Oxymatrine pretreatment protects H9c2 cardiomyocytes from hypoxia/reoxygenation injury by modulating the PI3K/Akt pathway

ZHONGBAI ZHANG[1], XUETING QIN[2], ZHENGHUI WANG[3], YANCHUN LI[4], FEI CHEN[5], RUNDU CHEN[6], CHUANG LI[6], WENCHENG ZHANG[7] and MEI ZHANG[6]

[1]The Fourth Detachment, Armed Police and Coastal Police Corps, Wenchang, Hainan 571300; [2]Department of Cardiology, The Third People's Hospital of Jingzhou, Jingzhou, Hubei 434000; [3]Department of Human Morphology Section, Logistics University of People's Armed Police Force, Tianjin 300162; [4]Department of Pharmacy, Heilongjiang Municipal Corps Hospital of Chinese People's Armed Police Force, Harbin, Heilongjiang 150076; [5]Department of Health Service, The Second Mobile Corps Hospital of Chinese People's Armed Police Force, Wuxi, Jiangsu 214000; [6]Department of Cardiac Thoracic Surgery, Characteristic Medical Center of People's Armed Police Force, Tianjin 300309; [7]Tianjin Key Laboratory of Hepatopancreatic Fibrosis and Molecular Diagnosis and Treatment, Characteristic Medical Center of People's Armed Police Force, Tianjin 300162, P.R. China

Received April 26, 2020; Accepted February 16, 2021

DOI: 10.3892/etm.2021.9988

Abstract. Ischemia-reperfusion (I/R) plays an important role in myocardial damage, which has been widely recognized as a key procedure in the cardiovascular disease. A hypoxia/reoxygenation (H/R) model was established using H9c2 cardiomyocytes to investigate the possible positive effect of oxymatrine (OMT), an alkaloid originating from the traditional Chinese herb *Sophora flavescens* Aiton, on cardiomyocytes exposed to H/R injury and the underlying molecular mechanisms. Cell viability was measured using the MTT assay, lactate dehydrogenase release measurements and hematoxylin and eosin staining. Oxidative stress was detected by measuring cellular malondialdehyde (MDA) content, as well as superoxide dismutase (SOD) and catalase (CAT) activities. Apoptosis was detected using TUNEL staining and flow cytometric analysis, and the underlying mechanism was investigated using reverse transcription-quantitative PCR and western blot analyses. The results revealed that OMT increased the viability of H9c2 cardiomyocytes exposed to H/R. The OMT pretreatment decreased the production of MDA by reactive oxygen species and increased the activities of SOD and CAT. Furthermore, the OMT pretreatment reduced the expression of Bax and caspase-3, while inducing Bcl-2 expression. In addition, the protective effect of OMT was shown to be associated with the PI3K/Akt signaling pathway, and the PI3K inhibitor LY294002 attenuated the effects of OMT on the H9c2 cardiomyocytes exposed to H/R. These findings indicate that OMT could be a potential therapeutic candidate for the treatment of myocardial ischemia/reperfusion injury.

Correspondence to: Dr Mei Zhang, Department of Cardiac Thoracic Surgery, Characteristic Medical Center of People's Armed Police Force, 222 Chenglin Road, Hedong, Tianjin 300309, P.R. China
E-mail: chyouyou@126.com

Key words: oxymatrine, pretreatment, cardiomyocytes, hypoxia/reoxygenation, PI3K/Akt

Introduction

Ischemic heart disease (IHD) has become a major public human issue, with a decreasing age of onset. Coronary heart disease (CHD) is a leading cause of death all over the world according to the World Health Organization (1). With the advent of cardiac surgery, including percutaneous coronary intervention (PCI) and coronary artery bypass graft (CABG), some clinical symptoms have been alleviated, but ischemia/reperfusion (I/R) injury induces arrhythmia, heart failure and cardiomyocyte death (2). Therefore, I/R injury is an important concern of doctors following cardiac surgery. According to previous studies, I/R injury is associated with calcium overload (3), oxidative stress (4) and myocardial apoptosis (5). Therefore, the search for a drug that is able to prevent or treat myocardial I/R injury is a popular focus of research.

Oxymatrine (OMT), an alkaloid that originates from the traditional Chinese herb *Sophora flavescens* Aiton, possesses numerous pharmacological properties, including anti-hepatic fibrosis (6), anti-inflammatory (7) and antitumor activities (8). The use of OMT in patients with cardiovascular diseases has attracted increasing attention, because studies have identified that OMT has a wide range of pharmacological properties, including activities against arrhythmia (9), shock (10) and hypertension (11). The nuclear factor erythroid-2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway and the

phosphatidylinositol 3-kinase (PI3K)/Akt/glycogen synthase kinase-3β (GSK3β) pathway are important pathophysiological mechanisms that are relevant to I/R injury, and previous studies have shown that OMT attenuates I/R injury in the brain through the p-Akt/GSK3β/HO-1/Nrf2 signaling pathway (12-14). However, the effects of OMT on I/R injury in cardiomyocytes, and the specific signaling pathways by which OMT exerts these effects have not yet been explored. Therefore, a hypoxia/reoxygenation (H/R) model of H9c2 cardiomyocytes was established in the present study to detect the potential effects and signaling pathways of OMT.

Materials and methods

Cell culture. The H9c2 cardiomyocyte cell line was provided by the Tianjin Key Laboratory of Hepatopancreatic Fibrosis and Molecular Diagnosis and Treatment, and were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) with 4,500 mg/l glucose containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. The H9c2 cardiomyocytes were grown in an incubator with 100% humidity containing 95% air and 5% CO_2 at 37˚C.

H/R treatment. The H/R model was established according to previously published methods (15,16). The H9c2 cardiomyocytes were cultured with Hank's balanced salt solution in an incubator containing 5% CO_2 and 95% N_2 at 37˚C for 2 h to establish hypoxia. Then, Hank's balanced salt solution was replaced with complete DMEM containing 10% FBS, and reoxygenation was conducted at 37˚C with 5% CO_2 for 4 h.

Cell grouping. Cell groups were established and the concentrations and durations of treatment chosen with reference to previously published methods (14,16,17). Also, the H9c2 cardiomyocytes were treated with different concentrations of OMT (0, 10, 30, 50, 70 and 90 μM) for 12 h under normoxic conditions to assess their cytotoxicity and select the concentrations for further analysis. OMT (cat. no. YM-0074) was purchased from Shanghai Yuanmu Biotech Co., Ltd. The H9c2 cardiomyocytes grown on plates were randomly divided into seven groups: i) Normally cultured H9c2 cardiomyocytes (control) group, in which the cells were cultured under standard conditions, without H/R or any additional treatments; ii) hypoxia/reoxygenation (model) group, in which the cells were exposed to hypoxia for 2 h followed by reoxygenation for 4 h; iii) model + 10 μmol/l OMT (10 μmol/l OMT) group, in which the cells were pretreated with 10 μmol/l OMT for 12 h and then exposed to H/R; iv) model + 30 μmol/l OMT (30 μmol/l OMT) group; v) model + 50 μmol/l OMT (50 μmol/l OMT) group; vi) model + LY294002 group, in which the cells were pretreated with 20 μmol/l LY294002(L9908, Sigma) for 1 h and then exposed to H/R; and vii) model + OMT + LY294002 (OMT + LY294002) group, in which the cells were maintained under the same conditions as those in the 50 μmol/l OMT group, but were also pretreated with 20 μmol/l LY294002 for 1 h beforethey were treated with OMT.

Cell viability assay. Cell viability was analyzed using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The H9c2 cardiomyocytes in the various groups were grown to a density of $1x10^4$ cells/well on 96-well plates, 20 μl MTT (5 mg/ml) was then added to each well, and the cells were incubated at 37˚C with 5% CO_2 for 4 h. The medium was removed, and 100 μl dimethylsulfoxide was added to the H9c2 cells in each well to dissolve the formazan crystals. Finally, the absorbance was read at 490 nm using a microplate reader (5200Multi; Tanon Science and Technology Co., Ltd.).

Observation of cell morphology. Cell morphology was observed with hematoxylin and eosin (H&E) staining. The cell supernatant of each group was discarded after 5 min of centrifugation at 132 x g at 4˚C. The H9c2 cardiomyocytes were sequentially washed with phosphate-buffered saline (PBS) and deionized water two or three times, incubated with hematoxylin for 5 min at room temperature, and then placed in eosin solution for 2 min at room temperature. Finally, the H9c2 cardiomyocytes were dried under ventilated conditions, and the cell morphology was observed using an optical microscope.

Detection of lactate dehydrogenase (LDH) levels. The severity of H9c2 cardiomyocyte injuries was evaluated by detecting the release of LDH into the cell supernatant. This was performed using an LDH kit (A020-2-2; Nanjing Jiancheng Bioengineering Institute), with measurement of the absorbance at 450 nm according to the manufacturer's instructions.

Detection of cellular malondialdehyde (MDA) levels, superoxide dismutase (SOD) activity and catalase (CAT) activity. The MDA levels, SOD activity and CAT activity of the cells were determined after the various treatments. H9c2 cardiomyocytes from the different groups were collected, washed three times with cold PBS, and then cell lysis buffer (RABLYSIS1; Sigma-Aldrich; Merck KGaA)was added for cell lysis. Following centrifugation at 206 x g for 5 min at 4˚C the supernatant was collected for detection. MDA levels, SOD activity and CAT activity were measured with corresponding kits (cat. nos. A003-3-1, A001-3-2 and A007-1-1; Nanjing Jiancheng Bioengineering Institute) at absorbances of 530, 450 and 405 nm, respectively, according to the manufacturer's instructions.

Cell apoptosis analysis with flow cytometric and TUNEL assays. The percentage of apoptotic cells in each group was analyzed using an Annexin V-FITC/PI apoptosis kit (Beijing 4A Biotech Co., Ltd.) for flow cytometry according to the manufacturer's instructions. Following treatment, the H9c2 cardiomyocytes from the different groups were collected and washed twice with cold PBS. Then, 5 μl Annexin V/FITC was added to the cells, which were then incubated for 5 min in the dark at room temperature for the labeling of early apoptotic cells. This was followed by incubation with 10 μl PI (20 μg/ml) for 10 min in the dark at room temperature to label late apoptotic cells. The analysis was performed using a flow cytometer (BeamCyte-1026; Changzhou Beamdiag Biotech Co., Ltd.), and quantitative processing was performed using FlowJo 10.6.2 software (FlowJo LLC).

The apoptosis of the H9c2 cardiomyocytes was also assessed using a TUNEL kit (KA4159; Abnova) according to

the manufacturer's instructions. The H9c2 cardiomyocytes were fixed with xylene for 10 min at room temperature and washed with PBS three times. The cells were then blocked with FBS in a humid atmosphere at 37˚C for 60 min and incubated with the antibody from the kit at 4˚C overnight. Afterwards, the slides were rinsed with PBS three times, the TUNEL reaction mixture was added and the slides were incubated for 1 h at 37˚C in the dark. The apoptotic cells were incubated in the mounting medium containing 0.05% DAPI for 10 min in the dark and then analyzed under a fluorescence microscope; green fluorescence was observed at 520 nm with a standard fluorescence filter and blue DAPI was observed at 460 nm. Image-Pro Plus 6.0 software (Media Cybernetics) was used for quantification.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to detect the expression of B cell lymphoma/leukemia-2 (Bcl-2), Bax, caspase-3, PI3K, Akt, GSK3β, Nrf2 and HO-1 in each group. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA, and UV spectrophotometry was used to measure the purity. Then, RNA was reverse transcribed into cDNAs using a HiFiScript cDNA Synthesis Kit (CoWin Biosciences) according to the manufacturer's instructions. The cDNA templates were analyzed by qPCR using the UltraSYBR Mixture (Low ROX) kit (CoWin Biosciences) under the following conditions: 40 cycles of 10 sec at 95˚C, 30 sec at 60˚C and 32 sec at 72˚C. The nucleotide sequences of the forward and reverse primers are shown in Table I. The relative expression level of each mRNA was calculated by using the $2^{-\Delta\Delta Cq}$ method (18).

Protein preparation and western blot analysis. H9c2 cardiomyocytes from the various groups were washed three times with PBS, and then lysed in complete RIPA buffer (R0020; Beijing Solarbio Science & Technology Co., Ltd.) at 4˚C for 20 min. The total protein concentrations were determined using a BCA kit (A045-4-2; Nanjing Jiancheng Bioengineering Institute). Equal amounts of protein from each group (30 μg) were loaded onto 10% polyacrylamide gels for electrophoresis and transferred to nitrocellulose membranes (EMD Millipore). Then, the membranes were blocked with Tris-buffered saline and 0.05%Tween 20 buffer containing 5% skimmed milk for 3 h at room temperature, followed by incubation with the following primary antibodies overnight at 4˚C: Bax (cat. no. 50599-2-Ig; 1:2,000;), Bcl-2 (cat. no. 60178-1-Ig; 1:2,000), pro caspase-3 (cat. no. 66470-2-Ig; 1:1,000), cleaved caspase-3 (cat. no. 66470-2-Ig; 1:1,000), PI3K (cat. no. 20584-1-AP; 1:1,000), Akt (cat. no. 10176-2-AP, 1:1000), GSK3β (cat. no. 22104-1-AP; 1:1,000), Nrf2 (cat. no. 16396-1-AP, 1:1,000), HO-1 (cat. no. 16396-1-AP, 1:1,000) and β-actin (cat. no. 4970S; 1:1,000), all from ProteinTech Group, Inc.; phosphorylated (p-)PI3K (cat. no. bs-3332R, 1:1,000; BIOSS); p-Akt (cat. no. 4060s; 1:2,000; Cell Signaling Technology, Inc.) and p-GSK3β (cat. no. 9327s; 1:1,000; Cell Signaling Technology, Inc.). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074V; 1:5,000; Cell Signaling Technology, Inc.) for 1 h at room temperature. Signals were observed using ECL reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Band densities were detected using ImageJ 1.52a software (National Institutes of Health).

Table I. Sequences of the primer pairs used for quantitative PCR.

Primer	Sequence
Bcl-2	F: 5'-ATAACCGGGAGATCGTGATGA-3'
	R: 5'-CTCTCAGGCTGGAAGGAGAAG-3'
Bax	F: 5'-CCACCAGCTCTGAACAGATCA-3'
	R: 5'-GCTCCATGTTGTTGTCCAGT-3'
Caspase-3	F: 5'-GAGCAGAGTCAAAGGCTGGT-3'
	R: 5'-TGTCGTCATGTCCACCACT-3'
Nrf2	F: 5'-TCCTCTGCTGCCATTAGTCA-3'
	R: 5'-GTGCCTTCAGTGTGCTTCT-3'
HO-1	F: 5'-TCTGGAATGGAAGGAGATGC-3'
	R: 5'-AGTTCTGGGGCTCTGTTGC-3'
PI3K	F: 5'-GACTCCAAGATGAAGAAGATGTG-3'
	R: 5'-GAGCATTCGCAGGTCCAAGCC-3'
Akt	F: 5'-CGAGGCCCAACACCTTCATC-3'
	R: 5'-CCGGAAGTCCATCGTCTCCT-3'
GSK3β	F: 5'-CCAGGTGGAGGACCATTTGC-3'
	R: 5'-ACTCTACACCAGCAGCAGCC-3'
β-actin	F: 5'-TCAGGTCATCACTATCGGCAAT-3'
	R: 5'-AAAGAAAGGGTGTAAAACGCA-3'

Bcl-2, B cell lymphoma/leukemia-2; PI3K, phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase-3β; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; F, forward; R, reverse.

Statistical analysis. Results are presented as the mean ± SD (n=10). Multigroup comparisons of the means were performed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. SPSS version 25.0 (IBM Corp.) statistical software was used to perform the analysis. $P<0.05$ was considered to indicate a statistically significant result. All experiments were repeated three times.

Results

OMT increases the viability of H9c2 cardiomyocytes exposed to H/R. H9c2 cardiomyocytes were treated with different concentrations of OMT for 12 h under normoxic conditions to explore the effects of OMT on these cells. As evidenced by the MTT assay, OMT did not exert marked cytotoxic effects or reduce the viability of H9c2 cardiomyocytes pretreated with 10, 30 or 50 μM OMT under normoxic conditions (Fig. 1A). Therefore, 10, 30 and 50 μM OMT were chosen as the low, medium and high concentrations for subsequent experiments. The viability of H9c2 cardiomyocytes was significantly decreased compared with that of the control group after H/R injury, and concentration-dependently increased in the cells treated with OMT for 12 h prior to H/R injury compared with that of the model group ($P<0.05$; Fig. 1B). The LDH release assay revealed that the H/R injury-induced increase in LDH release was significantly reduced when the cells were pretreated with OMT ($P<0.05$; Fig. 1C). The results of the cell viability and LDH release assays indicate a protective effect of OMT

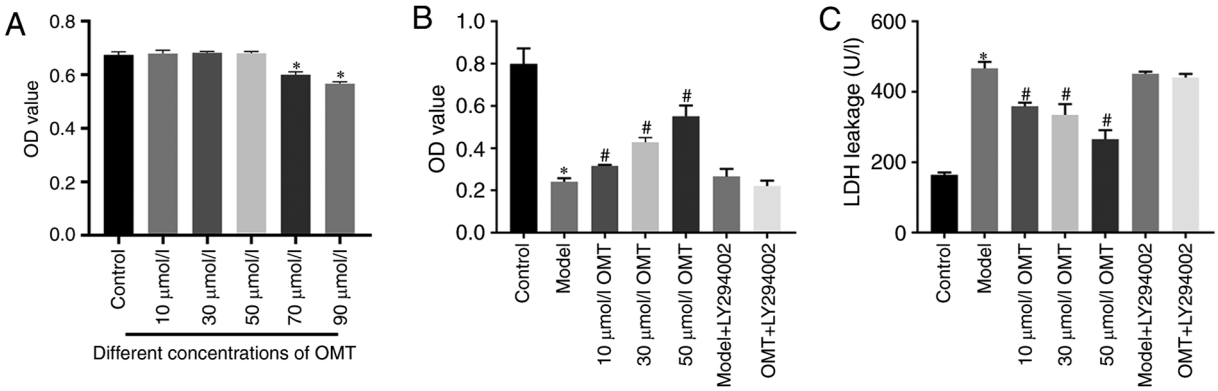


Figure 1. OMT increases the viability of H9c2 cardiomyocytes exposed to H/R. (A) MTT assay was performed to detect the effect of OMT on the viability of H9c2 cardiomyocytes cultured under normal conditions and after treatment with various concentrations of OMT. (B) H9c2 cardiomyocytes were pretreated with OMT at different experimental concentrations in the presence or absence of LY294002 prior to H/R injury, and cell viability was measured using the MTT assay. (C) Cytotoxicity was also measured by determining the LDH content. *P<0.05 compared with the control group; #P<0.05 compared with the model group. OMT, oxymatrine; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; OD, optical density at 490 nm.

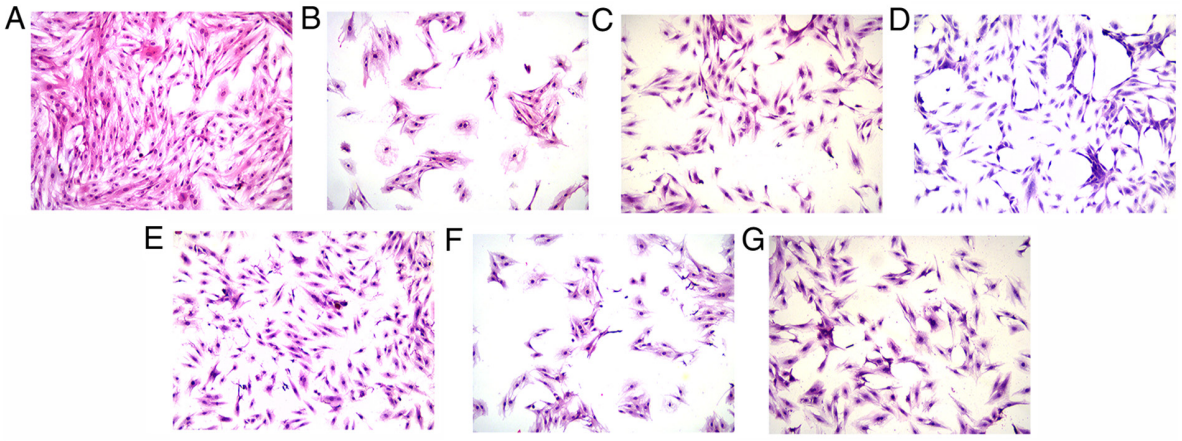


Figure 2. Representative images of H9c2 cardiomyocytes stained with hematoxylin and eosin. (A) The cells in the control group exhibited a long spindle-like morphology with a full cytoplasm and complete structure. (B) Cells in the model group exposed to H/R exhibited a marked loss of basic structure. After pretreatment with (C) 10, (D) 30 and (E) 50 μM OMT, the morphology of the cells gradually recovered in a concentration-dependent manner. (F) In the cells treated with LY294002 and exposed to H/R, no differences were observed compared the model. (G) LY294002 inhibited the protective effect of OMT on the cells. Magnification, x100. OMT, oxymatrine; H/R, hypoxia/reoxygenation.

against H/R injury. Furthermore, no difference in the viability and LDH release of the H9c2 cardiomyocytes was observed between the model group and the model group treated with the PI3K inhibitor LY294002, indicating that LY294002 was not toxic to cells. When LY294002 was added before the OMT pretreatment, the cell viability was significantly decreased and LDH release was significantly increased, indicating that the protection provided by OMT may be mediated by the PI3K/Akt signaling pathway.

OMT improves the morphology of H9c2 cardiomyocytes exposed to H/R. As shown in the images of H&E staining, the H9c2 cardiomyocytes in the control group (Fig. 2A) showed good growth and good adhesion to the well. The cells had an elongated spindle morphology with a full cytoplasm and intact structure. A large number of suspended cells were present in the model group (Fig. 2B), which exhibited a marked loss of basic structure and evident shrinkage. In addition, the cytoplasm appeared cloudy and the intracellular structures were unclear. The cell morphology was clearly ameliorated by the OMT pretreatment at different concentrations (Fig. 2C-E). Compared with the model group, the cells gradually recovered their spindle-like morphology, the cytoplasm became fuller, the intracellular structures became clearer and the intercellular space was significantly reduced, and thus the number of cells observed under the microscope increased. No differences were observed between the cells in the model group and model + LY294002 group (Fig. 2F), confirming the previous finding that LY294002 was not toxic to cells. However, the group treated with LY294002 prior to the OMT pretreatment exhibited cell morphology similar to that in the model group, suggesting that the protective effect of OMT on H9c2 cardiomyocytes subjected to H/R injury may be mediated by the PI3K/Akt signaling pathway (Fig. 2G).

OMT suppresses oxidative stress in H9c2 cardiomyocytes exposed to H/R. The activities of SOD and CAT and the quantity of MDA in each group were detected using the corresponding

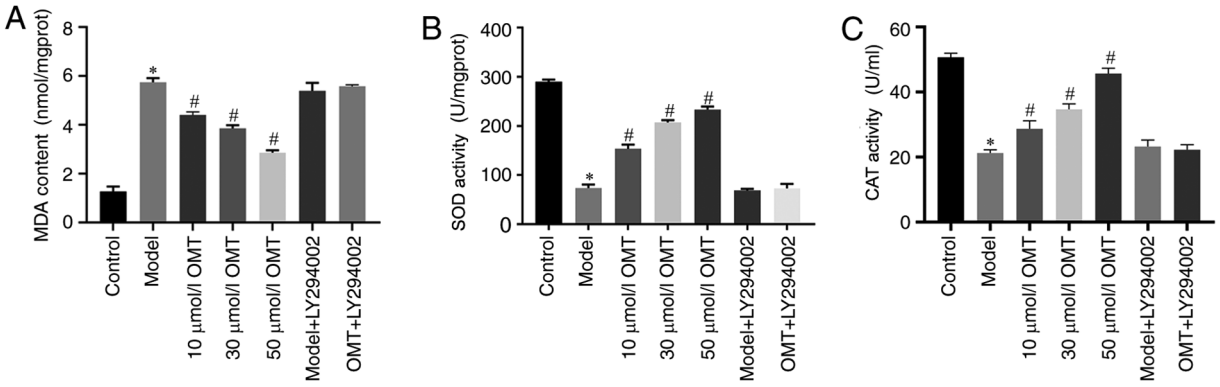
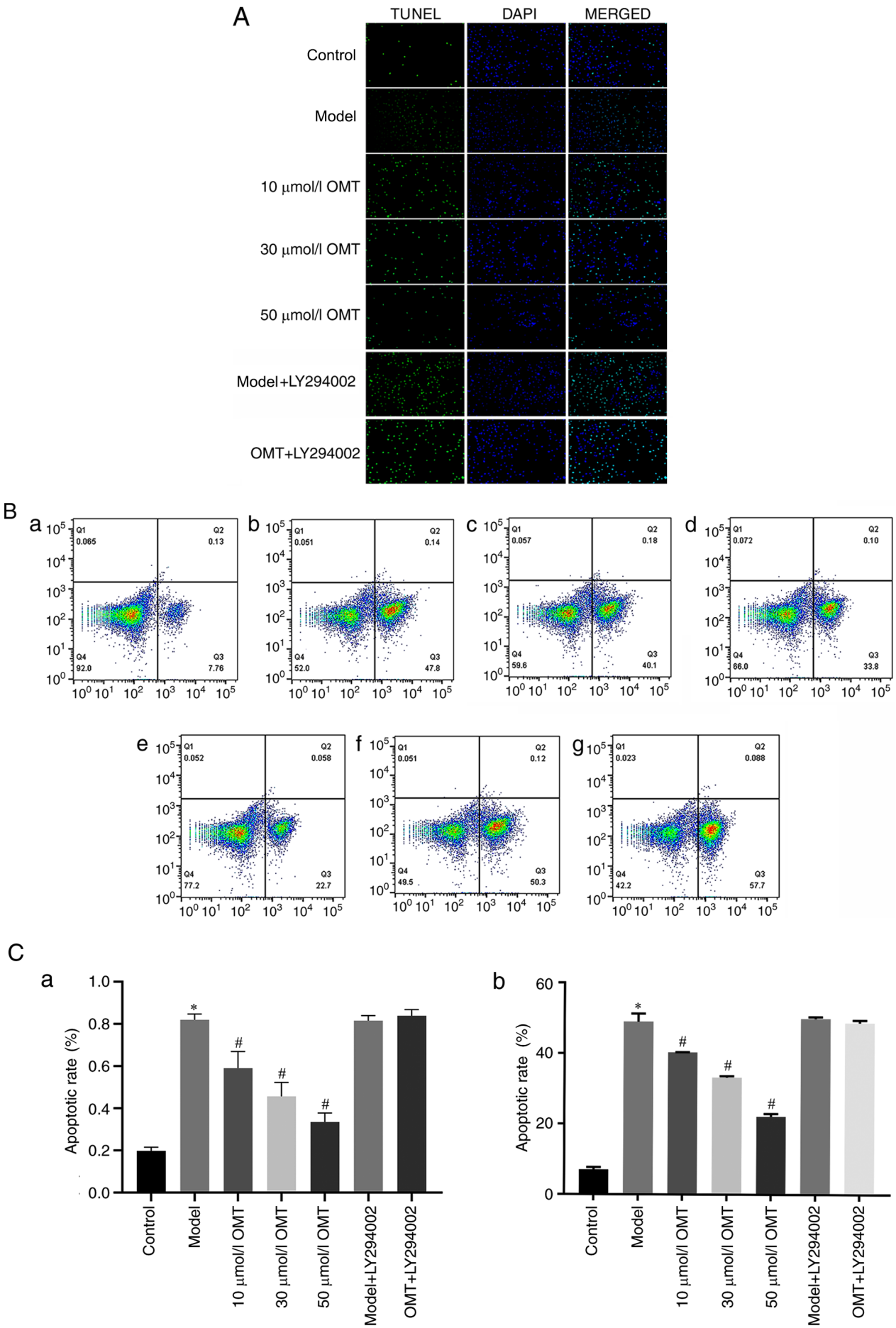


Figure 3. OMT suppresses oxidative stress in H9c2 cardiomyocytes exposed to hypoxia/reoxygenation. Markers of oxidative stress, namely (A) MDA content, (B) SOD activity and (C) CAT activity were detected using kits. After pretreatments with 10, 30 and 50 μM OMT, the SOD and CAT activities were increased and the MDA content was decreased in a concentration-dependent manner compared with those the model group. However, LY294002 inhibited the changes induced by OMT. *P<0.05 compared with the control group; #P<0.05 compared with the model group. OMT, oxymatrine; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase.

kits, to investigate whether the protective effect of OMT on H9c2 cardiomyocytes exposed to H/R was associated with the suppression of oxidative stress. Compared with the control group, the activities of the antioxidants SOD and CAT were decreased and the content of the lipid peroxide marker MDA was increased in H9c2 cardiomyocytes in the model group, indicating that H/R injury increased the oxidative stress response. No differences in results were detected between the model group and the model + LY294002 group, indicating that LY294002 had no effect on the cells. Compared with the model group, the H9c2 cardiomyocytes pretreated with 10, 30 and 50 μM OMT exhibited significantly increased SOD and CAT activities and significantly decreased MDA content, suggesting that the protective effect of OMT was associated with the suppression of oxidative stress. The SOD and CAT activities and MDA content in the cells treated with LY294002 prior to OMT pretreatment were comparable with those in the model group. These results indicate that the OMT pretreatment protected H9c2 cardiomyocytes from H/R injury by preserving their antioxidant capacity, which may be associated with the PI3K/Akt signaling pathway (P<0.05; Fig. 3).

OMT inhibits apoptosis in H9c2 cardiomyocytes exposed to H/R. TUNEL staining (Fig. 4A) and flow cytometry (Fig. 4B) were performed to evaluate the effect of OMT on the H/R-induced apoptosis of H9c2 cardiomyocytes, and the rates of apoptosis were determined (Fig. 4C). A significantly increased number of TUNEL-positive cells and apoptotic cells were detected in the model group compared with the control group, indicating that H/R injury promoted the apoptosis of H9c2 cardiomyocytes. Furthermore, the levels of Bcl-2 and Bax and caspase-3, biomarkers of mitochondrial apoptosis, were detected using western blotting and RT-qPCR (Fig. 4D and E). Regardless of whether mRNA or protein levels were analyzed, the results indicated that H/R injury accelerated the apoptosis of H9c2 cardiomyocytes by increasing the levels of the pro-apoptotic factors Bax and cleaved caspase-3, and reducing the level of the anti-apoptotic factor Bcl-2, which are mainly associated with the mitochondrial apoptotic pathway. No differences were observed between the model group and the model + LY294002 group, indicating that LY294002 did not alter apoptosis. However, compared with the model group, the apoptosis of H9c2 cardiomyocytes was significantly attenuated by the OMT pretreatment. Following treatment with increasing concentrations of OMT, the number of TUNEL-positive cells gradually decreased and the proportion of apoptotic cells also decreased, indicating that OMT exerts an anti-apoptotic effect on cells with H/R injury. However, the protective effects of OMT were markedly reduced by the addition of LY294002 prior to the OMT pretreatment, indicating that the anti-apoptotic effects of OMT were potentially mediated by the PI3K/Akt signaling pathway.

OMT protects H/R-exposed H9c2 cardiomyocytes by activating the PI3K/Akt signaling pathway. The PI3K/Akt/GSK3β and Nrf2/HO-1 signaling pathways were analyzed using western blotting and RT-qPCR to examine the molecular mechanism of OMT in H9c2 cardiomyocytes with H/R injury (Fig. 5). Western blots (Fig. 5A) revealed that the levels of p-PI3K, p-Akt and p-GSK3β in the model group were increased compared with those in the the control group, indicating that H/R injury activated the PI3K/Akt/GSK3β pathway. No difference was observed between the phosphorylated protein levels in the model group and the model + LY294002 group, suggesting that LY294002 did not modulate the activity of the PI3K/Akt/GSK3β pathway. Pretreatment with OMT concentration-dependently increased the levels of these phosphorylated proteins in H9c2 cardiomyocytes exposed to H/R injury, indicating that the protective effect of OMT was associated with the PI3K/Akt/GSK3β pathway. Furthermore, LY294002 attenuated the OMT-induced increases in the levels of p-Akt and p-GSK3β, confirming that the protective effect of OMT was mediated by the PI3K/Akt pathway. The expression of the PI3K and Akt mRNAs in different groups detected using RT-qPCR (Fig. 5B) were consistent with the protein levels. However, a difference was observed between the levels of the GSK3β mRNA and protein. Compared with the control group, the expression of the GSK3β mRNA was increased in the model group, indicating that H/R injury increased the expression of the GSK3β mRNA. However, expression of the GSK3β mRNA was markedly decreased in the 10, 30 and 50 μM OMT pretreatment groups compared with the model



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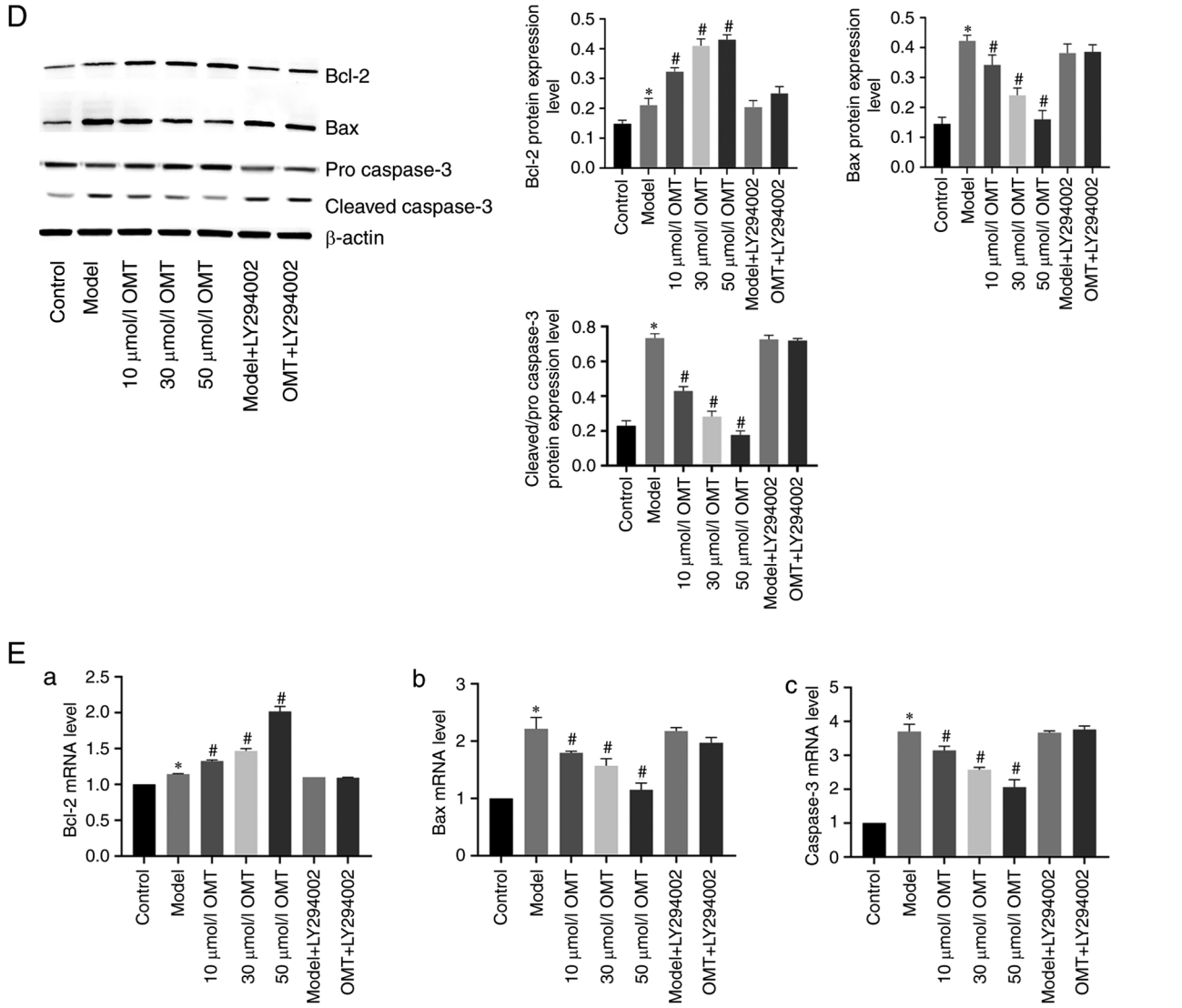

Figure 4. OMT inhibits apoptosis in H9c2 cardiomyocytes exposed to hypoxia/reoxygenation. (A) The apoptosis of H9c2 cardiomyocytes in different groups was determined using the TUNEL assay. TUNEL-positive cells are green, and nuclei are stained blue with DAPI (magnification, x100). (B) Flow cytometric analysis of the apoptosis of H9c2 cardiomyocytes in the (a) control, (b) model, (c) 10 μM OMT, (d) 30 μM OMT and (e) 50 μM OMT pretreatment, (f) model with LY294002 and (g) OMT + LY294002 pretreatment groups. (C) Apoptosis rates determined using (a) TUNEL assay and (b) flow cytometry. (D) The levels of apoptosis-associated proteins, namely Bax, Bcl-2, pro caspase-3 and cleaved caspase-3, were detected using western blot analysis. (E) The mRNA expression levels of the apoptosis-associated proteins (a) Bcl-2, (b) Bax and (c) caspase-3 were measured using RT-qPCR. *P<0.05 compared with the control group; $^{\#}$P<0.05 compared with the model group. OMT, oxymatrine; Bcl-2, B cell lymphoma/leukemia-2.

group, suggesting that the protective effect of OMT was related to a reduction in the expression of GSK3β mRNA.

The Nrf2/HO-1 pathway is a crucial component of the antioxidant defenses against H/R injury. Western blots (Fig. 5C) revealed increased levels of Nrf2 and HO-1 proteins in the model group compared with the control group. Thus, H/R injury activated the Nrf2/HO-1 pathway. Furthermore, no differences were observed between the cells in the model group and model + LY294002 group, indicating that LY294002 does not alter the Nrf2/HO-1 pathway. The levels of Nrf2 and HO-1 proteins were significantly increased in the 10, 30 and 50 μM OMT pretreatment groups compared with those in the model group, suggesting that the protective effect of OMT was associated with the Nrf2/HO-1 pathway. However, LY294002 reduced the levels of these factors, indicating that the protective effect of OMT was mediated by the activation of the Nrf2/HO-1 pathway via the PI3K/Akt pathway. The expression levels of the Nrf2 and HO-1 mRNAs in different groups were measured using RT-qPCR (Fig. 5D) and were consistent with the protein levels, confirming that OMT increased the expression of the Nrf2 and HO-1 mRNAs and proteins to function as an antioxidant.

Discussion

IHD is a serious threat to human health worldwide. Acute myocardial infarction (AMI) is one of the main diseases that constitute IHD. Patients with AMI often have a history of coronary atherosclerotic heart disease (CAD), and the basic pathological change in patients with CAD is atherosclerosis (19). In some patients with CAD, the rupture of atherosclerotic plaques in the coronary arteries due to fatigue,

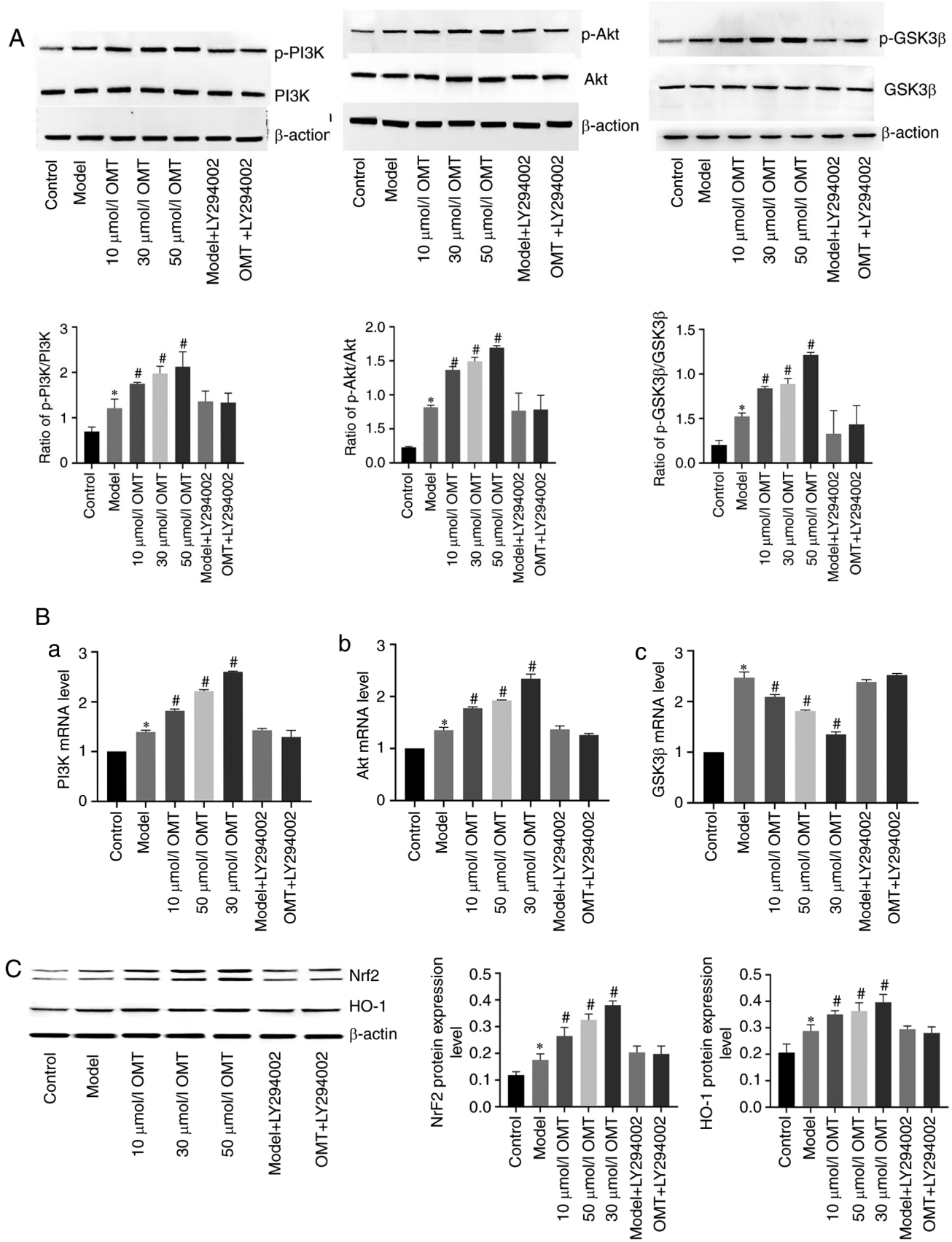

Figure 5. Continued.

stress and other factors leads to the rapid accumulation of platelets, neutrophils and macrophages, which form emboli and block the vascular cavity, leading to the necrosis of cardiomyocytes. Myocardial cells are non-renewable, and thus myocardial blood perfusion must be restored as soon as possible (20). However, although the continuous development

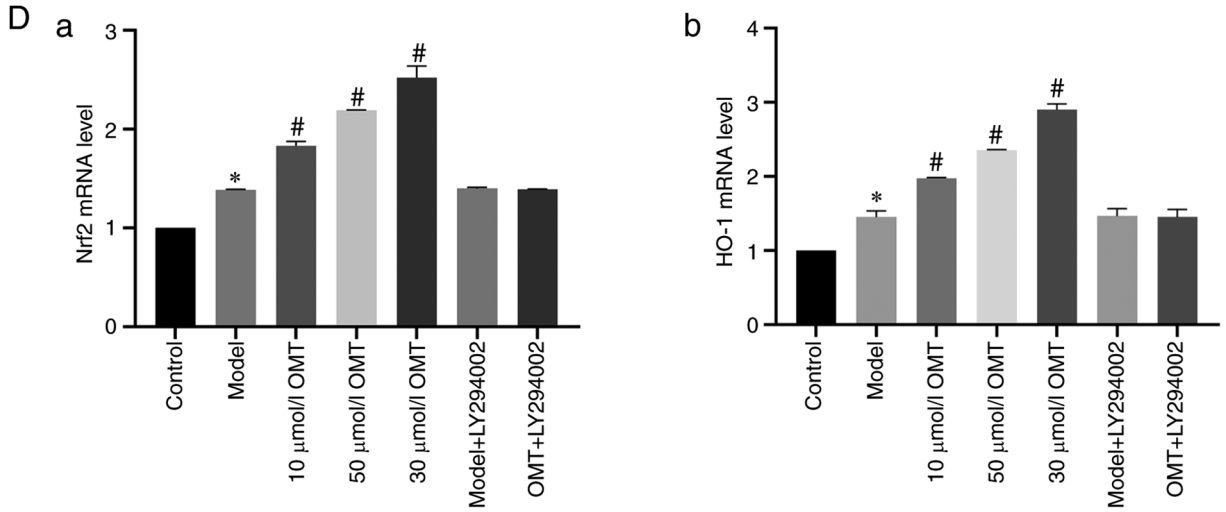

Figure 5. OMT protects H9c2 cardiomyocytes exposed to H/R by activating the Akt/GSK3β/Nrf2/HO-1 pathway. (A) After H/R injury and treatment with different concentrations of OMT, the levels of proteins involved in the PI3K/Akt/GSK3β pathway were detected using western blotting. (B) The expression of (a) PI3K, (b) Akt and (c) GSK3β mRNAs measured using RT-qPCR. (C) In addition, the levels of Nrf2 and HO-1 proteins, which are downstream targets of the PI3K/Akt/GSK3β pathway, were detected using western blotting and (D) the expression of (a) Nrf2 and (b) HO-1 mRNAs were measured using RT-qPCR. *P<0.05 compared with the control group; #P<0.05 compared with the model group. OMT, oxymatrine; H/R, hypoxia/reoxygenation; GSK3β, glycogen synthase kinase-3β; Nrf2, nuclear factor erythroid-2-related factor 2; HO-1, heme oxygenase-1; PI3K, phosphatidylinositol 3-kinase; RT-qPCR, reverse transcription-quantitative PCR.

of PCI, CABG and other technologies has effectively improved myocardial blood perfusion, I/R injury is a major problem that remains to be addressed. The apoptosis of cardiomyocytes mostly occurs during reperfusion and is mainly mediated by the mitochondrial apoptosis pathway (21). Therefore, according to the pathogenesis of I/R injury, the identification of a drug that protects cardiomyocytes exposed to I/R injury is the focus of the present study.

OMT is an alkaloid that has been widely used clinically and possesses various biological activities. OMT pretreatment has been shown to have a protective effect on cardiomyocytes exposed to I/R injury, but the protective mechanism has not been fully elucidated (22). Therefore, the present study simulated human myocardial I/R injury using an *in vitro* H9c2 cardiomyocyte H/R injury model, and an OMT pretreatment was administered to explore the protective effect of OMT on cardiomyocyte H/R injury. OMT effectively protected H9c2 cardiomyocytes with H/R injury. The protective mechanism may be associated with activation of PI3K/Akt signaling pathway and an increase in the expression of the downstream proteins GSK3β and Nrf2.

Under normal physiological conditions, the serum concentration of LDH is low, and LDH in cells is released only after cell membrane damage (23). Therefore, the degree of cell damage can be evaluated by measuring the LDH level. When cells were exposed to H/R in the present study, a large amount of LDH was released due to damage of the myocardial cell membrane, which increased the LDH content in the cell supernatant. When OMT was added to the cells before H/R injury, the LDH content of the cell supernatant decreased as the OMT concentration increased, suggesting that OMT exerted a protective effect on cell membranes and reduced cell damage. When LY294002 was added to the cells prior to OMT, the protective effect of OMT on the cell membrane was weakened, suggesting that the protective effect of OMT was mediated by the PI3K/Akt signaling pathway. This result also laid the foundation for the follow-up experiments.

Oxidative stress is an imbalance between oxidant levels and antioxidant activity in the body. It is often accompanied by the infiltration of a large number of inflammatory cells and increased lipid oxidation and decomposition. Oxidative stress is considered one of the pathological processes that promotes apoptosis in I/R injury (23,24). I/R injury causes the production of a large amount of hydrogen peroxide (H_2O_2) in cells. H_2O_2 interacts with iron in the nucleus to generate a large quantity of reactive oxygen species (ROS) and thereby accelerates cell damage. In addition, H_2O_2 also interacts with lipids to generate the lipid oxidation product MDA, which promotes protein polymerization and accelerates cell apoptosis (25). SOD and CAT are important endogenous antioxidants *in vivo*, which effectively remove excess oxygen free radicals, reduce mitochondrial damage and maintain cell homeostasis (26). As shown in the present study, cardiomyocytes exposed to H/R were extensively damaged, as evidenced by a significant reduction in intracellular SOD and CAT activities, and a significant increase in the MDA content, which prevents cells from removing excess ROS and results in excessive ROS deposition and the exacerbation of cell damage. When the cardiomyocytes were pretreated with OMT, their SOD and CAT activities were significantly increased and MDA content was significantly decreased, indicating that OMT increased the antioxidant capacity of the cells by increasing the activities of these antioxidant enzymes in cardiomyocytes and reducing lipid peroxide levels. However, this biological effect was weakened by LY294002, suggesting that OMT increased the antioxidant capacity of H9c2 cardiomyocytes exposed to H/R through the PI3K/Akt signaling pathway.

In-depth study of myocardial I/R injury has demonstrated that the Nrf2/HO-1 pathway, a downstream signaling pathway

of the PI3K/Akt pathway (27), plays an important role in oxidative stress. Under normal circumstances, Nrf2 exists in the cytoplasm in the form of an inactive complex with its inhibitor, Kelch-like ECH associated protein 1 (Keap1), and Nrf2 is degraded by the ubiquitin proteasome pathway. When myocardial tissue undergoes I/R injury and myocardial cells are exposed to ROS, the Nrf2-Keap 1 complex quickly separates and Nrf2 translocates to the nucleus, where it binds the antioxidant response element and initiates the transcription of downstream antioxidant genes and the phase II antioxidant enzyme HO-1 to activate antioxidant defenses (28). In addition, activation of the Nrf2/HO-1 pathway has been shown to upregulate the expression of the Bcl-2 gene and exert an anti-apoptotic effect (29). In the present study, RT-qPCR revealed that OMT significantly increased the expression of Nrf2 and the downstream gene HO-1 in H/R-injured cardiomyocytes. Western blot analyses of these proteins were consistent with the RT-qPCR analyses of mRNA expression, indicating that OMT activated the Nrf2/HO-1 signaling pathway to provide an antioxidant effect and concurrently increased the activity of antioxidant enzymes. When LY294002 was applied prior to IMP, the ability of OMT to upregulate Nrf2 and HO-1 was significantly attenuated, suggesting that OMT activated the Nrf2/HO-1 pathway via the PI3K/Akt pathway while simultaneously upregulating the expression of HO-1 to exert its antioxidant effect. In summary, the results indicate that OMT exerted antioxidant effects through multiple pathways to protect cardiomyocytes.

The PI3K/Akt/GSK3β signaling pathway is an important pathway involved in the intracellular transduction of signals from transmembrane receptors that serve key roles in cell survival (30), proliferation (31) and apoptosis (32). It is one of the more extensively investigated signaling pathways in clinical research. According to numerous studies, this pathway is activated following I/R injury, and effectively reduces the area of myocardial infarction, which is also the target of a number of biological molecules and drugs (14,33,34). Components of this signaling pathway were analyzed at the protein and mRNA levels to determine whether the protective effect of OMT on cardiomyocytes subjected to H/R injury was associated with this signaling pathway and to verify the pathway upstream of the Nrf2/HO-1 pathway. Western blotting revealed significantly increased levels of p-PI3K, p-Akt and p-GSK3β in the OMT group compared with the model group, suggesting that OMT protected H/R-injured cardiomyocytes via the activation of PI3K/Akt/GSK3β signaling. However, RT-qPCR revealed significant increases in the expression of PI3K and Akt mRNAs in the OMT pretreatment groups compared with the model group, whereas the expression of the GSK3β mRNA was significantly decreased. This finding differs from the western blotting results. According to previous studies, in this pathway, Akt phosphorylates GSK3β at Ser9, inactivating GSK3β and phosphorylating β-catenin, thereby promoting cell survival (35,36). Therefore, OMT may inhibit myocardial injury by activating the PI3K/Akt/GSK3β and Nrf2/HO-1 pathways.

Apoptosis is a type of programed death characterized by morphological changes, such as cell shrinkage, nucleolysis and DNA fragmentation (37). I/R injury activates the mitochondrial apoptosis pathway, and cardiomyocyte apoptosis is mainly mediated by the mitochondrial apoptosis pathway (21). A previous study by Sun *et al* (22) demonstrated that OMT is able to inhibit the mitochondrial apoptosis of cardiomyocytes injured by H/R *in vivo*. The mitochondrial apoptosis pathway mainly involves the Bcl-2 protein family, which is composed of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2. The extent of cell necrosis and apoptosis is determined by regulation of the permeability of the mitochondrial membrane (38). The caspase protein family also plays an important role in cell apoptosis. When Bax binds to the mitochondrial membrane, the gradient in the ion concentration between the inner and outer membrane of the mitochondria changes, and cytochrome *c* flows into the cytoplasm, forming apoptotic bodies with the apoptotic protein caspase-9 and activating caspase-3 to induce cell apoptosiss (39). In the present study, the expression of apoptosis-associated markers was detected at the mRNA and protein levels. OMT significantly increased the levels of the anti-apoptotic protein Bcl-2 and reduced those of the pro-apoptotic proteins Bax and cleaved caspase-3, suggesting that OMT inhibited the mitochondrial apoptosis pathway to protect cardiomyocytes injured by H/R. When the PI3K inhibitor LY294002 was added to cardiomyocytes prior to OMT, the ability of OMT to regulate the expression of the anti-apoptotic protein Bcl-2 was significantly reduced, indicating that the PI3K/Akt signaling pathway was involved in the anti-apoptotic effect of OMT. In addition TUNEL staining and flow cytometry assays were also performed in the present study to supplement and verify these conclusions. The results of these assays more comprehensively showed that OMT exerted its anti-apoptotic effects through the PI3K/Akt signaling pathway and protected H/R-injured cardiomyocytes. By analyzing different pathological mechanisms, the present study demonstrated that OMT protected cells from H/R injury and inhibited the mitochondrial apoptosis pathway in cardiomyocytes.

In summary, the present study provides new insights into the protective effects of OMT against myocardial I/R injury. The reperfusion injury salvage kinase signaling pathway, Nrf2/HO-1 signaling pathway and mitochondrial apoptosis pathway were used as entry points to clarify that the PI3K/Akt signaling pathway is involved in the protective effect of OMT on H9c2 cardiomyocytes subjected to H/R injury. The mechanism is hypothesized to be as follows: When H/R injury occurs in cardiomyocytes, upstream signaling activates PI3K via the stimulation of membrane receptors to transduce a signal in the cell. PI3K then transmits the extracellular signal to the downstream kinase Akt and activates it. Akt phosphorylates the downstream protein GSK3β to inactivate it, and finally apoptosis is inhibited via regulation of mitochondrial permeability. Concurrently, Nrf2, an important transcription factor downstream of the PI3K/Akt/GSK3β signaling pathway, is also activated by Akt, functions as an antioxidant and inhibits cell apoptosis by increasing the expression of the anti-apoptotic protein Bcl-2. When the H9c2 cardiomyocytes were exposed to H/R injury after OMT pretreatment, OMT significantly increased the expression of proteins involved in the Akt/GSK3β/Nrf2/HO-1 signaling pathway, while the PI3K inhibitor LY294002 blocked this biological effect. The occurrence of this phenomenon strongly suggests that the PI3K/Akt signaling pathway is involved in the protective effects of OMT.

The OMT pretreatment protected H9c2 cardiomyocytes from H/R-induced cell damage, oxidative stress and cell apoptosis via a common upstream PI3K/Akt pathway. Based on these findings, OMT might be a potential candidate treatment for myocardial I/R injury.

Acknowledgements

Not applicable.

Funding

The present study was supported by a grant from the Key Project of Tianjin Natural Science Foundation (grant no. 16JCZDJC31900).

Availability of data and materials

All data generated or used during the study are included in this published article.

Authors' contributions

ZZ, YL, WZ and MZ designed the experiments, conducted the experiments and wrote the manuscript. ZZ, FC and ZW performed RT-qPCR, MTT and H&E staining assays. XQ, RC and CL performed flow cytometry and TUNEL assays. ZZ, CL, ZW, RC and WZ analyzed the datasets and supervised the project. All authors reviewed the data and provided feedback on the manuscript. ZZ and MZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing Interests

The authors declare that they have no competing interests.

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