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Regular paper

Oxypaeoniflorin improves myocardial ischemia/reperfusion injury by activating the Sirt1/Foxo1 signaling pathway

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Myocardial ischemia/reperfusion (MI/R) injury is a leading cause of damage to cardiac tissues and is associated with high mortality and disability rates worldwide. Oxypaeoniflorin (OPA) has been found to be the main constituent of Paeonia veitchii Lynch. This study was conducted to explore the effect of OPA on MI/R injury and its potential mechanism. An *in vivo* MI/R injury model was established by transient coronary ligation in BALB/c mice, and an *in vitro* hypoxia/reoxygenation (H/R) injury model was established with rat cardiomyocyte H9c2 cells. Echocardiographic assessments demonstrated that OPA significantly reduced disruption of cardiac function and improved the indicators of ejection fraction (EF) and fractional shortening (FS). The enzyme-linked immunosorbent assay (ELISA) results suggested that OPA significantly reduced the release of myocardial infarction-related factors, such as the creatine kinase (CK-MB), cardiac troponin I (cTnI) and cardiac troponin T (cTnT). Additionally, hematoxylin-eosin (H&E) staining demonstrated that OPA markedly inhibited the myocardial apoptosis and necrosis caused by MI/R. Consistently, the results obtained from the cell counting kit-8 (CCK-8) and flow cytometry assays revealed that OPA obviously reversed the H/R-induced decrease in cell activity and increase in apoptosis of H9c2 cells. Furthermore, western blot assays indicated that OPA inhibited apoptosis by activating the Sirt1 (silent information regulator factor 2 related enzyme 1)/Foxo1(forkhead transcription factor FKHR) signaling pathway in myocardial tissues and H9c2 cells. Collectively, these novel findings are the first to provide strong evidence that OPA attenuates MI/R injury by activating the Sirt1 (silent information regulator factor 2 related enzyme 1)/Foxo1(forkhead transcription factor FKHR) signaling-mediated anti-apoptotic pathway.

Key words: Oxypaeoniflorin, myocardial ischemia/reperfusion, apoptosis, Sirt1/Foxo1

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Abbreviations: CCK-8, cell counting kit-8; CK-MB, creatine kinase; cTnI, cardiac troponin I; cTnT, cardiac troponin T; EF, ejection fraction; ELISA, immunosorbent assay; H/R, hypoxia/reoxygenation; FS, fractional shortening; MI/R, Myocardial ischemia/reperfusion

INTRODUCTION

Ischemic heart disease, also known as coronary atherosclerotic heart disease (referred to as coronary heart disease), has become the leading cause of death worldwide (Finegold *et al.*, 2013). According to the World Health Organization, coronary heart disease caused a total of 7.4 million deaths worldwide in 2012, and its incidence has increased annually in China (Zhu *et al.*, 2016). Mounting evidence has demonstrated that the occlusion of blood vessels by coronary arteriosclerotic plaques due to myocardial ischemia can lead to myocardial tissue damage; after the vascular occlusion is removed, the process of regaining blood supply to the myocardial tissue causes further myocardial tissue damage, which is widely known as myocardial ischemia/reperfusion (MI/R) injury (Buja, 2013). MI/R injury can lead to many adverse complications, including cardiomyocyte necrosis and apoptosis, ultimately destroying the cardiac contractile function (Salas *et al.*, 2010; Wang *et al.*, 2019). Naturally, protecting cardiomyocytes from apoptosis and necrosis could be a reasonable method of ameliorating the MI/R injury (Wang *et al.*, 2015; Zhu *et al.*, 2015; Xu *et al.*, 2019; Wang *et al.*, 2016). Therefore, for the prevention and treatment of ischemic heart disease, it is of great significance to explore the mechanisms that regulate MI/R injury and those that protect against MI/R injury.

Paeonia lactiflora Pall. is a well-known Chinese herbal medicine of the Ranunculaceae family that is used to relieve abdominal cramps, reduce pain and improve blood circulation (Chen *et al.*, 2013). The main active chemical components of *Paeonia lactiflora* include paeoniflorin and oxypaeoniflorin. Among these components, paeoniflorin has been reported to protect against the MI/R injury by inhibiting inflammation and apoptosis (Nizamutdinova *et al.*, 2008; Qian *et al.*, 2015; Chen *et al.*, 2015; Li *et al.*, 2012; Han *et al.*, 2016b). Oxypaeoniflorin (OPA), also known as [1a-(hexopyranosyloxy)-5-hydroxy-2-methyltetrahydro-1H-2,5-methano-3,4-dioxacyclobuta[cd]pentalen-5b(3aH)-yl]methyl 4-hydroxybenzoate, has a molecular formula of $C_{23}H_{28}O_{12}$ and a molecular weight of 496.46 (Feng *et al.*, 2010). OPA is a monoterpene glycoside compound very similar to paeoniflorin and has been reported to alleviate advanced glycation end product-induced oxidative damage and inflammation in mesangial cells (Zhang *et al.*, 2013). In addition, a recent study revealed that OPA attenuated inflammatory effects *via* regulation of the toll-like receptor (TLR), extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases signaling pathways in LPS-stimulated RAW264.7 cells (Yoo *et al.*, 2018b). However, the effect of OPA on MI/R injury remains unclear and needs to be clarified and studied. Based on the data described above, we hypothesized that OPA could alleviate the MI/R injury. Therefore, MI/R injury models were designed to investigate the effects of OPA on the apoptosis of cardiomyocytes both, *in vivo* and *in vitro,* and studies of

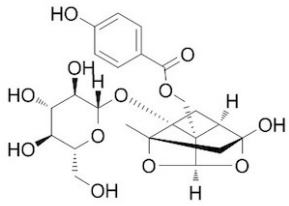

Figure 1. Chemical structure of oxypaeoniflorin (OPA).

the molecular mechanism focused on the Sirt1/Foxo1 signaling.

MATERIALS AND METHODS

Reagents. Oxypaeoniflorin (purity >98%) was purchased from Shanghai Hengfei Biological Technology Co., Ltd., and the molecular structure is shown in Fig. 1. The antibodies against Sirt1 (#ab12193), Bcl-2 (#ab59348), Bax (#ab32503), caspase-3 (#ab13847), cleaved caspase-3 (#ab49822), and GAPDH (#ab49822) and the HRP-conjugated goat anti-rabbit IgG (#ab6721) were all purchased from Abcam (Cambridge, UK). The antibodies against acetylated Foxo1 (Ac-Foxo1, #sc-49437) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dulbecco's modified Eagle's medium, penicillin, streptomycin, and fetal bovine serum were all purchased from Sigma Aldrich (St. Louis, MO, USA). An enzyme-linked immunosorbent assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). A hematoxylin and eosin staining kit, CCK-8 solution, BCA detection kit and ECL reagent were purchased from Beyotime Biotechnology (Shanghai, China). The Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Becton Dickinson (Rutherford, NJ, USA). The reagents used to make the ischemic buffer (137 mM NaCl, 12 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4.0 mM HEPES, 10.0 mM deoxyglucose, 0.75 mM sodium dithionate, and 20.0 mM lactate) were all of analytical or reagent grade.

Animals. A total of 30 C57BL/6 male mice, 6–8 weeks of age (body weight 20–25 g), were obtained from the Shanghai Slack Laboratory Animal Co., Ltd. (production license no. CXK(HU)2017-0015), raised in the animal experimental research center of ZheJiang Chinese Medical University, and used in this study. All the procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication NO. 85-23, revised in 1996). The protocol was reviewed and approved by the Ethical Committee of ZheJiang Chinese Medical University before the animal study (No. ZSLL-2017-059). All the mice were maintained in diurnal lighting conditions (12 h/12 h) at a constant temperature of 25 ± 2 °C for 7 days before the experiment.

***In vivo* MI/R model and treatment**. The MI/R injury model was established as previously described (Yu *et al.*, 2014; Guan *et al.*, 2016). In brief, a mouse model of MI/R injury was established by ligation of the left anterior descending coronary artery for 0.5 hours, followed by reperfusion for 2 hours. The mice in the sham operation group were not ligated. The mice were randomly assigned to the following groups (6 mice for each group): (1) Sham group; (2) MI/R group; (3) MI/R + OPA (10 mg/kg) group; (4) MI/R + OPA (20 mg/kg) group, and (5) MI/R + OPA (40 mg/kg) group. OPA was administered intragastrically at the concentrations described above (diluted in sterile saline containing less than 1% dimethyl sulfoxide (DMSO)) every day for 30 days before the surgery. Finally, blood samples from the sacrificed mice were collected for biochemical analysis, and the hearts of the sacrificed rats were collected for pathological and biochemical examination.

***In vitro* H/R cell model and treatment**. H9c2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA) with 1% penicillin/streptomycin and 10% fetal bovine serum (Sigma-Aldrich, USA). To establish the H/R injury model, the procedure was performed as previously described (Li *et al.*, 2017). Briefly, H9c2 cells were cultured in an ischemic buffer. The pH of the buffer was 6.5, and the cells were incubated for 2 hours in a humidified cell incubator containing 21% oxygen and 5% CO_2 at 37°C. Reperfusion was initiated by culturing the cells in normal medium for 4 hours under the above incubation conditions. Then, the cells were treated with OPA at different concentrations, and the experimental groups were as follows: (1) Control group; (2) H/R group; (3) H/R group + 0.1 μM OPA; (4) H/R group + 1 μM OPA; and (5) H/R group + 10 μM OPA. The cells were cultured for 8 hours in serum-free medium under the conditions described above before establishing the ischemia-reperfusion injury model.

Echocardiography. Two-dimensional echocardiography was performed using the VisualSonics Vevo 770 machine (VisualSonics, Toronto, ON, Canada) as previously described (Zhang *et al.*, 2018). In brief, the mice were anesthetized by inhalation of 1.5% isoflurane 72 hours after MI/R surgery, and motion (M)-mode echocardiographic images were used to determine the left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD). The ejection fraction (EF) and fractional shortening (FS) were calculated with the following equations:

$$EF\ (\%) = [(LVEDD^3 - LVESD^3)/LVEDD^3] \times 100$$

$$FS\ (\%) = [(LVEDD - LVESD)/LVEDD] \times 100$$

Detection of the myocardial enzyme levels in the serum. The mice were sacrificed after the peripheral blood was collected, and serum was separated to evaluate indicators of the heart muscle damage, including creatine kinase (CK-MB), cardiac troponin I (cTnI) and cardiac troponin T (cTnT), using an enzyme-linked immunosorbent assay kit (Thermo Fisher Scientific, USA).

Hematoxylin-eosin (H&E) staining. Two hours after MI/R surgery, the chests of mice were opened again. The hearts were separated from the aortic roots and washed with prechilled phosphate-buffered saline at 4°C, followed by fixation in 4% paraformaldehyde for 24 hours. Then, the hearts were embedded and cut into 4-μm sections by a paraffin slicing machine (Leica Microsystems, Germany). For H&E staining, the sections were deparaffinized in xylene, hydrated in serially diluted ethanol, and stained with a hematoxylin and eosin staining kit for 10 min. Representative photomicrographs were captured by a light microscope (Olympus Corp. Tokyo, Japan).

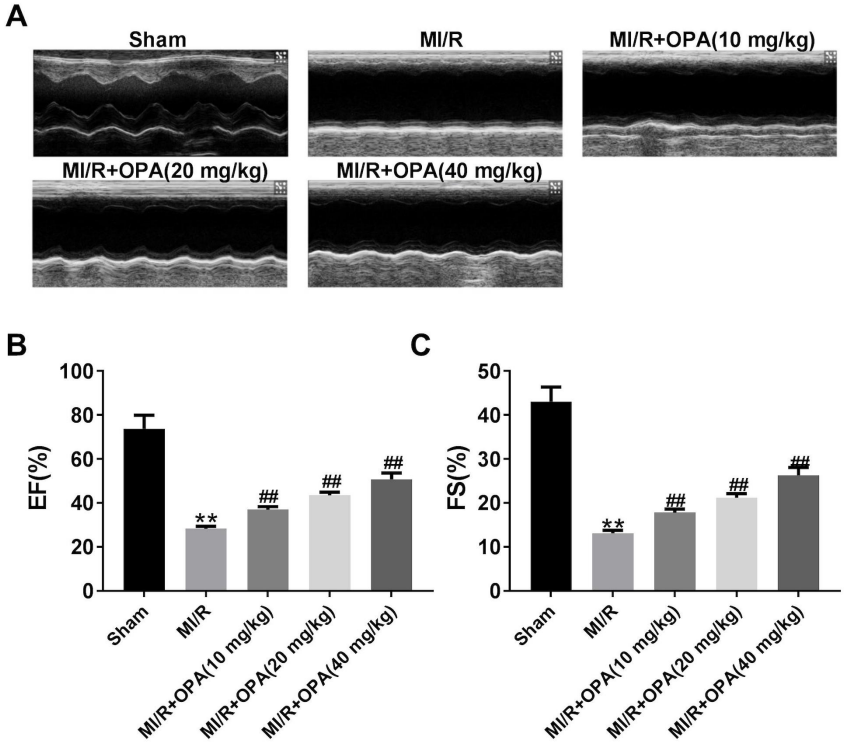

Figure 2. Effects of OPA on the cardiac functions in MI/R mice observed by echocardiographic assessment.
(**A**) Representative motion (M)-mode images obtained by echocardiography. (**B**) Ejection fraction (EF). (**C**) Fractional shortening (FS). **P*<0.05, ***P*<0.01 compared with the Sham group; #*P*<0.05, ##*P*<0.01 compared with the MI/R group.

Cell viability. Cultured H9c2 cells (1×10^4 per well) were plated into 96-well plates. Ten microliters of CCK-8 solution was added to each well after reperfusion, which was followed by incubation for 4 h; then, the absorbance was measured at 450 nm using a microplate reader.

Flow cytometry. The Annexin V-FITC/PI Apoptosis Detection Kit was used to quantify cell apoptosis. The cells were resuspended in 200 μL Annex binding buffer with 10 μL Annexin V-FITC and 5 μL PI, in the dark for 10 min at 25°C. Flow cytometry (Abcam, USA) was used to analyze the double-stained cells.

Western blot. The proteins were obtained from the tissues and cells, and the concentration was determined with a BCA detection kit. A total of 30 μg protein was separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, USA). After blocking in 5% skim milk for 1 h, the membranes were incubated in primary antibodies, such as anti-Sirt1 (1:2000), anti-Ac-FoxO1 (1:500), anti-Bcl-2 (1:800), anti-Bax (1:2000), anti-caspase-3 (1:500), anti-cleaved caspase-3 (1:500), and anti-GAPDH (1:2500), at 4°C overnight. Then, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (1:2000) for 45 min at room temperature. Finally, the bands were examined with an ECL reagent. The signals were analyzed *via* Image Lab™ Software (NIH Image, Bethesda, USA).

Statistical analyses. The GraphPad 7.0 software was used to analyze the data. All the experiments were repeated at least three independent times, and the data are expressed as the mean ± standard deviation (SD). Student's *t*-test and one-way ANOVA were used to compare the significance of the differences among experimental groups. (**P*<0.05, ***P*<0.01, ****P*<0.001), and *P*<0.05 were considered significant.

RESULTS

Effects of OPA on the cardiac functions in MI/R mice observed by echocardiographic assessment

To investigate the effect of OPA on the cardiac function of MI/R mice, the left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD) were measured by echocardiography to calculate the ejection fraction (EF) and fractional shortening (FS), which are indexes of LV systolic function. The results shown in Fig. 2A–C confirm that the mouse model of MI/R was successfully established, as shown by the following evidence. Compared to the mice in the Sham group, the mice in the MI/R group showed a decreased cardiac function, and the EF and FS of the MI/R group were decreased by approximately 40.48% and 29.98%, respectively (Fig. 2A–C). In addition, OPA (10, 20 and 40 mg/kg) observably reversed the abnormal EF and FS values in the mice subjected to MI/R injury in a dose-dependent manner (Fig. 2A–C). Specifically, compared with those in the MI/R group, the EF and FS in the MI/R+OPA (10 mg/kg) group increased by approximately 8.72% and 4.72%, the EF and FS in the MI/R+OPA (20 mg/kg) group increased by approximately 15.16% and 8.10%, and the EF and FS in the MI/R+OPA (40 mg/kg) group increased by approximately 22.55% and 13.21%, respectively. These data demonstrated that the MI/R injury model was suc-

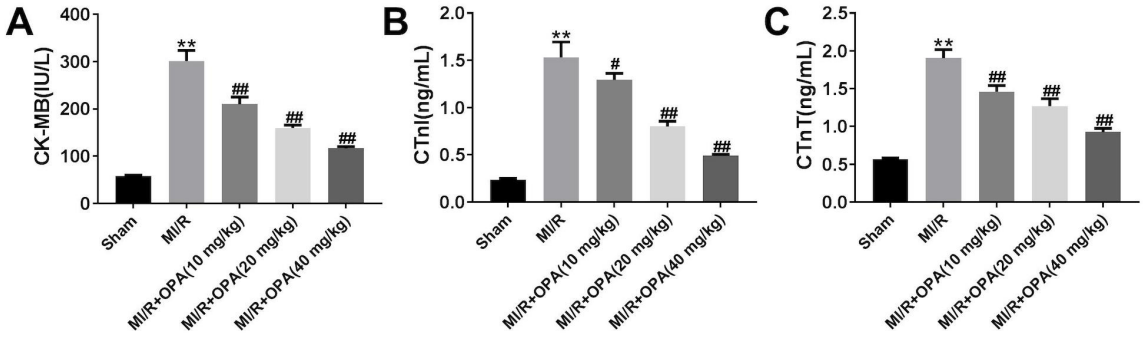

Figure 3. Effects of OPA on the myocardial enzyme markers in MI/R mice.
(**A**) Serum creatine kinase-MB (CK-MB) levels. (**B**) Serum cardiac troponin I (cTnI) levels. (**C**) Serum cardiac troponin T (cTnT) levels. **P*<0.05, ***P*<0.01 compared with the Sham group; #*P*<0.05, ##*P*<0.01 compared with the MI/R group.

cessfully established and that OPA could significantly improve cardiac function in the MI/R mice.

Effects of OPA on the myocardial enzyme markers in MI/R mice

To further explore the protective effects of OPA against MI/R injury, the levels of the myocardial enzyme CK-MB and the structural proteins cTnI and cTnT were evaluated in the serum of mice in different groups (Fig. 3A–C). Significantly increased levels of CK-MB, cTnI and cTnT were observed in the MI/R group compared to those in the Sham group. Compared with those in the Sham group, the levels of CK-MB, cTnI and cTnT in the MI/R group were significantly increased by 243.48 IU/L, 1.29 ng/mL and 1.34 ng/mL, respectively. OPA treatment dramatically reduced the MI/R-induced increase in the levels of CK-MB, cTnI and cTnT in a dose-dependent manner. Specifically, compared with those in the MI/R group, the levels of CK-MB, cTnI and cTnT in the MI/R+OPA (10 mg/kg) group decreased by approximately 91.25 IU/L, 0.24 ng/mL and 0.44 ng/mL, the levels of CK-MB, cTnI and cTnT in the MI/R+OPA (20 mg/kg) group decreased by approximately 141.66 IU/L, 0.73 ng/mL and 0.63 ng/mL, and the levels of CK-MB, cTnI and cTnT in the MI/R+OPA (20 mg/kg) group decreased by approximately 191.47 IU/L, 1.04 ng/mL and 0.98 ng/mL, respectively. These results suggested that OPA could ameliorate cardiac damage in the MI/R mice.

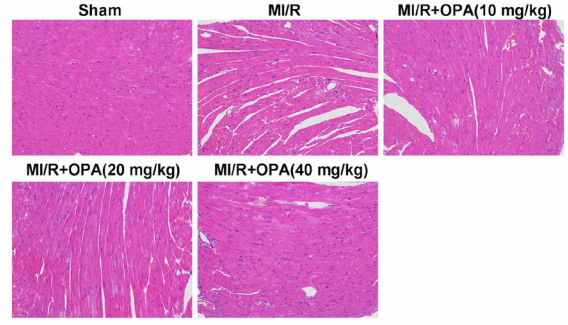

Figure 4. Effects of OPA on the histochemical alterations in MI/R mice.
Histopathological changes in mouse cardiac tissues observed by H&E staining (200×, bar=100 μm).

Effects of OPA on the histochemical alterations in MI/R mice

In the Sham group, the cardiomyocytes were well arranged, the myocardial fiber structure was normal, and no degeneration or myocardial necrosis was observed (Fig. 4). However, in contrast to the Sham group, the

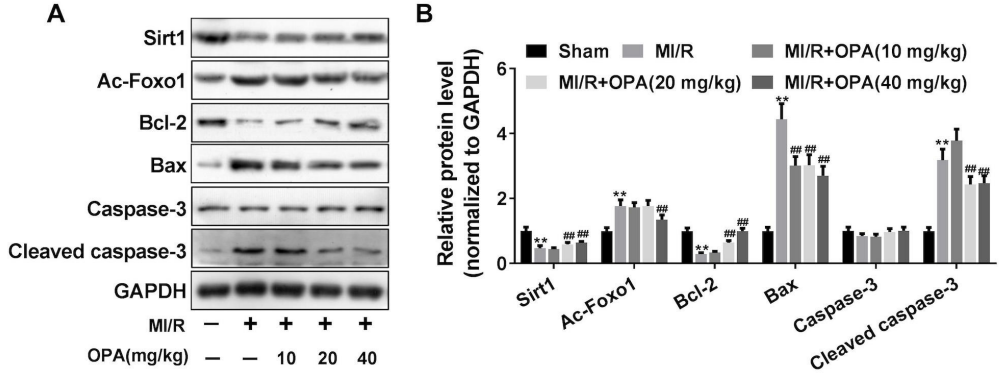

Figure 5. OPA activates the Sirt1/Foxo1 signaling pathway and inhibits the apoptosis of cardiomyocytes in MI/R mice.
(**A**) Representative images of the western blot results. (**B**) Quantitative densitometric analysis of the proteins. **P*<0.05, ***P*<0.01 compared with the Sham group; #*P*<0.05, ##*P*<0.01 compared with the MI/R group.

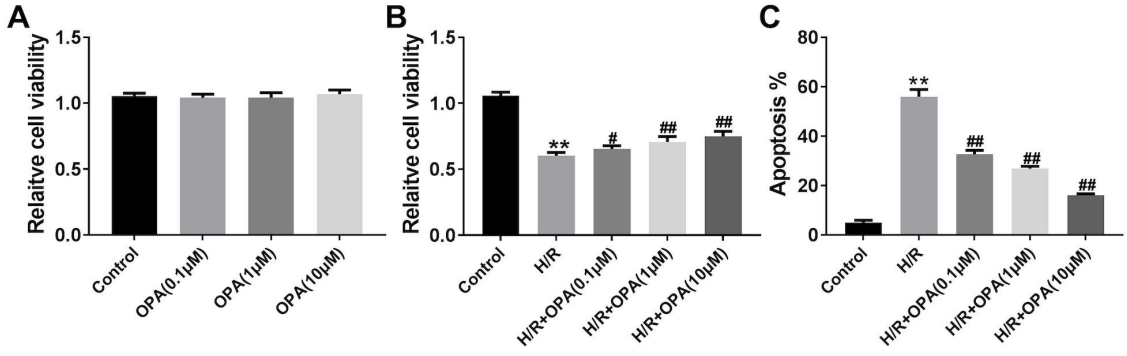

Figure 6. Effects of OPA on the viability and apoptosis of H/R–treated H9c2 cells.
(A) The cell viability of H9c2 cells without H/R treatment was measured by CCK-8 assay. (B) The cell viability of H/R–treated H9c2 cells was measured by CCK-8 assay. (C) Cell apoptosis was detected by flow cytometry assay. *P<0.05, **P<0.01 compared with the Control group; #P<0.05, ##P<0.01 compared with the H/R group.

MI/R group presented with ruptured myocardial fibers, karyopyknosis and fragmentation, and a large area of cardiac necrosis (Fig. 4). However, treatment with OPA significantly mitigated the MI/R-induced pathological alterations in a dose-dependent manner (Fig. 4). These data showed that OPA could protect cardiomyocytes from MI/R-induced necrosis.

OPA activates the Sirt1/Foxo1 signaling pathway and inhibits the apoptosis of cardiomyocytes in MI/R mice

Considering that the above results have clearly demonstrated that OPA can protect against the MI/R injury, we explored a potential mechanism that regulates this effect. We focused on the Sirt1/Foxo1-mediated apoptosis signaling pathway, which is related to the MI/R injury. Western blot assays (Fig. 5A–B) showed that Sirt1 expression was significantly decreased, but ac-Foxo1 expression was obviously increased in the MI/R group compared with the Sham group. Notably, these effects induced by MI/R were obviously reversed by OPA treatment; that is, Sirt1 expression significantly increased after OPA treatment, and ac-Foxo1 expression significantly decreased after OPA treatment. The detection of apoptosis-related proteins showed that compared with the mice subjected to MI/R alone, the mice subjected to MI/R and treated with OPA exhibited obviously decreased expression of Bax and cleaved caspase-3 and increased expression of Bcl-2, indicating that OPA could reduce the MI/R-induced apoptosis of cardiomyocytes. Therefore, OPA could activate the Sirt1/Foxo1 signaling pathway and inhibit the apoptosis of cardiomyocytes in MI/R mice.

Effects of OPA on the viability and apoptosis of H/R–treated H9c2 cells

An H/R injury model was established in H9c2 cells to confirm the effects of OPA on MI/R injury and the underlying mechanism *in vitro*. A CCK-8 assay was performed to evaluate cardiomyocyte viability in the different groups. As shown in Fig. 6A, OPA treatment had no significant effect on the viability of cardiomyocytes in which H/R was not induced. In Fig. 6B, compared to that of the control cells, the cell viability of the H/R-induced H9c2 cells was decreased by approximately 45.61%. Treatment with OPA markedly increased the H/R-mediated decrease in cell viability in a dose-dependent manner. Specifically, compared with that in the H/R group, cell viability in the H/R+OPA (0.1 μM) group, H/R+OPA (1 μM) group and H/R+OPA (10 μM) group were increased by approximately 5.05%, 10.41% and 14.53%, respectively. In addition, the flow cytometry results suggested that cell apoptosis in the H/R group was significantly higher than that in the Control group by 50.84%. Nevertheless, compared with that in the H/R group, the cell viability in the H/R+OPA (0.1 μM) group, H/R+OPA (1 μM) group and H/R+OPA (10 μM) group were decreased by approximately 23.26%, 29.05% and 40.50%, respectively (Fig. 6C). These results

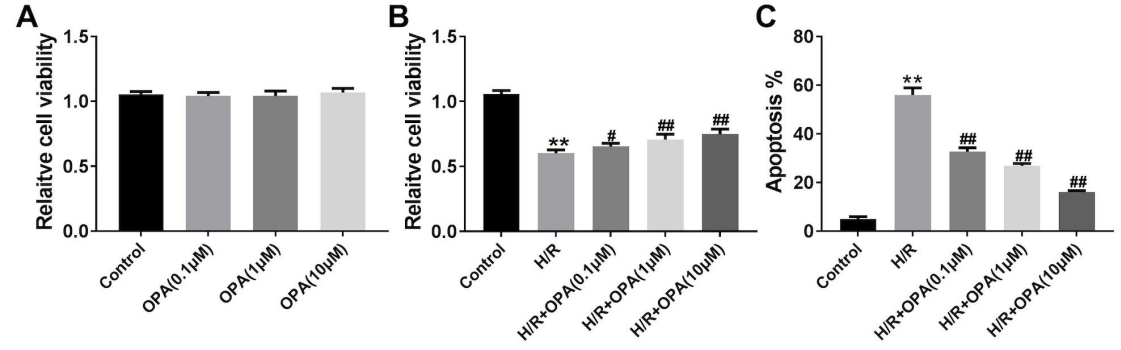

Figure 7. OPA activates the Sirt1/Foxo1 signaling pathway and inhibits the apoptosis of cardiomyocytes in H/R–induced H9c2 cells.
(**A**) Representative images of the western blot results. (**B**) Quantitative densitometric analysis of the proteins. *P<0.05, **P<0.01 compared with the Control group; #P<0.05, ##P<0.01 compared with the H/R group.

indicated that OPA could increase the cardiomyocyte viability and inhibit the cardiomyocyte apoptosis induced by H/R *in vitro*.

OPA activates the Sirt1/Foxo1 signaling pathway and inhibits apoptosis of cardiomyocytes in H/R–treated H9c2 cells

The effects of OPA on the expression levels of Sirt1, ac-Foxo1, Bcl2, Bax and cleaved Caspase3 in H/R–treated H9C2 cells were analyzed by western blot to elucidate the regulatory mechanism (Fig. 7A–B). Consistent with the *in vivo* results, the Sirt1 and Bcl-2 levels were notably reduced, but the ac-FOXO1, Bax and cleaved Caspase-3 levels were significantly increased in the H/R group compared with the Control group. However, when compared with those in the Control group, the Sirt1 and Bcl-2 levels were notably increased but the ac-FOXO1, Bax and cleaved Caspase-3 levels were significantly decreased in the H/R+OPA (0.1 μM) group, H/R+OPA (1 μM) group and H/R+OPA (10 μM) group, indicating that OPA could promote the Sirt1/Foxo1 pathway and inhibit apoptosis of cardiomyocytes in an *in vitro* MI/R injury model.

DISCUSSION

With the development of the social economy and changes in people's lifestyles, the risk factors for cardiovascular disease continue to increase. Tissue injury caused by myocardial ischemia is an important cause of fatal diseases and is commonly observed in clinical practice. Many mechanisms, including reactive oxygen species (ROS) production, inflammation, apoptosis, mitochondrial dysfunction, intracellular calcium overload, etc., are involved in the pathophysiological processes of myocardial ischemia/reperfusion injury (Song *et al.*, 2017; Mastrocola *et al.*, 2016; Jin *et al.*, 2018). Understanding the complexity of pathological reperfusion injury and its mechanisms may lead to the development of a promising therapeutic strategy for cardiac I/R injury. In our current study, we found that OPA protected mouse hearts (*in vivo*) and H9c2 cells (*in vitro*) against I/R injury by regulating the Sirt1/Foxo1-mediated apoptosis pathway.

The mouse model of MI/R was established by ligating the anterior descending branch of the left coronary artery, and the cardiac function, and hemodynamic and histopathological changes were evaluated. The EF and FS are important indicators of cardiac function (Bai *et al.*, 2017). In this study, treatment with OPA significantly improved the EF and FS of the MI/R mice. Necrosis is also one of the most common forms of cell death in the cardiac tissue after I/R injury. Damage to the myocardial cell membrane leads to the release of myocardial enzymes and proteins, including CK-MB, cTnI and cTnT, into the peripheral blood, and these enzymes and proteins are widely used as biomarkers of myocardial injury in clinical practice (Guo *et al.*, 2019). Therefore, the levels of CK-MB, cTnI and cTnT in peripheral blood can reflect the degree of myocardial ischemia-induced necrosis. Here, OPA treatment reduced the MI/R-induced increase in the levels of CK-MB, cTnI and cTnT, indicating that OPA mitigated MI/R injury. Furthermore, the clear pathological changes in the myocardial tissue observed by H&E staining also reflected the protective effect of OPA against MI/R injury.

Sirt1 is a member of the Sirtuin family. Sirtuins are class III histone deacetylated transferases that rely on nicotinamide adenine dinucleotide (NAD+) for deacetylation (Sundaresan *et al.*, 2011). Sirt1 can regulate many transcription factors by deacetylation to extensively regulate various processes, such as cell survival, apoptosis, growth, metabolism and aging (Higashida *et al.*, 2013). Mounting evidence has proven that Sirt1 is involved in MI/R injury (Ding *et al.*, 2015; Potenza *et al.*, 2019). In particular, Sirt1 regulates the downstream protein Foxo1 by deacetylation, which inhibits apoptotic pathways and protects against the MI/R-induced injury (Yang *et al.*, 2013; Yamamoto *et al.*, 2011; Yu *et al.*, 2016). Consistent with previous research, the present study found that Sirt1 expression was significantly downregulated in the MI/R group, which was accompanied by enhanced Ac-Foxo1 expression and increased cardiac apoptosis. Both, the *in vivo* and *in vitro* studies showed that OPA treatment promoted an obvious increase in Sirt1 expression and Foxo1 deacetylation. The activated Sirt1 pathway was also related to the increased expression of anti-apoptotic proteins (Bcl-2) and decreased expression of pro-apoptotic proteins (Bax and cleaved Caspase-3). Together, these data demonstrate that the myocardial protection achieved by OPA treatment is mediated by activating Sirt1 signaling, and OPA-induced Sirt1 activation can inhibit Foxo1 acetylation, thus promoting anti-apoptotic effects.

Based on the abundant evidence showing the protective effect of paeoniflorin (a structural analog of OPA) against MI/R injury (Chen *et al.*, 2018; Han *et al.*, 2016a; Wu *et al.*, 2019), and the reported inhibitory effects of OPA on oxidative damage and inflammation in mesangial cells and LPS-stimulated RAW264.7 cells (Yoo *et al.*, 2018a), we speculate that OPA may protect against the MI/R injury. Our experiments successfully verified the above hypothesis and elucidated the mechanism of action by which OPA protects against MI/R injury, namely, by regulating the Sirt1/Foxo1-mediated apoptosis signals. However, whether this mechanism affects apoptosis that is caused by oxidative damage, mitochondrial dysfunction or inflammation remains to be further studied.

CONCLUSION

Overall, our findings revealed for the first time that OPA plays a protective role in MI/R injury. More importantly, the Sirt1/Foxo1-mediated apoptotic pathway was found to play a critical role in regulating the cardioprotective effects of OPA. These results emphasize that OPA may serve as a novel agent for the treatment of MI/R injury in clinical cardiac surgery and ischemic heart diseases.

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Not applicable.

Funding

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available.

Ethics approval and consent to participate

All the procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication NO. 85-23, revised in 1996). The protocol was reviewed and approved by the Ethical Com-

mittee of ZheJiang Chinese Medical University before the animal study (No. ZSLL-2017-059).

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