dehydrogenase (LDH), malondialdehyde (MDA), catalase (CAT), Na^+K^+-ATPase and Ca^{2+}-ATPase were determined using a LDH Assay Kit (cat. no. ab102526), MDA Assay Kit (cat. no. ab118970) (both from Abcam, Cambridge, UK), CAT Assay Kit (cat. no. BC0205), Na^+K^+-ATPase assay kit (cat. no. BC0065) and Ca^{2+}-ATPase assay kit (cat. no. BC0960) (all from Beijing Solarbio Science & Technology Co., Ltd.), respectively, in accordance with the manufacturers' protocol.

Apoptosis assay. Annexin V is a phospholipid binding protein, which has a high affinity for phosphatidylserine. Annexin V is a sensitive indicator for detecting early apoptosis of cells. Propidium iodide (PI) is a type of nucleic acid dye that is not able to penetrate an intact cell membrane. PI penetrates the cell membrane as cell membrane permeability increases in the late stage of apoptosis. Therefore, Annexin V and PI may be used to distinguish cells in different apoptotic periods. Flow cytometry (FCM) was conducted to assess the apoptosis of H9C2 cells. Following washing with PBS, cultured H9C2 cells were trypsinized with 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China). The supernatant was collected and the H9C2 cells were prepared for assessment by suspension in an incubation buffer at a density of $1x10^6$ cells/ml. H9C2 cells were subsequently incubated with Annexin V-fluorescein isothiocyanate and PI (XiLong Scientific Co., Ltd., Shantou, China) in the dark at room temperature for 15 min. A flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, CA, USA) with CellQuest software version 3.3 was used to measure cellular apoptosis.

Evaluation of mitochondrial membrane potential (MMP) and reactive oxygen species (ROS). PBS was added to the cultured H9C2 cells until the cell density reached $1x10^6$/ml. FL1-H [Multisciences (Lianke) Biotech Co., Ltd., Hangzhou, China] was added to the H9C2 cells. H9C2 cells were incubated in the dark at room temperature for 10 min. The supernatant was collected and 100 μl PBS was added to the H9C2 cells. ROS have the ability to transform dichloro-dihydro-fluorescein diacetate (DCFH-DA) into 2',7'-dichlorofluorescein (DCF). The fluorescence of DCF indicates the ROS level. The DCFH-DA dye (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to detect ROS generation, according to previous literature (23,24). The fluorescence dye Rhodamine-123 (Rho-123) is able to penetrate the cell membrane. As described in previous studies (25,26), Rho-123 (Sigma Aldrich; Merck KGaA) was used to detect the MMP in the present study. FCM was performed with an excitation wavelength of 488 nm to assess the MMP and ROS levels in H9C2 cells. Cells ($1x10^4$) were collected from each sample, and the data were analyzed using BD CellQuest™ Pro software (version 3.3; BD Biosciences).

Western blot analysis. Cultured cells were lysed on ice in a radioimmunoprecipitation assay lysis buffer (Thermo Scientific Inc.; cat. no. 89900). The cells were fragmented following treatment with an ultrasonic cell disruptor. Following centrifugation at 5,000 x g at 4˚C, fractionation occurred and the supernatant was collected. The protein expression level was measured using a protein assay reagent (Bio-Rad Laboratories,

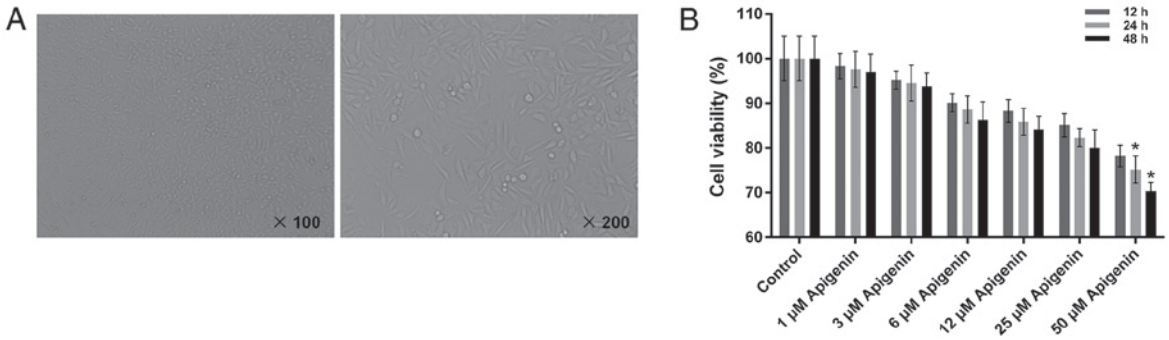

Figure 1. Growth characteristics and cell viability of H9C2 cells treated with apigenin. (A) H9C2 cells were observed with an inverted microscope. (B) A cell counting kit-8 assay was performed to assess the cell viability of H9C2 cells treated with different concentrations of apigenin. *P<0.05 vs. control.

Inc.) and was performed following the manufacturer's protocol. From each sample, an equal quantity of protein (50 μg) was separated using 5% SDS-PAGE. The proteins were obtained and transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) for 1.5 h. Nonspecific proteins were blocked by immersing the membranes into 5% low fat dried milk at room temperature for 2 h following washing with PBS. The membranes were incubated with primary antibodies: Anti-poly ADP-ribose polymerase (PARP; 1:5,000; cat. no. ab32138); anti-cleaved-caspase-3 (1:500; cat. no. ab49822); tumor necrosis factor receptor superfamily member 6 (Fas; 1:2,000; cat. no. ab133619); tumor necrosis factor ligand superfamily member 6 (Fasl; 1:8,000; cat. no. ab186671); anti-phospho (p)-PI3K (1:800; cat. no. ab182651); anti-PI3K (1:1,000; cat. no. ab189403); anti-p-Akt (1:500; ab38449); anti-Akt (1:6,000; cat. no. ab81283); anti-apoptosis regulator Bcl-2 (Bcl-2; 1:1,000; cat. no. ab59348); anti-p-serine/threonine-protein kinase mTOR (mTOR; phospho S2448; 1:1,000; cat. no. ab109268); anti-mTOR (1:1,000; cat. no. ab32028); and anti-GAPDH (1:2,500; cat. no. ab9485) (all from Abcam) overnight at 4˚C. Horseradish peroxidase-conjugated secondary antibodies (1:5,000; Abcam; cat. no. ab205718; goat anti-rabbit) were added and incubated at room temperature for 1 h. Enhanced chemiluminescent reagents (EMD Millipore) with an enhanced chemiluminescence system (GE Healthcare Life Sciences, Little Chalfont, UK) were performed to assess the results. Densitometric analysis was performed using Quantity One software (version 4.6.9; Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. TRIzol® reagent (Beyotime Institute of Biotechnology) was used to extract the total RNA from the cultured H9C2 cells. RNA was reverse transcribed to cDNA using an RT kit (Beyotime Institute of Biotechnology), according to the manufacturers' protocol. The temperature protocol used for RT was as follows: 25˚C for 10 min, 42˚C for 50 min and 70˚C for 5 min. RT-qPCR analysis was performed using SYBR-Green PCR Master Mix (Thermo Fisher Scientific, Inc.) on an ABI 7500 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR cycling conditions were as follows: Pretreatment at 95˚C for 10 min; 96˚C for 15 sec; 65˚C for 45 sec (45 cycles); 96˚C for 15 sec; 65˚C for 1 min; 95˚C for 15 sec; and a final extension at 75˚C for 10 min, maintained at 4˚C. The results were quantified using the $2^{-\Delta\Delta Cq}$ method (27). The primers were purchased from Invitrogen (Thermo Fisher Scientific, Inc.): PARP forward, 5'-CTGTTA ATGCTAATCGTGAT-3' and reverse, 5'-AACTGACTCCTA CATATTAG-3' (product, 223 bp); Fas forward, 5'-AGTACA GCCGGGAAGACAAT-3' and reverse, 5'-TTTCTGGGCCAT GCTTCTCT-3' (product, 269 bp); Fasl forward, 5'-CAGAAA GCATGATCCGCGAC-3' and reverse, 5'-GGTCTGGGCCAT AGAACTGA-3' (product, 215 bp); GAPDH forward, 5'-TCT GAACTCCAACGATGCCT-3' and reverse, 5'-TCTTGTCCT TAAGCCTGGGG-3' (product, 225 bp). GAPDH was used as the control to normalize the input RNA level.

Statistical analysis. The results are presented as the mean ± standard deviation. The data was evaluated using one-way analysis of variance followed by Tukey's multiple comparisons. Statistical analyses were performed using GraphPad Prism software (version 6.0; GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference. All experiments were performed in triplicate.

Results

Characterization of the H9C2 rat myocardial cell line. The cultured cardiomyocytes were observed with an inverted microscope (Fig. 1A). The shape of the H9C2 cells was round prior to adhesion. However, after 4-6 h, the majority of the cardiomyocytes began to adhere to the wall of culture bottle, exhibiting a round or spindle shape. Following culture for 24 h, the cells were arrayed in long fusiform, with a few arranged in triangular or irregular polygonal form. Additionally, cell nuclei were small and the cytoplasm was dark. After 48 h, cells dispersed gradually at the bottom of culture bottle, forming a cell monolayer. The cardiomyocytes were subsequently harvested for further experiments.

Apigenin has limited cytotoxic activity on the cell viability of H9C2 cells. To evaluate the cytotoxic activity of apigenin on normal H9C2 cells, a CCK-8 assay was performed to determine the cell viability of H9C2 cells. The present results demonstrate that apigenin at concentrations of 1, 3, 6, 12 and 25 μM did not induce significant cytoxicity in H9C2 cells. The cell viability of H9C2 cells treated with apigenin was >80% with the exception that the cell viability of H9C2 cells was significantly lower compared with the control when the concentration of apigenin was 50 μM (Fig. 1B; P<0.05). Thus,

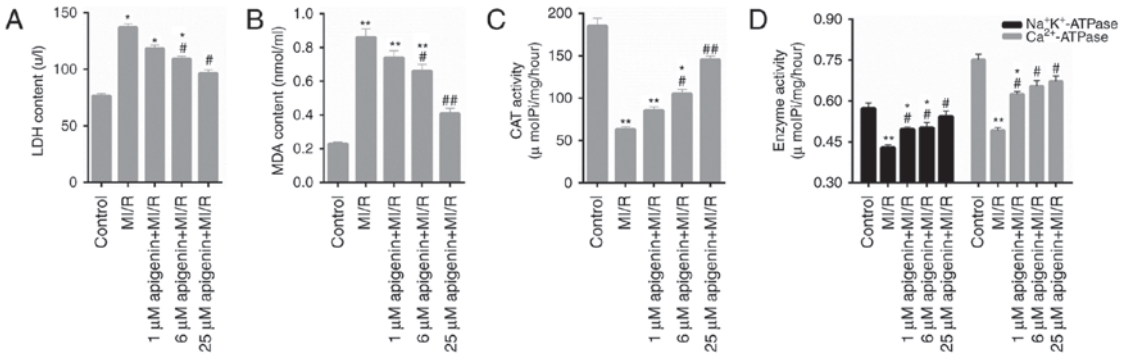

Figure 2. Apigenin decreases the oxidative stress in MI/R-induced H9C2 cells. H9C2 cells were treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. The levels of (A) LDH, (B) MDA, (C) CAT (D) and ATPase in H9C2 cells was determined using commercial kits. *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MI/R. MI/R, myocardial ischemia-reperfusion; LDH, lactate dehydrogenase; MDA, malondialdehyde; CAT, catalase.

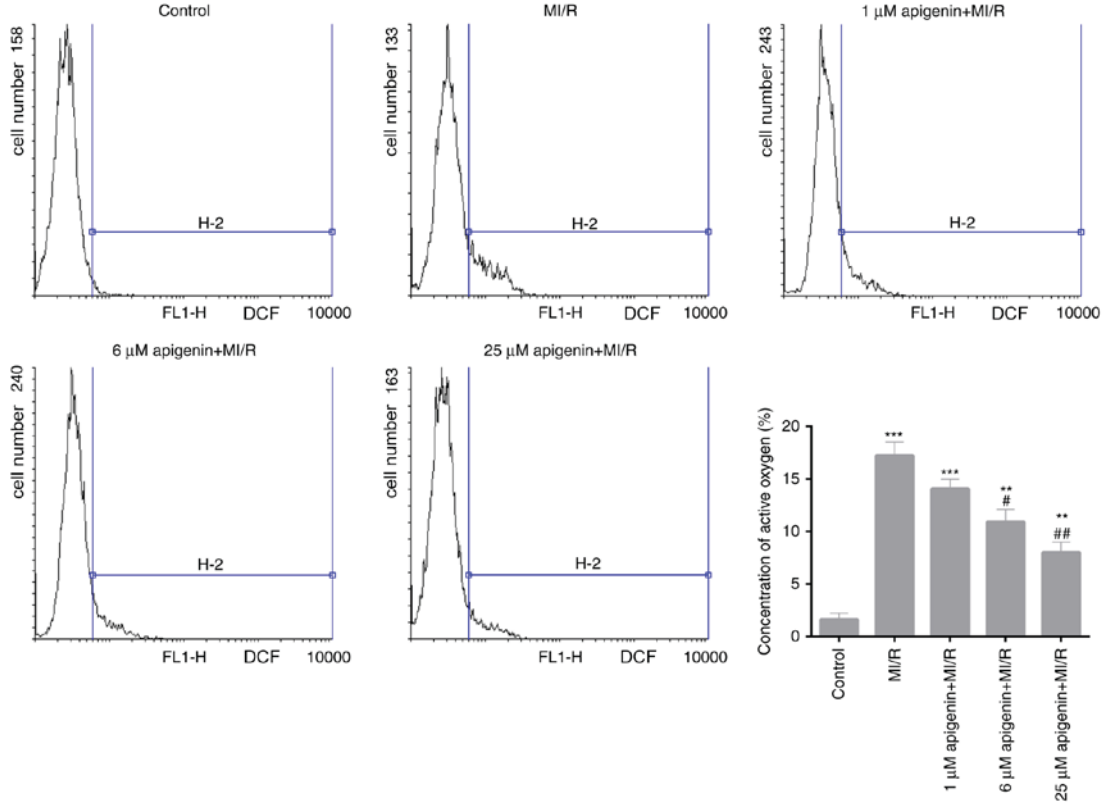

Figure 3. Apigenin reduces the ROS production in MI/R-induced H9C2 cells. Flow cytometry was conducted to evaluate the ROS content in H9C2 cells treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MI/R. ROS, reactive oxygen species; MI/R, myocardial ischemia-reperfusion; DCF, 2',7'-dichlorofluorescein.

we selected 1, 6 and 25 μM to treat the H9C2 cells for 12 h for the subsequent experiments.

Oxidative stress in MI/R-induced H9C2 cells is decreased by apigenin. Numerous oxidative stress markers in H9C2 cells, including LDH, MDA, CAT and ATPase activity, were assessed in the present study. The data indicated that the LDH content was significantly increased by MI/R injury (Fig. 2A; P<0.05); however, reduced upon treatment with apigenin. MDA content in H9C2 cells additionally exhibited similar trends following treatment with MI/R injury and apigenin (Fig. 2B; P<0.05) at different concentrations. The CAT activity in H9C2 cell treated with MI/R injury was significantly decreased compared with the control group (Fig. 2C; P<0.01). However, apigenin markedly enhanced the activity of CAT in MI/R-induced H9C2 cells (Fig. 2C). It was additionally observed that apigenin significantly increased the activities of $Na^{+}K^{+}$-ATPase and Ca^{2+}-ATPase in H9C2 cells induced by MI/R injury (Fig. 2D; P<0.05).

Apigenin reduces ROS production in MI/R-induced H9C2 cells. The ROS content was measured in H9C2 cells from each treatment group. According to the FCM data, MI/R injury significantly increased the ROS production in H9C2 cells (Fig. 3; P<0.001). However, following treatment with apigenin at different concentration levels, a dose-dependent

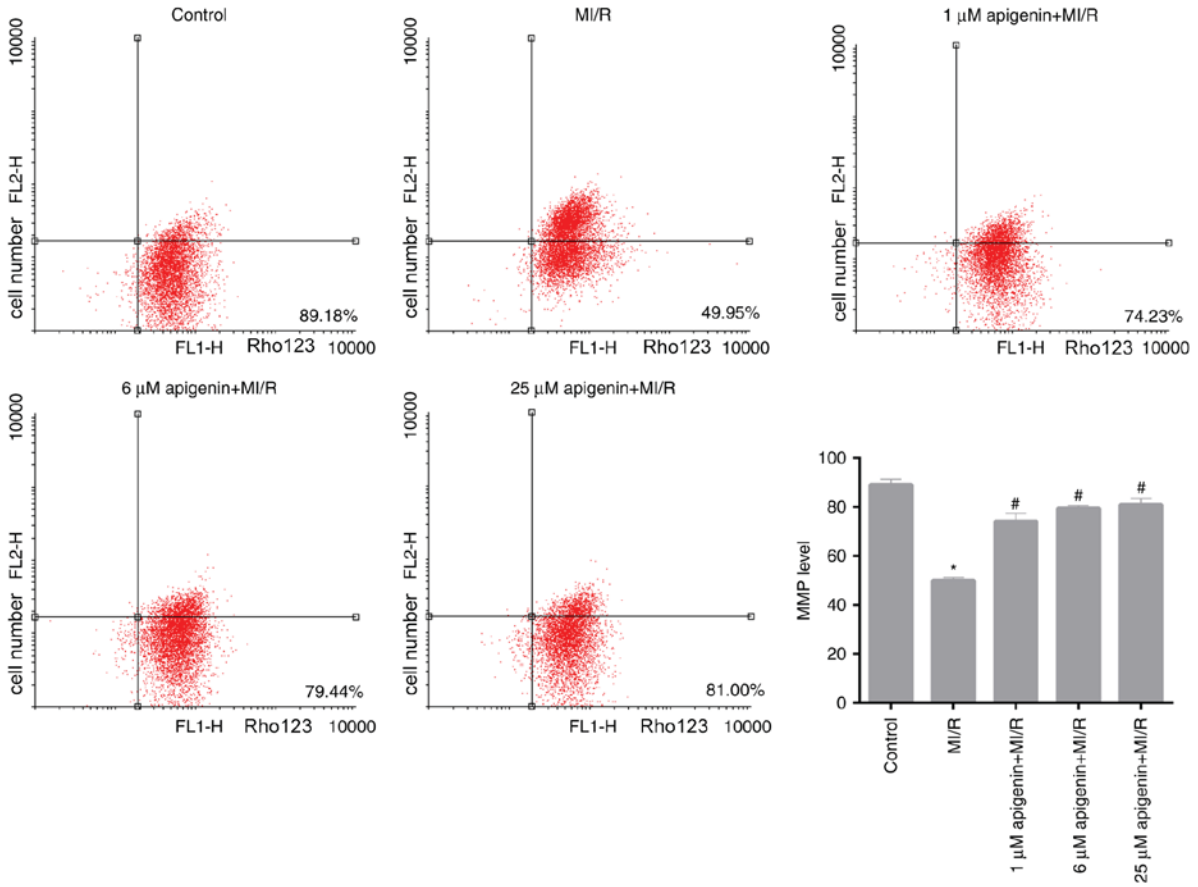

Figure 4. Apigenin modulates the MMP level in MI/R-induced H9C2 cells. Flow cytometry was performed to assess the MMP level in H9C2 cells treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. *P<0.05 vs. control; #P<0.05 vs. MI/R. MMP, mitochondrial membrane potential; MI/R, myocardial ischemia-reperfusion; Rho123, Rhodamine-123.

decrease in the ROS content in MI/R-induced H9C2 cells was observed, which was significant at 6 and 25 μM compared with MI/R injury (Fig. 3; P<0.05).

Apigenin modulates the MMP level in MI/R-induced H9C2 cells. Furthermore, in the present study, MMP level in H9C2 cells, which were treated with MI/R and apigenin at different concentrations, was measured. Based on the FCM data, it was observed that MI/R significantly reduced the MMP level in H9C2 cells compared with the control (Fig. 4; P<0.05). Following treatment with apigenin at different concentrations, the MMP level in MI/R-induced H9C2 cells significantly recovered (Fig. 4; P<0.05).

Apigenin suppresses the MI/R-mediated apoptosis of H9C2 cells. As the aforementioned data suggested that apigenin may decrease the oxidative stress in MI/R-induced H9C2 cells, the apoptosis capacity of MI/R-induced H9C2 cells that treated with apigenin was evaluated. The FCM data demonstrated that the percentage of apoptotic H9C2 cells in the MI/R group was 16.27%, which was significantly increased compared with the control (Fig. 5; 1.77%; P<0.001). Furthermore, following treatment with apigenin at different concentrations of 1, 6 and 25 μM, the apoptosis rate of MI/R-induced H9C2 cells decreased from 16.27 to 13.18, 10.43 and 9.74%, respectively (Fig. 5; P<0.01). These data indicated that apigenin suppressed the apoptosis capacity of MI/R-induced H9C2 cells in a dose-dependent manner.

Expression of apoptosis-associated proteins is regulated by apigenin. It was demonstrated that apigenin inhibited apoptosis of H9C2 cells induced by MI/R; therefore, apoptosis-associated protein expression in H9C2 cells was measured. According to the RT-qPCR data, PARP, Fas and Fasl expression in H9C2 cells from the MI/R group was significantly higher than the control group (Fig. 6A; P<0.001). The PARP, Fas, and Fasl expression in MI/R-induced H9C2 cells was markedly decreased upon treatment with apigenin in a dose-dependent manner (Fig. 6A). Furthermore, western blot analysis additionally demonstrated that the expression levels of PARP, cleaved caspase-3, Fas and Fasl in H9C2 cells were significantly upregulated upon MI/R injury compared with the control (Fig. 6B; P<0.0001); whereas, the expression of these molecules was markedly decreased with the addition of apigenin at different concentrations (Fig. 6B).

Apigenin affects the PI3K/Akt pathway. In addition, the mechanisms of apigenin in protecting H9C2 cells against MI/R induced injury were examined. The p-PI3K, PI3K, p-Akt, and Akt level in H9C2 cells was measured. Among the H9C2 cells from MI/R groups, the level of p-PI3K was significantly decreased compared with the control (Fig. 7A; P<0.001). However, significant increases were observed in the p-PI3K level in MI/R-induced H9C2 cells treated with apigenin at different concentrations (Fig. 7A; P<0.01). Furthermore, there was no significant difference in the expression of

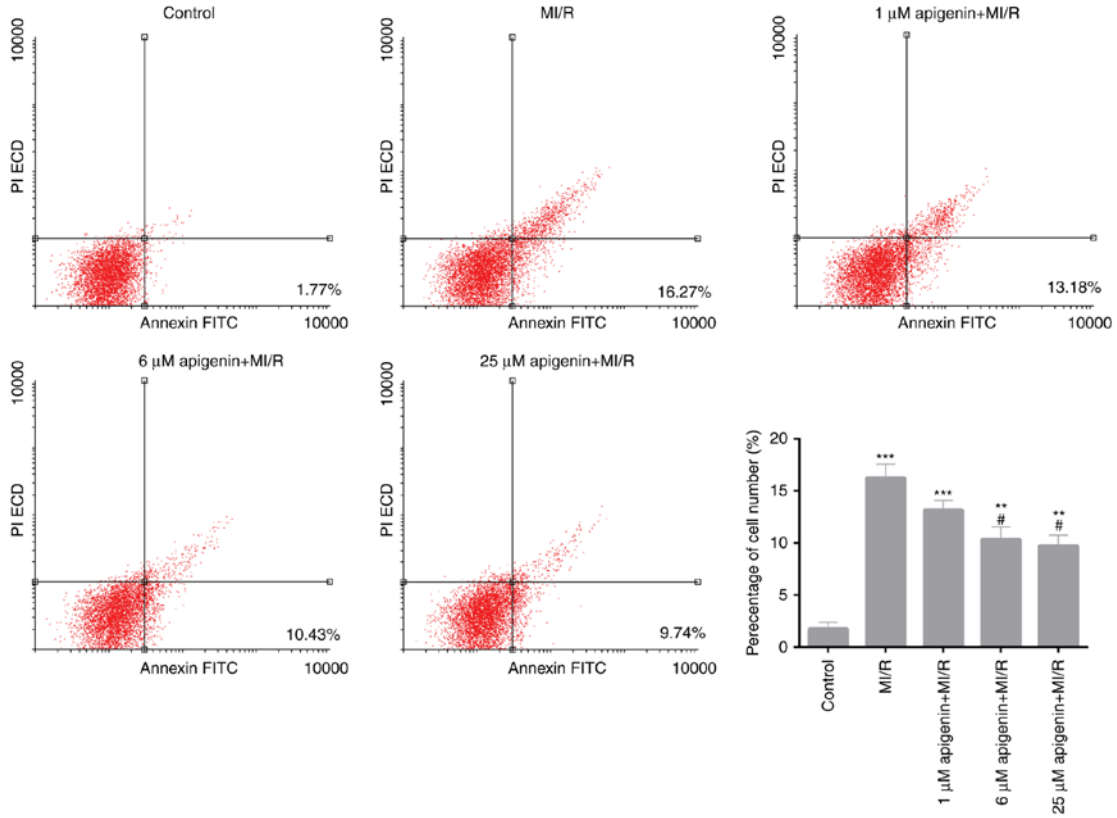

Figure 5. Apigenin suppresses the MI/R-mediated apoptosis of H9C2 cells. Flow cytometry was performed on the cell apoptosis of H9C2 cells treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 vs. MI/R. MI/R, myocardial ischemia-reperfusion; PI, propidium iodide; FITC, fluorescein isothiocyanate.

PI3K in H9C2 cells between each treatment group (Fig. 7A; P>0.05). Additionally, the level of p-Akt in H9C2 cells was significantly downregulated by MI/R injury (Fig. 5B; P<0.001). The decreased level of p-Akt in MI/R-induced cardiomyocytes was significantly recovered upon treatment with apigenin (Fig. 5B; P<0.001). Additionally, there was no significant difference in the expression level of Akt in H9C2 cells among the treatment groups (Fig. 7B; P>0.05).

LY294002 reverses the anti-apoptotic effect of apigenin. To further confirm the role of PI3K/Akt in the present study model, LY294002 was used to block the activity of PI3K. It was revealed that the apoptosis rate was significantly increased in the MI/R + apigenin + LY294002 group compared with the MI/R + apigenin group (Fig. 8A; P<0.05). Furthermore, the expression of Bcl-2 and p-mTOR decreased upon treatment with LY294002 compared with the apigenin + MI/R group (Fig. 8B; P<0.01).

Discussion

Myocardial ischemia is common in patients with CHD (28,29). Myocardial ischemia and hypoxia lead to myocardial apoptosis and necrosis, resulting in heart damage. Early restoration of blood flow and reperfusion is important for the primary treatment of CHDs (30). Secondary impairment caused by reperfusion, including arrhythmia, myocardial stunning and myocardial energy metabolism disorders, has become a major clinical problem. There are numerous potential factors contributing to CHD, including increased levels of oxygen free radicals, neutrophil infiltration, calcium overload, complement involvement, myocardial apoptosis, gene expression changes and the impact of enzymes (31). However, to recover the dying myocardium and reduce the myocardial infarction area, MI/R is necessary (32). Therefore, the prevention and therapy for MI/R injury and search for effective therapeutic agents are of particular significance.

Apigenin was previously hypothesized to be involved in the protection of anoxia/reoxygenation-induced myocardium injury in a previous study (33). Therefore, apigenin was selected as the focus for the present study, and its role and mechanism in MI/R injury of cardiomyocytes was examined. In the present study, the cytotoxicity of apigenin was evaluated. A CCK-8 assay was performed to determine the cell viability of H9C2 cells. The present results demonstrated that apigenin had limited cytotoxicity to H9C2 cells. Previous studies suggested that apigenin exhibits the function of suppressing oxidative stress in early brain injury and Parkinson's disease (34,35). LDH, MDA and CAT are considered to represent oxidative stress markers, and are involved in the regulation of the intracellular redox state (36). Therefore, the expression levels of LDH, MDA and CAT in H9C2 cells following I/R injury and apigenin treatment at different concentrations was assessed. Apigenin reduced the LDH and MDA contents in MI/R-induced H9C2 cells. Furthermore, apigenin markedly enhanced the activity of CAT in MI/R-induced H9C2 cells. ATPase is a type of protease that exists in tissue cells and organelle membranes, which serves an important role

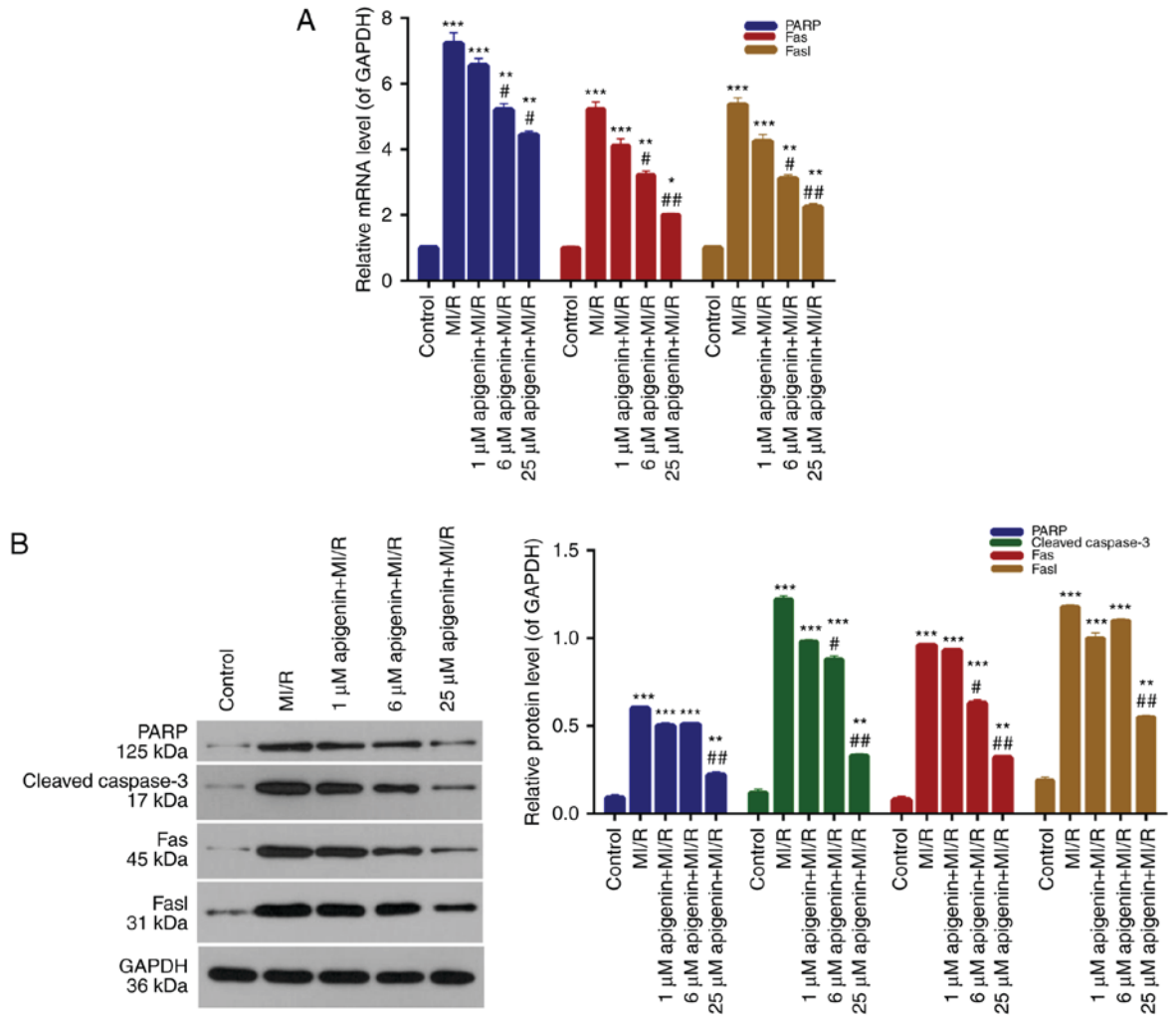

Figure 6. Apigenin regulates the expression levels of apoptosis-associated proteins. (A) Reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were conducted to evaluate the expression levels of PARP, caspase-3, Fas, and Fasl in H9C2 cells treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. MI/R. MI/R, myocardial ischemia-reperfusion; PARP, poly ADP-ribose polymerase; Fas, tumor necrosis factor receptor superfamily member 6; Fasl, tumor necrosis factor ligand superfamily member 6.

in material transport, energy conversion and information transfer (37). In particular, Na^+K^+-ATPase and Ca^{2+}-ATPase serve crucial roles in the modulation of oxidative stress (38,39). Therefore, the activities of Na^+K^+-ATPase and Ca^{2+}-ATPase in H9C2 cells treated with MI/R injury and apigenin at different concentrations were examined. It was observed that apigenin may enhance the activities of Na^+K^+-ATPase and Ca^{2+}-ATPase in MI/R-induced H9C2 cells. Therefore, apigenin may be involved in reducing oxidative stress in MI/R injury.

Previous studies have suggested that ROS content in cells is associated with typical characteristics of oxidative stress (40,41). In the current study, MI/R resulted in ROS overproduction in H9C2 cells, whereas, apigenin markedly reduced the ROS production in MI/R-induced H9C2 cells. These results suggested that apigenin alleviated the oxidative stress in H9C2 cells that was induced by MI/R injury, potentially by regulating the levels of LDH, MDA, CAT, ATPases and ROS. Therefore, it was demonstrated that apigenin may reduce the oxidative stress in H9C2 cells induced by MI/R injury.

Mitochondrial dysfunction has been reported to participate in the induction of apoptosis and has even been suggested to be important in the apoptotic pathway (42,43). Indeed, opening of the mitochondrial permeability transition pore was hypothesized to induce depolarization of the transmembrane potential, release of apoptogenic factors and loss of oxidative phosphorylation (44). Furthermore, a previous study demonstrated that a close association exists between the mitochondria and oxidative stress (45). As apigenin may reduce the oxidative stress in H9C2 cells induced by MI/R injury, it was investigated whether apigenin affected the MMP of H9C2 cells subjected to MI/R injury. According to the present results, the MMP level in H9C2 cells was decreased upon MI/R injury, and apigenin markedly increased the MMP level in MI/R-induced H9C2 cells. Additionally, it has been reported that apigenin attenuated the apoptosis of adriamycin-induced cardiomyocytes (46). However, the impact of apigenin on myocardial apoptosis induced by MI/R injury remains unclear.

As apigenin reduced the oxidative stress and enhanced the MMP level in MI/R-induced H9C2 cells, it was hypothesized that apigenin may suppress myocardial apoptosis induced by MI/R injury. The apoptotic capacity of H9C2 cells treated with MI/R and apigenin at different concentrations was studied. The FCM data indicated that apigenin markedly decreased the

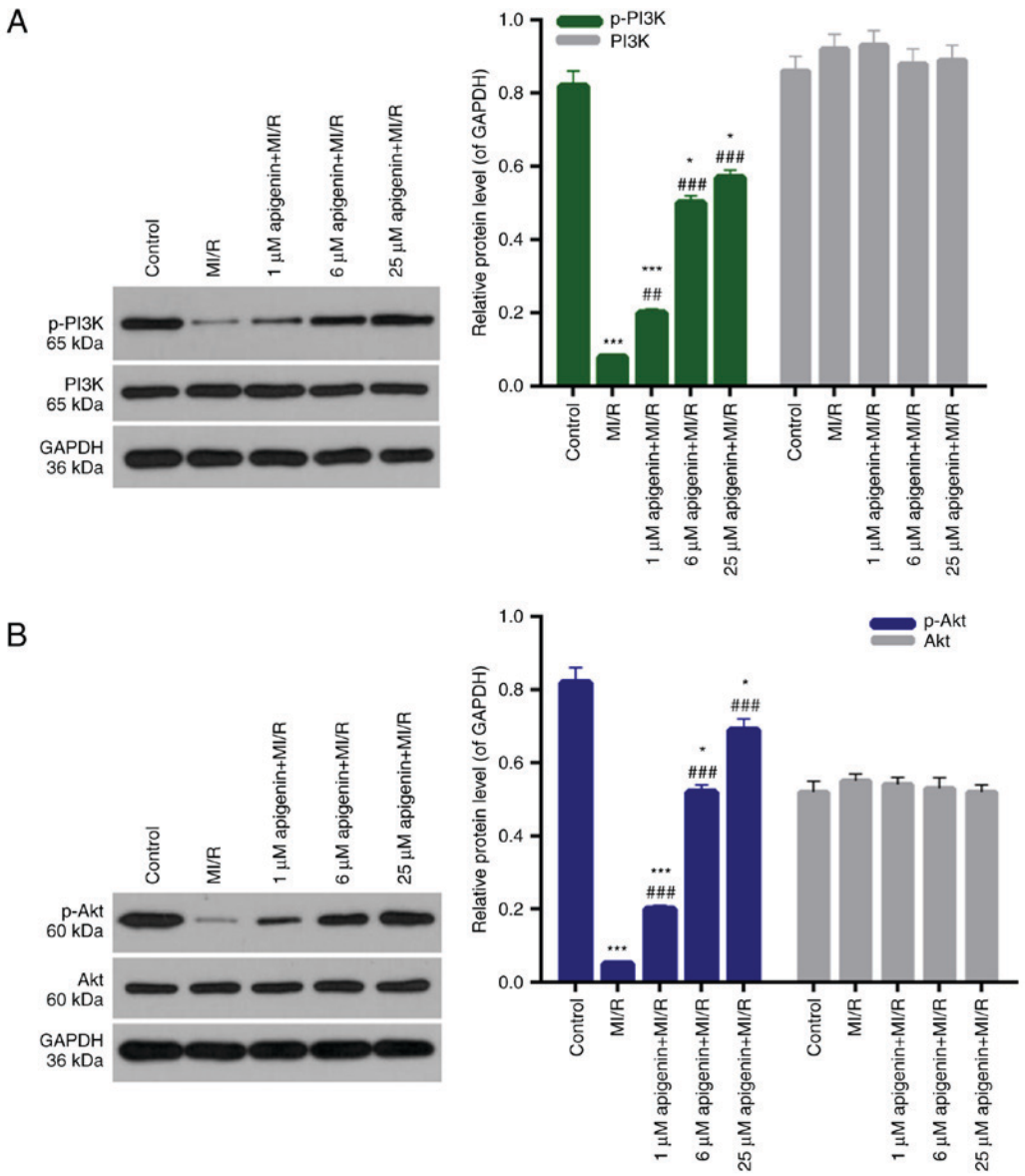

Figure 7. Apigenin affects the PI3K/Akt pathway. H9C2 cells were treated with MI/R, and 1, 6 and 25 μM apigenin + MI/R. Western blot analysis was conducted to measure the expression levels of (A) p-PI3K and PI3K and (B) p-Akt and Akt in H9C2 cells. *P<0.05 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MI/R. PI3K, phosphatidylinositol 3'-kinase; Akt, RAC-α serine/threonine-protein kinase; MI/R, myocardial ischemia-reperfusion; p-, phospho-.

apoptotic capacity of MI/R-induced H9C2 cells. Furthermore, the apoptosis-associated mechanisms in MI/R-induced H9C2 cells that affected by apigenin were investigated. The present results demonstrated that apigenin significantly downregulated the expression levels of PARP, cleaved caspase-3, Fas, and Fasl in MI/R-induced H9C2 cells. Therefore, apigenin suppressed the MI/R-induced apoptosis of H9C2 cells and modulated the expression levels of oxidative stress markers, MMP and apoptosis-associated proteins.

Previous studies suggested that the PI3K/Akt pathway is involved in the apoptotic process of cardiomyocytes (47-49). However, the role and mechanism of apigenin in the regulation of the PI3K/Akt pathway in MI/R-induced H9C2 cells remains unclear. The possible mechanism of the PI3K/Akt pathway in the suppression of MI/R-induced H9C2 cell apoptosis was investigated. According to the western blot analysis, it was observed that apigenin enhanced the phosphorylation of PI3K and Akt in MI/R-induced H9C2 cells. These results suggested that apigenin affects the phosphorylation of PI3K and Akt in MI/R-induced H9C2 cells. Furthermore, the inhibition of PI3K mediated by LY294002 reversed the anti-apoptotic effect of apigenin by suppressing cellular apoptosis, indicated by the decreased expression level of Bcl-2, which is an anti-apoptotic protein. As a downstream target of PI3K/Akt, mTOR may be phosphorylated. The inhibition of PI3K/Akt/mTOR is associated with apoptosis (50). Treatment with LY294002 additionally decreased the phosphorylation of mTOR in the current study. It was confirmed that apigenin inhibited the apoptosis of MI/R-induced H9C2 cells by regulating the phosphorylation levels of PI3K and Akt. The present study demonstrated that apigenin suppressed the apoptosis of H9C2 cells subjected to MI/R injury by affecting the PI3K/Akt pathway; however, the exact molecular mechanisms underlying this apoptotic effect remain unclear.

In conclusion, the present study demonstrated that apigenin suppressed the apoptosis of H9C2 cells subjected to

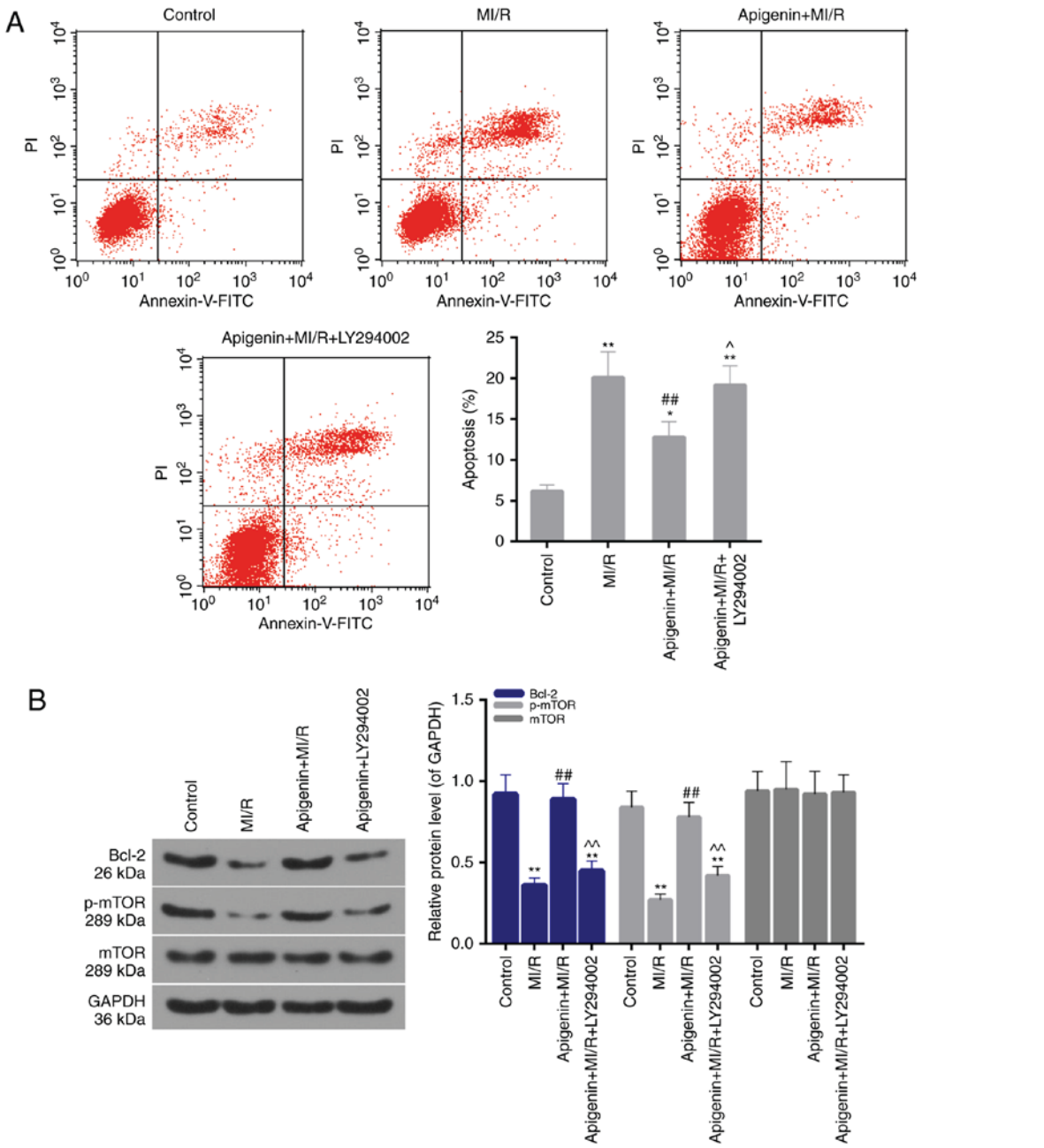

Figure 8. LY294002 reverses the effect of apigenin (6 μM). Effect of LY294002 on (A) apoptosis and (B) expression of Bcl-2, p-mTOR, and mTOR. *P<0.05 and **P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. MI/R, $^{\wedge\wedge}$P<0.01 and $^{\wedge}$P<0.05 vs. MI/R + apigenin. MI/R, myocardial ischemia reperfusion; Bcl-2, apoptosis regulator Bcl-2; mTOR, serine/threonine-protein kinase mTOR; PI, propidium iodide; FITC, fluorescein isothiocyanate.

MI/R injury by affecting the PI3K/Akt pathway. The results provided a novel insight for understanding the pathogenesis of MI/R and the mechanisms of apigenin in cardiomyocytes. The potential effects of apigenin on the suppression of myocardial apoptosis suggest that apigenin may be an effective MI/R therapeutic.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated and/or analyzed during this study are included in this published article.

Authors' contributions

JZ designed the study. YZ and LL performed the experiments. ZZ wrote the manuscript and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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