YiQiFuMai Powder Injection Attenuates Ischemia/Reperfusion-Induced Myocardial Apoptosis Through AMPK Activation

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Abstract

The YiQiFuMai powder injection (YQFM), a traditional Chinese medicine (TCM) prescription re-developed based on the well-known TCM formula Sheng-maisan, showed a wide range of pharmacological activities in cardiovascular diseases in clinics. However, its role in protection against myocardial ischemia/reperfusion (MI/R) injury has not been elucidated. The present study not only evaluated the cardioprotective effect of YQFM from MI/R injury but also investigated the potential molecular mechanisms both in vivo and in vitro. The myocardium infarct size, production of lactate dehydrogenase (LDH), creatine kinase (CK), cardiac function, TUNEL staining, and caspase-3 activity were measured. Cell viability was determined, and cell apoptosis was measured by Hoechst 33342 staining and flow cytometry. Mitochondrial membrane potential (ΔΨm) was measured, and ATP content was quantified by bioluminescent assay. Expression of apoptosis-related proteins, including Caspase-3, Bcl-2, Bax, AMPKα, and phospho-AMPKα, was analyzed by western blotting. AMPKα siRNA transfection was also applied to the mechanism elucidation. YQFM at a concentration of 1.06 g/kg significantly reduced myocardium infarct size and the production of LDH, CK in serum, improved the cardiac function, and also produced a significant decrease of apoptotic index. Further, combined treatment with compound C partly attenuated the anti-apoptotic effect of YQFM. In addition, pretreatment with YQFM ranging from 25 to 400 μg/mL markedly improved cell viability and decreased LDH release. Moreover, YQFM inhibited H9c2 apoptosis, blocked the expression of caspase-3, and modulated Bcl-2 and Bax proteins, leading to an increased mitochondrial membrane potential and cellular ATP content. Mechanistically, YQFM activated AMP-activated protein kinase (AMPK) signaling pathways whereas pretreatment with AMPK inhibitor Compound C and application of transfection with AMPKα siRNA attenuated the anti-apoptotic effect of YQFM. Our results indicated that YQFM could provide significant cardioprotection against MI/R injury, and potential mechanisms might suppress cardiomyocytes apoptosis, at least in part, through activating the AMPK signaling pathways.

Introduction

Ischemia and reperfusion occur in a wide range of situations, including trauma, thrombolysis treatment, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, organ transplantation, and hypovolemic shock with resuscitation.1 The effects of reperfusion for ischemia heart diseases are favorable in most cases, but reperfusion can also cause harmful effects to cardiomyocytes. Myocardial ischemia/reperfusion (MI/R), one of the leading causes of human death worldwide, is due to blood restoration after a critical period of coronary artery obstructions, which is closely associated with cardiac dysfunctions, in particular myocardial infarction, left ventricular remodeling, and heart failure.2,3 It is widely accepted that MI/R injury is a pathological process that induces extensive cardiomyocytes death.4,5 Apoptosis, programmed cell death, is one of the main causes of MI/R injury and therapeutic strategies that aim at preventing or delaying cardiomyocytes apoptosis may be an advisable choice for the treatment of related heart disease, especially on MI/R injury.6,7 AMP-activated protein kinase (AMPK) is a multifunctional cytosolic protein that plays important roles in cell survival and apoptosis. AMPK is a serine-threonine kinase that plays an essential role in the regulation of energy balance and myocardial signaling in the heart,8 and it meanwhile exerts significant physiological functions such as regulation of inflammation, mediation of reactive oxygen species...
(ROS), and protection from apoptosis.\(^9\) Previous studies have revealed that AMPK is cardioprotective during MI/R by enhancing glucose uptake and glucose transporter translocation, decreasing apoptosis, and limiting myocardial infarction.\(^{10-12}\) Activation of the AMPK pathway is essential to promote cardiomyocytes survival in the damaged heart. Thus, these studies emphasize the potential utility of targeting AMPK pathway in the treatment of MI/R.

Nowadays, many traditional Chinese medicines (TCMs) have been claimed to be effective for the control of problems due to reperfusion and associated pathologies.\(^{13}\) It has been reported that diverse pathological factors, such as oxidative stress, inflammation, calcium overload, and apoptosis, are all involved in the pathological mechanisms underlying MI/R injury.\(^{14-16}\) TCMs offer many advantages in these respects because of multiple bioactive ingredients aimed at multiple targets, exerting a systemic effect.

The YiQiFuMai powder injection (YQFM) has been re-developed based on the well-known TCMs formula Shengmaisan, which is composed of Ginseng Radix Et Rhizoma Rubra, Ophiopogonis Radix, and Schisandrae Chinensis Fructus. Considerable clinical trials have indicated that YQFM can be widely used for the treatment of chronic heart failure with better efficacy and fewer side effects compared with standard medical treatments.\(^{17,18}\) It has also been suggested that the three herbs of YQFM have beneficial effects in the treatment of cardiovascular diseases, regulation of blood vessel function, immunity modulation, and anti-lipid peroxidation.\(^{19-21}\) Both clinical and experimental studies demonstrate the prophylactic and/or therapeutic efficacy of YQFM; the mechanisms underlying the cardioprotective effects are mainly focused on enhancing myocardial systole, scavenging hydroxyl radicals, and suppressing inflammation.\(^{18,22,23}\)

In our previous study, a network pharmacology approach was employed to identify the YQFM’s potential pathways and targets against cardio-cerebral ischemia.\(^{24}\) Twelve main functional annotation clusters and main signaling pathways were established by Biocarta analysis, including the NF-κB signaling pathway, the MAPKinase signaling pathway, anti-apoptotic signaling pathways, and so on. No report is available for the protective effect and underlying molecular mechanisms of YQFM on MI/R-induced cardiomyocytes apoptosis.

In the current study, therefore, YQFM was tested \textit{in vivo} in an MI/R-injured mouse model, and \textit{in vitro} in an H9c2 cardiomyocyte cell line subjected to simulated ischemia/reperfusion (SI/R), investigating the therapeutic effect of YQFM and furthermore exploring the possible underlying mechanisms of YQFM against cardiomyocytes apoptosis, especially focusing on whether AMPK signaling is critical for the myocardial protection of YQFM.

Materials and Methods

Materials

YQFM was provided by Tasly Pharmaceutical Co., Ltd. with the batch number of 20121210. The quality of YQFM was confirmed by HPLC-DAD-ELSD analysis (Supplementary Figs. S1–S3 and Supplementary Table S1). Dulbecco’s modified Eagle medium (DMEM) was obtained from GIBCO/BRL (Life Technologies). Fetal bovine serum (FBS) was from ScienCell; 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) was from AM-RESCO. Compound C was purchased from Sigma-Aldrich.

The kits for determination of lactate dehydrogenase (LDH), creatine kinase (CK), caspase-3 activity, and fluorescence kit for Hoechst 33342, probe JC-1 were obtained from Beyotime Institute of Biotechnology. Fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit was from Becton Dickinson Co. RIPA lysis buffer, protease inhibitor, and enhanced chemiluminescence (ECL) reagent were from Va-zyme Biotech. AMPK\(_{z}\) siRNA kit was from Santa Cruz Biotechnology. Antibody against β-actin was from Bio-world Technology; antibodies against caspase-3, Bcl-2, Bax, AMPK\(_{z}\), and phospho-AMPK\(_{z}\) (Thr-172) were obtained from Cell Signaling Technology.

Animals and MI/R injury model

ICR mice (22–25 g) were purchased from Model Animal Research Center of Yangzhou University (Yangzhou, Jiangsu, China). The animals were housed in a standard vivarium with free access to food and water. All procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

The MI/R model was produced as previously described.\(^{25}\) Briefly, mice were anesthetized intraperitoneally with chloral hydrate (400 mg/kg). Myocardial ischemia was produced by exteriorizing the heart with a left thoracic incision followed by making a slipknot (6-0 silk) around the left anterior descending coronary artery. After 30 minutes of ischemia, the slipknot was released and followed by 24 hours of reperfusion. Mice were divided randomly into four groups: the sham group, the MI/R group, the YQFM group (1.06 g/kg), and the verapamil group (1.85 mg/kg). The sham group was given the same surgical procedures without ligation the left anterior descending coronary artery. Both the sham and the MI/R mice received the same volume of saline alone. All drugs were administered via intraperitoneal injection with beginning of reperfusion.

Measurement of myocardial infarct size

Twenty-four hours after reperfusion, the heart was rapidly excised and frozen at –70°C; then, the ventricular tissue was cut into five slices perpendicular to the long axis of the heart. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C. Red parts in the heart stained by TTC indicated ischemic but viable tissue. However, staining negative areas represented infarcted myocardium. Areas of infarct size were measured by computerized planimetry. The size of infarction area was expressed as a percentage of the total left ventricular area.

Measurement of LDH, CK release in serum, and histopathologic examination

Myocardial damage was evaluated by measuring plasma concentrations of LDH and CK. At the end of the experiment, blood was collected and serum was separated by centrifugation; LDH and CK were measured by following the manufacturer’s instructions, respectively. At the end of the experiment, the heart tissue was removed, fixed by 10% formalin, embedded with paraffin, sliced into pieces of 5 μm thickness, eosin-stained, and mounted. The histopathological changes were detected by an optical microscope.
Echocardiography

In vivo cardiac function was assessed noninvasively by using a high-frequency, high-resolution echocardiographic system consisting of a Vevo 2100 Imaging System (Visual Sonics) that was equipped with a 30 MHz transducer. For echocardiographic assessment, each mouse was anesthetized in an induction chamber with inhalant isoflurane at 2.5% in 100% oxygen. When fully anesthetized, the mouse was transferred to dorsal recumbency and placed on a heated imaging platform. LV ejection fraction (EF) and fractional shortening (FS) were measured.

TUNEL staining for apoptosis in vivo

At 24 hours after MI/R, the ischemic reperfused myocardium was harvested and fixed in 4% paraformaldehyde solution. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed using the Fluorescein In Situ Cell Death Detection Kit (Roche Diagnostics) by following the manufacturer’s instructions. Cardiomyocytes, apoptotic nuclei, and total cardiomyocyte nuclei were labeled with anti-F-actin antibody, green fluorescein staining, and DAPI, respectively. For each slice, five fields were randomly obtained under a confocal scanning microscope (LSM700; Zeiss). The extent of cell apoptosis was expressed as the ratio of TUNEL positive nuclei over DAPI-stained nuclei.

Caspase-3 activity assay in vivo

Caspase-3 activity was determined using a colorimetric activity assay kit according to the manufacturer’s protocol. Heart tissue samples for determination of myocardial caspase-3 activity were obtained from the margin of infarct areas, lysed in ice-cold lysis buffer for 30 minutes, and finally centrifuged at 4°C for 10 minutes at 12,000 rpm. The supernatant was incubated with the caspase-3 substrate (Ac-DEVD-pNA) after determining the protein concentration. The caspase-3 activity was determined using a Microplate Reader at 405 nm.

Cell culture

Rat H9c2 cardiomyocyte cell line was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The H9c2 cells were maintained in DMEM that was supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The culture medium was replaced every 2 days, and cells were subcultured or subjected to experimental procedures at 80%–90% confluence.

SI/R injury model in vitro

To mimic the ischemic injury in vitro, the oxygen and glucose deprived (OGD) technique was based on a previously described protocol. In our study, the OGD injury was produced by incubating with none-glucose DMEM and exposed to a hypoxic environment of 94% N2, 5% CO2, and 1% O2 for 6 hours at 37°C in a humidified N2/CO2 incubator and then in a standard incubator with 5% CO2 in normal atmosphere at 37°C for 6 hours. Cells were treated with YQFM (25–400 µg/mL) or N-acetyl cysteine (NAC, 500 µmol/L) during the hypoxia. At the onset of simulated reperfusion, cardiomyocytes were re-exposed to YQFM and NAC.

Cell viability and LDH assays

Cell viability was determined using the MTT assay. Cells were seeded at a density of 8000 cells/well in 96-well plates. After different treatments, cells were incubated with MTT at a final concentration of 0.5 mg/mL for 3 hours at 37°C. Then, the medium was discarded and 150 µL of DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm with a reference wavelength of 650 nm using a microplate reader, and cell viability was expressed as percentage of absorbance to control values. To further measure the extent of cell injury, release of LDH was also tested. At the end of the incubation period, the culture supernatants were collected. The activity of LDH was detected at 490 nm according to the manufacturer’s instructions.

Hoechst 33342 staining assay

Briefly, H9c2 cardiomyocytes were washed with ice-cold phosphate-buffered saline (PBS), then exposed to Hoechst 33342 (5 µg/mL), and finally incubated for 30 minutes at room temperature. The H9c2 cardiomyocytes were then washed three times in PBS and examined under a fluorescence microscope (Zeiss). The nuclei of apoptotic cells appeared irregularly shaped and hyper-condensed (brightly stained).

Flow-cytometric analysis for apoptosis

FITC-conjugated Annexin V and propidium iodide (PI) were applied to identify apoptotic cells. Double staining with FITC-Annexin V and PI was performed by following the manufacturer’s instructions. Briefly, H9c2 cardiomyocytes were harvested, washed with PBS, resuspended with binding buffer, and incubated with Annexin-V and PI in a final concentration of 100 ng/mL at room temperature in the dark for 15 minutes. Cellular fluorescence was then analyzed with a flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software.

Measurement of cellular ATP content

Intracellular ATP levels were measured using the ATP bioluminescent assay kit according to the manufacturer’s instructions.
protocol. After experiments, the cells were washed with PBS and lysed, and the supernatants were collected. An aliquot (100 μL) of an ATP detection working solution was added to each well of a black 96-well culture plate, and then the plate was incubated for 3 minutes at room temperature. One hundred microliter samples of the cell lysate were added to the wells, and the luminescence was immediately evaluated. ATP content was determined by comparison to a concurrent standard curve, then normalized by protein concentration, and finally expressed as nmol/mg protein.

siRNA transfection

H9c2 cells were transfected with siRNA directed against AMPKα subunits or scrambled siRNA as a control.Briefly, cells were transfected with 80 nmol/L of total siRNA by using Transfection Reagent according to the manufacturer’s instructions. To assess the AMPKα siRNA efficiency, total cell lysates were subjected to SDS-PAGE for immunoblotting analysis with β-actin as a reference.

Western blot analysis

As reported,27 after washing with the ice-cold PBS, the cells were lysed with RIPA lysis buffer that was supplemented with protease inhibitor. The whole cell lysates were centrifuged at 12,000 rpm for 10 minutes at 4°C, and protein concentrations were analyzed by the BCA method (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of proteins (35 μg) were loaded onto a 12.5% SDS-PAGE and transferred to PVDF membranes (Millipore Corporation) by electroblotting. The membranes were blocked with 3% BSA in TBS/T and stained with primary antibodies against caspase-3, Bax, Bcl-2, AMPK, p-AMPK, and β-actin (dilution 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:4000, respectively; Cell Signaling Technology) overnight at 4°C. Membranes were then probed with peroxidase-conjugated secondary antibody at a 1: 8000 dilution (Bioworld Technology). The antigen–antibody complexes were then detected with ECL reagent, visualized by ChemiDoc™ MP System (Bio-Rad), and analyzed using Image Lab™ Software (version 4.1; Bio-Rad).

Statistical analysis

All experiments were performed in triplicate, and data were expressed as the mean±SEM. Statistical analysis was carried out using Student’s two-tailed t-test for a comparison between two groups and one-way analysis of variance, followed by Dunnett’s test when the data involved three or more groups. p <0.05 was defined as significant.

Results

YQFM decreased myocardial injury and improved the cardiac function in MI/R mice

To examine how YQFM influenced MI/R injury, infarct size and the release of LDH and CK in serum were measured. ST-segment elevation on an electrocardiogram monitor represented success in MI/R model performance. As shown in Figure 1, 30 minutes of ischemia and 24 hours of reperfusion resulted in myocardial injury, as evidenced by increased infarct size and LDH, CK activities. However, YQFM and verapamil treatment groups markedly suppressed the increase of infarct size (Fig. 1A, B); decreased the LDH and CK release (Fig. 1C, D). Simultaneously, the histopathological examination from MI/R mice heart tissues showed widespread myocardial structural disarray, increased necrosis and fusion area, and a large number of inflammatory cells infiltrating the myocardial tissue. After treatment with YQFM and verapamil, the histological features became typical of normal cardiac structure or mild architectural damage with marked amelioration (Fig. 1E). Further, MI/R injury induced decreases in EF and FS. After administered with YQFM and verapamil, the deficient systolic and diastolic LV function were alleviated (Fig. 2).

YQFM inhibited myocardial apoptosis in MI/R mice

Anti-apoptotic effects of YQFM were detected by TUNEL assay. As shown in Figure 3A and B, the number of TUNEL-positive (apoptotic) cells was significantly increased in the MI/R group compared with that of the sham group, whereas the apoptosis index was markedly decreased by YQFM treatment compared with that in the MI/R group. As illustrated in Figure 3C, MI/R resulted in a noticeable increase in caspase-3 activity, whereas YQFM suppressed caspase-3 activity significantly compared with that of the MI/R group, indicating lower pro-apoptotic enzyme activity in this group.

YQFM inhibited myocardial apoptosis correlating to AMPK activation in MI/R mice

After confirming the anti-apoptotic role of YQFM in myocardium under MI/R conditions, we tried to explore the underlying mechanism. AMPK has been reported to protect the heart against ischemic injury11; therefore, we investigated whether AMPK was involved in the treatment of YQFM on MI/R-induced myocardial apoptosis in vivo. As shown in Figure 4A, YQFM treatment increased the phosphorylation of AMPKα at Thr172 under MI/R conditions, whereas AMPKα activation was inhibited by compound C. Moreover, YQFM suppressed the expression of active caspase-3, but this effect was abolished by compound C (Fig. 4B, C). These results demonstrated that YQFM reduced myocardial apoptosis after MI/R, probably, at least in part, through AMPKα activation.

YQFM attenuated SI/R-induced injury and apoptosis in H9c2 cardiomyocytes

We first examined the viability of H9c2 cardiomyocytes after treatment with YQFM, and we found that YQFM at concentrations of 25–1000 μg/mL did not significantly affect cell viability (Supplementary Fig. S4A). On the basis of the results of the previous step, three concentrations (25, 100, and 400 μg/mL) were selected for further investigation. Exposure of H9c2 cardiomyocytes to SI/R led to a decrease in cell viability, whereas treatment with 25–400 μg/mL YQFM maintained cell viability at roughly 78.3%, 87.6%, and 94.3%, respectively (Supplementary Fig. S4B). As LDH release was an acknowledged marker for cell damage, cardiomyocyte injury was also assessed by determining
FIG. 2. YQFM improved the cardiac function in MI/R mice. (A) Cardiac function was assessed by using an echocardiographic system. (B) YQFM alleviated the deficient ejection fraction. (C) YQFM alleviated the deficient fractional shortening. Results were obtained from three independent experiments and were presented as mean ± SEM. ##p < 0.01 versus Sham group without MI/R, *p < 0.05, **p < 0.01 versus group treated with MI/R alone. Color images available online at www.liebertpub.com/rej

FIG. 1. YQFM decreased myocardial injury in MI/R mice. (A, B) 2,3,5-triphenyltetrazolium chloride (TTC) staining determined the effect of YQFM on myocardial infarct area. (C) YQFM suppressed the LDH release. (D) YQFM suppressed the CK release. (E) YQFM ameliorated the histopathological changes. Results were obtained from three independent experiments and were presented as mean ± SEM. ##p < 0.01 versus Sham group without MI/R, **p < 0.01 versus group treated with MI/R alone. CK, creatine kinase; MI/R, myocardial ischemia/reperfusion; LDH, lactate dehydrogenase; YQFM, YiQiFuMai powder injection. Color images available online at www.liebertpub.com/rej
the release of LDH in culture medium (Supplementary Fig. S4C). As compared with the control group, LDH release significantly increased to 29.3% after SI/R, whereas treatment with 25–400 μg/mL YQFM decreased to 22.5%, 20.3%, and 18.2% at the end of reperfusion, respectively. Nuclear morphological change was observed by Hoechst 33342 staining, which made it appear that the control group exhibited uniformly dispersed chromatin, normal organelles, and intact cell membranes. After exposure to SI/R injury, the nuclei appeared irregularly shaped and hyper-condensed (brightly stained). However, with YQFM treatment, the morphological changes were significantly attenuated, and the number of cells with nuclear condensation and fragmentation was markedly decreased (Fig. 5A). Quantitative analysis using flow cytometry confirmed that most H9c2 cardiomyocytes were viable in the control group, and after SI/R injury, the apoptosis index was markedly increased compared with that of the control group. However, the apoptosis index was significantly decreased by YQFM treatments (Fig. 5B, C).
YQFM restored SI/R-induced loss of mitochondrial membrane potential and abrogated the depletion of cellular ATP in H9c2 cardiomyocytes

As shown in Figure 6A, B, untreated cells exhibited bright-staining mitochondria that emitted red fluorescence. Cells subjected to SI/R caused the formation of monomeric JC-1, which was indicative of loss of membrane potential. YQFM treatment, however, blocked the SI/R-induced formation of JC-1 monomers, suggesting that YQFM could restore SI/R-induced loss of mitochondrial membrane potential. Moreover, SI/R significantly depleted cellular ATP content in H9c2 cells, whereas YQFM treatment largely abrogated the depletion of cellular ATP, which was apparently favorable to the cell survival (Fig. 6C).

YQFM regulated the expression of apoptosis-related proteins and inhibited the caspase-3 activity in SI/R-induced H9c2 cardiomyocyte injury

The balance of anti- and pro-apoptotic proteins in the Bcl-2 family plays an essential role in the control of cell survival against ischemia/reperfusion injuries.28 As presented in Figure 7A, the Bcl-2/Bax ratio was markedly increased in YQFM pretreated cells compared with the SI/R group. Caspase-3 was also an important downstream enzyme of the final step of apoptotic pathway, and it could cause DNA degradation and apoptosis when it was activated. As illustrated in Figure 7B and C, SI/R resulted in a noticeable increase in caspase-3 expression and activity compared with that of the control group, whereas treatment of YQFM significantly reduced the level of caspase-3. These results supported the anti-apoptosis effect elicited by YQFM, and they validated that YQFM up-regulated Bcl-2 expression and down-regulated Bax, active caspase-3 expression, and activity in H9c2 cardiomyocytes injured by SI/R.

YQFM inhibited SI/R-induced H9c2 cardiomyocyte apoptosis through AMPKα activation

As illustrated in Figure 8A, we found that YQFM could also promote the phosphorylation of AMPKα under SI/R conditions and show no AMPKα phosphorylation on H9c2 cardiomyocytes without SI/R treatment. There was no difference in total AMPKα protein abundance among all groups tested.

To further understand whether the beneficial effects of YQFM was AMPKα dependent, AMPKα activation was inhibited using compound C, an AMPK inhibitor. As shown in Figure 8B, the expression of p-AMPKα was inhibited by compound C. Meanwhile, co-incubation with compound C.
FIG. 5. YQFM attenuated SI/R-induced H9c2 cell apoptosis. H9c2 cells were pretreated with YQFM at the concentration of 25–400 μg/mL for 6 hours and then exposed to hypoxia of 6 hours followed by 6 hours of reoxygenation. (A) Cell apoptosis was detected by Hoechst 33342 staining. (B, C) SI/R-induced H9c2 apoptosis rate was quantified by flow cytometry. Results were obtained from three independent experiments and were presented as mean ± SEM. ##p < 0.01 versus control group without SI/R, **p < 0.01 versus group treated with SI/R alone. SI/R, simulated ischemia/reperfusion. Color images available online at www.liebertpub.com/rej
partly abrogated the anti-apoptotic effect of YQFM, evidenced by increased expression of caspase-3 and increased caspase-3 activity (Fig. 8C, D). We further tested whether AMPKα siRNA could compromise the ability of YQFM on apoptosis inhibition. The siRNA transfection was optimized; total cell lysates were subjected to SDS-PAGE for immunoblotting analysis with β-actin as a reference to assess the AMPKα siRNA efficiency; and AMPKα was successfully knocked down by an siRNA approach (inhibition efficacy larger than 60%; Supplementary Fig. S5). As shown in Supplementary Figure S6, AMPKα siRNA led to a significant increase in caspase-3 expression and activity, resulting in apoptosis in H9c2 cardiomyocytes, which indicated that AMPKα played an essential role in cardioprotection. In addition, when AMPKα siRNA was employed, YQFM significantly reduced the level of caspase-3 activity, whereas the inhibitory effect was partly attenuated (Fig. 8E). These results suggested that YQFM was likely to affect multiple targets in the complex cardiovascular diseases, and the protective mechanism was, at least in part, through the activation of the AMPK signaling pathway.

Discussion
There have been a number of studies illustrating the clinical usefulness of YQFM treatment in cardiovascular diseases,17,18 and evidence points to the possibility that YQFM may act on multiple targets and pathways leading to synergistic positive benefits. However, the underlying mechanisms and particular pathways have yet to be elucidated. The main findings of the present study were that YQFM protected the myocardium from MI/R injury and inhibited cardiomyocyte apoptosis via the AMPK-dependent signaling pathway. To our knowledge, this is the first evidence on the molecular mechanism of YQFM on protecting ischemia and reperfusion-induced apoptosis.

Accumulating evidence indicates that the loss of cardiomyocytes as a result of apoptosis is significant in various heart diseases and inevitably leads to heart failure.29 Myocardial apoptosis is a significant pathogenic event in MI/R injury. It is widely acknowledged that the intervention of the apoptosis process could prevent the loss of cardiomyocytes, minimize cardiac injury, and, finally, slow down or even prevent the occurrence and development of MI/R injury.30
Our present study showed that MI/R (30 min/24 h) in vivo and SI/R (6 h/6 h) in vitro significantly aggravated myocardial injury, decreased the cell viability, and increased cardiomyocyte apoptosis, which were demonstrated with decreased LV function, increased infarct size, LDH, CK releases in serum, condensed and fragmented chromatin in the nuclei, and enhanced the percentage of apoptotic cells. However, treatment with different concentrations of YQFM significantly restored the cardiac function, decreased infarct size, cell viability loss, and LDH, CK activities. In addition, the anti-apoptotic effect was also confirmed by the results of TUNEL staining, Hoechst 33342 staining, and Annexin-V/PI staining. These results strongly indicated that YQFM might exert a protective effect against MI/R injury and inhibit cardiomyocyte apoptosis in response to MI/R.

I/R injury also caused significant changes in cell signaling, including cell apoptosis rate, disturbance of mitochondria membrane potential, caspase-3 activation, and imbalance of anti- and pro-apoptotic proteins in Bcl-2 family proteins, indicating an activation of the mitochondria apoptosis pathway. The present observation is consistent with previous studies on apoptosis signaling pathways in cardiomyocytes under I/R conditions. Mitochondria not only provide energy for cardiomyocytes but also play a vital role in the cardiomyocyte apoptotic signaling pathway. Mitochondria dysfunction is a predominant cause of I/R-induced cardiomyocyte apoptosis. The observed cell apoptosis and changes in signaling molecules are most likely to be associated with mitochondrial dysfunction, as evidenced by decreased mitochondrial potential as an indicator of MPTP opening, as previously reported. The disturbance may be due to the altered mitochondria transport of proton in the electron transport chain (ETC) and manifested as de- and hyper-polarization. YQFM treatments, however, restored mitochondrial membrane potential. It had been reported that depletion of ATP during hypoxia and reoxygenation could contribute to cell death via apoptosis, which was associated with mitochondrial dysfunction. In the presence of YQFM, ATP production was significantly increased. The increase of ATP level by YQFM might be related to its protection of mitochondrial ETC function, as ATP was mostly generated by oxidative phosphorylation, a process translocating, uptaking protons, and accompanying the synthesis of ATP in mitochondrial ETC. These data demonstrated that YQFM protected cardiomyocytes from I/R-induced apoptosis by maintaining mitochondria membrane integrity and function.

It is well known that Bcl-2 family proteins play an important role in maintaining mitochondrial function and mediating cardiac protections of various therapeutic agents. Modern pharmacological research demonstrates that overexpression of Bcl-2 has been related to protection of cell

FIG. 7. YQFM regulated the expression of apoptosis-related proteins and inhibited the caspase-3 activity. H9c2 cells were pretreated with YQFM at the concentration of 25–400 μg/mL for 6 hours and then exposed to hypoxia of 6 hours followed by 6 hours of reoxygenation. (A) Bcl-2 and Bax expression was detected by western blot. (B) Caspase-3 protein expression was detected by western blot. (C) YQFM decreased caspase-3 activity in H9c2 cardiomyocytes subjected to SI/R. Results were obtained from three independent experiments and were presented as mean ± SEM. *p < 0.05, **p < 0.01 versus control group without SI/R, *p < 0.05, **p < 0.01 versus group treated with SI/R alone.
apoptosis via inhibition the release of cytochrome c.\textsuperscript{38} whereas depletion of Bax protein has been appeared to re-
duce cell death in ischemia/reperfusion injury in mice.\textsuperscript{39} Thus, the ratio of Bcl-2 to Bax may be a critical factor in the cellular threshold for apoptosis. In the present study, we validated that YQFM markedly inhibited Bax expression but increased Bcl-2 expression under the SI/R condition, eventually resulting in an increased Bcl-2/Bax ratio and cell survival rate. In addition, caspase cascade also plays an essential role in apoptosis.\textsuperscript{40} To further support our findings, the expression of caspase-3 and caspase-3 activity was de-
termined. Our results indicated that YQFM resulted in active caspase-3 expression and downregulated activity. Based on these results, it could be reasonably speculated that YQFM might attenuate apoptosis.

On the basis of the obtained results that YQFM protected cardiomyocytes against apoptosis induced by I/R, further investigation was performed with focus on the possible mechanisms involved in the anti-apoptotic effects of YQFM. Many pathways have been demonstrated to participate in apoptosis regulation; AMPK is an essential positive regulator when specifically activated at the time of myo-
cardial reperfusion, and it provides an amenable pharmacological target for cardioprotection.\textsuperscript{41} AMPK consists of an \( \alpha \) catalytic subunit, and regulatory \( \beta \) and \( \gamma \) subunits. The activation of AMPK occurs due to phosphorylation of the \( \alpha \) subunit at threonine-172, and phosphorylation at this site is essential for AMPK’s kinase activity.\textsuperscript{42} Recent studies showed that AMPK deficiency aggravated both myocardial necrosis and apoptosis after I/R in transgenic mice expressing a dominant negative kinase dead form of the \( \alpha \) subunit.\textsuperscript{10} Moreover, AMPK is ubiquitously expressed and functions as an intracellular energy sensor by facilitating ATP production and suppressing unnecessary ATP use in energy-
stressed cells. It has been known that AMPK is associated with energy homeostasis, mitochondrial function, and cell survival.\textsuperscript{43} Therefore, we speculate that the anti-apoptotic action of YQFM may be responsible for activation of AMPK. Our data demonstrated that AMPK was activated by YQFM in response to MI/R. When blocked with compound C, an AMPK inhibitor, the expression of p-AMPK was decreased and cardiomyocyte apoptosis was increased in the YQFM treatment group. And AMPKz siRNA knockdown signifi-
cantly increased H9c2 cardiomyocyte apoptosis. Our results

FIG. 8. YQFM inhibited SI/R-induced cardiomyocyte apoptosis through AMPKz activation, and AMPKz siRNA attenu-
tuated the anti-apoptotic effect of YQFM. H9c2 cells were pretreated with YQFM at the concentration of 25–400 \( \mu \)g/mL for 6 hours and then exposed to hypoxia of 6 hours followed by 6 hours of reoxygenation. (A) YQFM phosphorylated AMPKz in H9c2 cells. (B) Compound C inhibited the phosphorylation of AMPKz. (C) Compound C attenuated the anti-apoptotic effect of YQFM on caspase-3 expression. (D) Compound C attenuated the anti-apoptotic effect of YQFM on caspase-3 activity. (E) AMPKz siRNA attenuated the inhibition of caspase-3 activity of YQFM. Results were obtained from three independent experiments and were presented as mean ± SEM. \# \( p < 0.05 \), \## \( p < 0.01 \) versus control group without SI/R and AMPKz siRNA, \* \( p < 0.05 \), \** \( p < 0.01 \) versus group treated with SI/R alone or with AMPKz siRNA alone or with SI/R and AMPKz siRNA, \$ \( p < 0.05 \), \$$ \( p < 0.01 \) versus group treated with YQFM and SI/R, @@ \( p < 0.01 \) versus group treated with YQFM and SI/R.
proved the critical role of AMPKz in regulating cardiomyocyte apoptosis and suggested that AMPKz mediated, at least in part, the function of YQFM, which was consistent with previous studies, though in different settings. Russell et al. have reported that increased AMPK activity prevented post-ischemic cardiac dysfunction and apoptosis. Some studies have also shown that AMPK protected proximal tubular cells from stress-induced apoptosis and was beneficial in reducing hepatocyte apoptosis. As a major active component of YQFM, ginsenoside Rb1 has been reported to stimulate AMPK activation. Without doubt, TCM formulae offer the advantage in which several active ingredients in one prescription are aimed at numerous targets and work together to provide therapeutic benefits in treating complex diseases. Certainly, there must be some other essential targets apart from AMPKz in cardioprotection of YQFM, which needs our further investigation.

Increasing evidence demonstrates that there are multiple bioactive components that contribute to YQFM’s cardioprotection, especially the ginsenosides, such as ginsenoside Rg1, Rb1, Rg3, etc. All of them show the properties to protect the heart against cardiac injury. Some other studies have shown that ophiopogonins could increase myocardial nutritional blood flow and enhance myocardial contractility, and lignans protect the heart mainly by regulation of energy metabolism and oxidation–anti-oxidation level. Nowadays, the safety and efficacy of TCM injections have been a focus of attention for the medical community and studies on their bioactive ingredients and mechanisms of action are essential for determining quality control of TCM injections. Further studies are necessary to develop some safer and more efficient compositions based on these effective components. Previous studies have reported that the mechanisms underlying the cardioprotective effects of YQFM are focused on scavenging the hydroxyl radical, enhancing myocardial systole, and suppressing inflammation. It is possible that the anti-apoptotic actions of YQFM observed in the present study are associated with not only direct action on the intrinsic apoptotic pathway but also the anti-oxidant, anti-inflammatory actions of YQFM. Further investigation should be performed with focus on connections of different mechanisms due to multiple active components. Moreover, the limitation of our study is that the H9c2 cardiomyocyte cell line cannot accurately represent cardiac myoblasts although it was widely applied in research of heart diseases, and utilization of primary cultured cardiomyocytes that sustain the anti-apoptosis effect of YQFM is in progress.

Conclusions

In summary, we have demonstrated that YQFM protected the myocardium from MI/R injury and inhibited MI/R-induced cardiomyocyte apoptosis via increased phosphorylation of AMPKz both in vivo and in vitro. These results indicated that YQFM protected myocardial cells from apoptosis, at least in part, through activating the AMPK signaling pathway in I/R-induced cardiac injury. These findings might provide important insights for the understanding of the molecular mechanisms involved in the cardioprotective effect of YQFM.

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Author Disclosure Statement

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