

Baoyuan decoction alleviates myocardial infarction through the regulation of metabolic dysfunction and the mitochondria-dependent caspase-9/3 pathway

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Abstract

Objective: Baoyuan decoction (BYD) is a traditional Chinese formula with myocardial protection efficacy validated by modern pharmacological tests. The present study aimed to investigate the effect and mechanism of BYD on alleviating myocardial infarction (MI).

Methods: Nuclear magnetic resonance-based serum and urinary metabolomics were employed to explore the metabolic regulation effects of BYD in rats with MI induced by left anterior descending ligation. Oxygen-glucose deprivation/recovery (OGD/R) model in H9c2 cells and multiple molecular biology approaches were used to clarify the underlying action mechanisms of BYD.

Results: BYD treatment recovered the serum and urinary metabolite profiles of the MI rats toward normal metabolic status and significantly improved mitochondrial energy metabolism and apoptosis pathways perturbed by MI. Analysis of the molecular mechanism of BYD indicated that it suppressed OGD/R-induced H9c2 cell apoptosis in a concentration-dependent manner by inhibiting the mitochondria-dependent caspase-9/3-poly ADP-ribose polymerase pathway.

Conclusions: Our results demonstrate that BYD protects against myocardial apoptosis *via* the mitochondrial metabolic and apoptosis pathways. They also provide novel insights into the clinical application of BYD for the treatment of ischemic heart diseases.

Keywords: Baoyuan decoction, Caspase-9/3 pathway, Mechanisms, Metabolomics, Mitochondrial energy metabolism, Myocardial infarction

1 Introduction

Cardiovascular diseases (CVD) are major global death risk factors^[1]. Myocardial infarction (MI) is a severe form of CVD in which sudden blockage of a coronary artery, and subsequent myocardial ischemia, results in damage to the surrounding myocardium. Insufficient coronary blood flow and inadequate oxygen supply may induce serious ischemic cardiac muscle necrosis^[2–3]. Numerous drugs have been developed to prevent or treat MI, such as renin-

angiotensin-aldosterone system inhibitors, β -blockers, etc, unfortunately, their curative efficacy is unsatisfactory, MI still remains a major public health problem worldwide^[4]. Therefore, the quest for effective drugs to treat MI-related pathological injury of myocardial cells has become popular in medical research^[5].

It has been found that prolonged myocardial ischemic injury can induce myocardial cell apoptosis and lead to MI and heart failure^[6]. The mitochondrial apoptotic, death receptor, extrinsic or cytoplasmic, and endoplasmic reticulum apoptotic pathways are involved in cardiac ischemia^[7–8]. As the energy factories of the cell, mitochondria play vital roles in cell proliferation, differentiation, metabolism, and death^[9]. MI injury may induce mitochondrial dysfunction by differentially regulating the expression of mitochondria-related proteins including Bcl-2, Bax, Bad, Bim, and Bid^[10].

Traditional Chinese medicine (TCM) is a multi-component, multi-dimensional pharmacological system for the treatment of complex clinical syndromes and disorders^[11–12]. Various TCM combinations have displayed therapeutic efficacy in the clinical treatment of CVD. In general, TCMs have significant advantages such as few side effects and low cost^[13]. Baoyuan decoction (BYD) is a classic TCM formula consisting of astragalus root, ginseng, processed licorice, and cinnamon^[14]. It has been well established for the clinical treatment of various heart diseases with a long usage history since the Ming dynasty. Our previous studies demonstrated that BYD

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may exhibit cardioprotective effects by attenuating myocardial ischemia, hypertrophy, necrosis, and fibrosis and show more comprehensive protective activities compared with fosinopril and propranolol^[15–17]. However, the precise mechanisms of BYD have not been elucidated as it is a complex multi-component system.

Metabolomics is a powerful, unbiased approach toward the discovery of molecular perturbations. It is used to formulate biological hypotheses and is widely applicable for the interpretation of disease, drug action, and toxicology^[18]. Nuclear magnetic resonance (NMR)-based metabolomics is a robust, non-invasive technique that provides important prognostic and diagnostic information. It supports dynamic disease progression monitoring and elucidates drug mechanisms by measuring multiple metabolites in biological samples^[19].

In the present study, we proposed proton NMR (¹H NMR)-based urinary and serum metabolomics to analyze the effects of BYD on the metabolic changes that occur in MI model rats induced by left anterior descending (LAD) ligation. We also established a cellular oxygen-glucose deprivation/recovery (OGD/R) model to explore the cardioprotective effects and molecular mechanisms of BYD.

2 Materials and methods

2.1 Chemicals and reagents

K₂HPO₄, NaN₃, D₂O, KOH, and 3-(trimethylsilyl) propionic-2,2,3,3-*d*₄ acid sodium salt (TSP-*d*₄) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM, minimum essential) and fetal bovine serum (FBS) were acquired from HyClone Laboratories (Logan, UT, USA). A lactate dehydrogenase (LDH) activity assay kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Curcumin was procured from J&K (Beijing, China). Hoechst 33258 was purchased from Leagene Biotech (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-phenyltetrazolium bromide (MTT) and Rhodamine 123 were acquired from Sigma-Aldrich Corp. (St. Louis, MO, USA). Acridine orange (AO) and ethidium bromide (EB) were obtained from Solarbio Science & Technology (Beijing, China). Primary antibodies against Bax, Bcl-2, caspase-3, cleaved caspase-3, caspase-9, poly ADP-ribose polymerase (PARP), and GAPDH were procured from Cell Signaling Technology (Danvers, MA, USA). Western chemiluminescent horseradish peroxidase (HRP) substrate was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Strata-X SPE columns were acquired from Phenomenex (Torrance, CA, USA). The 0.22 μm filters were obtained from Millipore Corp (Tullagreen, Carrigtwohill, County Cork, Ireland). All other reagents used were commercially available and of the highest purity.

2.2 BYD preparation

All raw herbs in BYD were purchased from Anguo TCM Market (Hebei, China) and authenticated by Professor Pengfei Tu to be the roots of *Astragalus membranaceus* (Fisch.) Bunge var. *mongolicus* (Bunge) Hsiao, *Panax ginseng* C. A. Mey., prepared *Glycyrrhiza uralensis* Fisch., and the bark of *Cinnamomum cassia* Presl. according to the Chinese Pharmacopoeia (Chinese Pharmacopoeia

Commission, 2020). BYD preparation was described in a previous report^[16]. Briefly, *A. membranaceus* var. *mongolicus* (15 kg), *P. ginseng* (5 kg), prepared *G. uralensis* (5 kg), and *C. cassia* (2.5 kg) were mixed, soaked in tenfold volume water overnight, and refluxed thrice for 2 h each time. The solutions were combined, evaporated *in vacuo* (TAITEC, Osaka, Japan), and mixed with sufficient 95% (v/v) ethanol to a final concentration of 65% (v/v) ethanol, and left to stand for 2 h. The supernatant was freeze-dried (Savant SpeedVac, Thermo Scientific, Massachusetts, USA) into lyophilized BYD powders (8.5 kg) for use in the subsequent experiments.

2.3 Animal study

Serum and urine samples were collected from recent experimental research for the metabolomics study^[20]. All animal studies followed the relevant national legislation and were approved by the Animal Care Committee of Beijing University of Chinese Medicine (BUCM-4-2018001201-1014). All animal experiments were performed in accordance with the guidelines on the humane use and care of laboratory animals for biomedical research published by the National Institutes of Health, Sham, model, and BYD groups (*n*=8) were assigned. The MI model was produced in rats by ligating the LAD aorta and promoting cardiac ischemia. The sham-operated rats underwent the same surgical procedure but without aortic ligation. The rats in the BYD treatment group were administered BYD at 2.57 g/kg. The rats in the model and sham groups were administered 0.9% (w/v) physiological saline. The drug and vehicle were orally administered once daily for 4 consecutive weeks. After the final treatment, all rats fasted for 12 h and their urine was collected from metabolic cages. The samples were centrifuged at 3,000 rpm and 4°C for 5 min to remove particulate contaminants. The supernatants were collected in clean, dry tubes. Serum samples were collected from the abdominal aortae and centrifuged at 4,000 rpm and 4°C for 10 min. The supernatants were collected in clean, dry tubes. All samples were frozen at –80°C until subsequent analysis.

2.4 Serum and urinary sample preparation and NMR spectroscopy

Serum samples were prepared by mixing 150 μL thawed serum with 400 μL saline solution (0.9% NaCl, 50% D₂O, and 0.1% TSP; pH 7.4). The mixtures were homogenized by vortexing for 30 s and centrifuged at 14,000 g and 4°C for 10 min. A 500 μL aliquot of each serum supernatant was subsequently transferred to a 5 mm NMR tube. Each 400 μL thawed urine sample was mixed with 200 μL of 1.5 M KH₂PO₄ buffer (0.05% NaN₃, 100% D₂O, and 0.1% TSP; pH 7.4) and centrifuged at 16,000 g and 4°C for 10 min. A 500 μL aliquot of each urinary supernatant was transferred to a 5 mm NMR tube.

All NMR spectra were acquired at 300 K on a 500 MHz NMR spectrometer system (Varian, Palo Alto, CA, USA). The 90° pulse length was adjusted to 10 μs. The 1D ¹H NMR spectra of the serum samples were acquired using a water-suppressed Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. A total of 128 transients were collected by irradiation during a 4 s relaxation delay and 80 ms mixing time (32 kb data points; 16 ppm spectral width). The 1D ¹H NMR spectra of the urine samples were

recorded with a nuclear Overhauser enhancement spectroscopy (NOESY) pulse sequence and water suppression during relaxation delay (4s) and mixing time (100ms). Sixty-four transients were collected into 32 kb data points over a 16-ppm spectral width. All ^1H NMR spectra for the serum and urinary samples were manually phased, baseline-corrected, and calibrated to TSP (δ 0.0) in MestReNova 6.1.1 (Mestrelab Research S.L., La Coruña, Spain). Resonances were assigned according to the Human Metabolome Database (<http://www.hmdb.ca/>), the Madison Metabolomics Consortium Database (<http://mmcd.nmr.fam.wisc.edu/>), and the chemical shift data of chemical standards. Standard 2D TOCSY, HSQC, and HMBC assays were performed to assign metabolite resonances to the selected samples.

2.5 Multivariate statistical analysis of the metabolomic data

The δ 4.68–5.19 resonance regions of the serum samples were removed to eliminate the influence of imperfect water saturation. The regions containing water (δ 4.67–5.20) and urea (δ 5.52–6.00) resonances were removed from the urine samples. Adaptive-intelligent binning (AI-binning) in the MVAPACK toolbox was used for ^1H NMR spectral binning. Pareto-scaled NMR matrix bins were submitted for MVA with SIMCA-P 14.1 (Umetrics, Umeå, Sweden). The quality of the MVA models was controlled by introducing R^2 and Q^2 values. An unsupervised principal component analysis (PCA) was used to identify clustering trends among the various groups. A supervised orthogonal partial least squares discriminate analysis (OPLS-DA) was performed to discriminate classes and characterize alterations. CV-ANOVA P values were calculated to verify OPLS-DA model reliability. S -plot was used to identify differentially altered metabolites among groups based on the NMR-acquired metabolomic data. The significance of the difference among groups in terms of these metabolic alterations was evaluated by the Student t test ($P < 0.05$). Heatmap cluster analyses of the alterations were plotted with TBtools v. 0.6738.

2.6 OGD/R model establishment and treatment

H9c2 rat myocardial cells were purchased from the Institute of Biochemistry and Cell Biology, Shanghai, China. Cultured in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated FBS, penicillin G (100 U/mL), and streptomycin (100 mg/mL) under a 5% $\text{CO}_2/95\%$ air (v/v) atmosphere at 37°C and 90% RH. The H9c2 rat myocardial cells were seeded in culture dishes or plates at $1.0 \times 10^5/\text{mL}$ before each experiment. The culture medium was removed and replaced with glucose-free DMEM and the cells were incubated under a 5% $\text{CO}_2/95\%$ N_2 (v/v) atmosphere at 37°C for 4 h. Freshly lyophilized BYD was mixed with deionized water to prepare a standard 10 mg/mL stock solution and sterilized by passing through a $0.22 \mu\text{m}$ filter (Merck Millipore Ltd., Tullagreen, Carrigtwohill, County Cork, Ireland). The BYD filtrate was diluted to the desired concentrations with DMEM before the experiment. The glucose-free culture medium was removed and replaced for 24 h with DMEM containing BYD. The cells were cultured under a 5% $\text{CO}_2/95\%$ air (v/v) atmosphere at 37°C for 24 h to induce OGD reperfusion. The control group was not subjected to OGD/R.

2.7 MTT assay

Cell viability was measured by the MTT colorimetric assay^[20]. Supernatants from the treated cultures were removed and replaced with medium containing 0.5 mg/mL MTT and the cells were cultured at 37°C for 4 h. The supernatants were removed and 100 μL dimethyl sulfoxide was added to each well for 5 min. Absorbance was measured in a microplate reader (Tecan, Crailsheim, Germany) at 570 nm. Cell viability was expressed as the mean % absorbance of the cells treated with drugs *vs.* that of the control group. The relative viability of the control group was set to 100%. Each experiment was performed five times.

2.8 LDH assay

Cell membrane integrity was assessed using a LDH activity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Supernatants from the treated cells were collected and the LDH release reagent was added. The mixtures were then incubated under a 5% CO_2 atmosphere at 37°C for 1 h and centrifuged at 1,300 rpm for 5 min. Then, 120 μL supernatant was added to each well in a 96 well plate. The samples were incubated in the dark at 37°C for 30 min. The absorbance was measured in an enzyme-linked immunosorbent assay (ELISA) reader (Tecan, Crailsheim, Germany) at 450 nm.

2.9 Mitochondrial membrane potential (MMP) assay

The MMP assay was performed with Rhodamine 123 staining as previously described^[21]. Briefly, H9c2 cells were cultured in 24 well plates at $5.0 \times 10^4/\text{well}$. After OGD/R treatment, the cells were washed thrice with phosphate-buffered saline (PBS) and incubated with 1 μM Rhodamine 123 in the dark at 37°C for 30 min. The cells were then washed thrice with PBS and the images were captured under a fluorescence microscope (IX73, Olympus, Tokyo, Japan) at 488 nm excitation, 525 nm emission, and 200 magnification. The relative MMP value for each group was calculated by comparing it against the control group. At least 500 cells were counted per group.

2.10 Transmission electron microscopy (TEM) assay

Cells were prepared in ultrathin sections as previously described^[22]. Briefly, the cells were collected and perfused with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer at room temperature for 2 h. The cells were then washed with phosphate buffer for 30 min and post-fixed with 1% (w/v) osmium tetroxide for 1 h. The cells were then dehydrated with ethanol, embedded in Spurr resin, and prepared as ultrathin sections using LEICA CM1950 (Leica biosystems, Wetzlar, German) for examination under TEM at 200 magnification (JEM1400, Tokyo, Japan).

2.11 Hoechst 33258 staining

Treated cells were fixed with 4% (v/v) paraformaldehyde for 30 min, washed thrice with PBS, and stained with Hoechst 33258 in the dark at 37°C for 30 min. Fluorescent staining was examined by fluorescence microscopy (IX73, Olympus, Tokyo, Japan) at 200 magnification. Apoptotic cells exhibited bright fluorescence, chromatin condensation, and nuclear and cytoplasm shrinkage. At least 500

cells were counted per group and each assay was performed in triplicate. The percentages of apoptotic cells were expressed as mean $\pm$ SD and the apoptosis ratios were calculated from the proportion of apoptotic cells.

2.12 AO/EB staining

The treated cells were incubated with 1 mL PBS containing 20 μ L/well AO/EB. Equal volumes of 100 μ g/mL AO and 100 μ g/mL EB were combined and incubated in the dark at room temperature for 2 min. Fluorescence images were captured under a fluorescence microscope (IX73, Olympus, Japan) at 200 magnification^[23]. At least 500 cells were counted per group within 20 min. Each assay was conducted in triplicate. The percentages of apoptotic cells were reported as mean $\pm$ SD and the apoptosis ratios were calculated from the proportion of apoptotic cells.

2.13 Western Blot (WB) analysis

WB was performed as previously described^[24]. The treated H9c2 cells were collected and lysed in radio-immunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA) plus protease inhibitor cocktail (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's instructions. Equal amounts of protein lysate (20 μ g) were separated by 6%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free milk powder and then primary antibodies (1:500 dilution) were added to them. They were incubated at room temperature for 2 h and with anti-rabbit IgG conjugated with HRP (1:1,000 dilution) at room temperature for 1 h. They were then washed thrice with PBS plus Tween-20 and visualized with an Immobilon Western chemiluminescent HRP substrate. The relative optical density of each blot was determined using a Kodak digital imaging system (Gel Logic 2200Pro; Kodak, Tokyo, Japan). This assay was normalized to GAPDH. The results were analyzed with Gel-Pro Analyzer 4.0 (Sigma-Aldrich Corp., St. Louis, MO, USA) and the target protein: GAPDH OD ratio was obtained.

2.14 Statistical analysis

All data are presented as mean $\pm$ SD of ≥ 3 independent experiments. Data were analyzed with SPSS 26.0 (SPSS Inc., Chicago, USA)^[25]. Pairwise group comparisons were made with Student *t* test. Comparisons between treatments were performed by one-way ANOVA and Tukey multiple comparison post-test. *P* < 0.05 was considered statistically significant.

3 Results

3.1 BYD ameliorated MI-induced metabolic changes

Our report indicated that BYD significantly improved echocardiographic, pathological, and biochemical perturbations in LAD aortic ligation induced-MI rats^[20]. To clarify the effects of BYD on the metabolic profiles of serum and urinary samples, an untargeted metabolome analysis was conducted using ¹H NMR. Typical ¹H NMR CPMG spectra for the serum and ¹H NMR NOESY spectra for the

urine are depicted in Supplementary Figure S1, <http://links.lww.com/AHM/A2>. PCA-based score plots were constructed to identify differences in the serum and urinary metabolite profiles among the sham, model, and BYD groups and investigate the therapeutic efficacy of BYD against MI. As shown in Supplementary Figure S1A and S1B, <http://links.lww.com/AHM/A2>, points for the sham and BYD groups were clustered separately from those of the model group. Hence, BYD had strong efficacy according to the MI-induced serum and urinary metabolic profiles. Moreover, the PCA score plot classification was satisfactory ($R^2 = 0.64$, $Q^2 = 0.57$ for serum samples; $R^2 = 0.58$, $Q^2 = 0.52$ for urine samples).

3.2 BYD recovered MI-induced metabolic changes

The unsupervised PCA revealed that BYD treatment significantly reversed the perturbations in the serum and urine metabolic profiles caused by MI (Figure 1A and 1B). Then, a supervised OPLS-DA-based approach was employed to maximize differences among groups and screen for the key metabolic alterations associated with MI. The OPLS-DA models were significant as they had high goodness-of-fit parameter values ($R^2Y = 0.84$, $Q^2 = 0.93$ for serum samples; $R^2Y = 0.76$, $Q^2Y = 0.84$ for urine samples) and significant CV-ANOVA *P* values (2.43×10^{-6} for serum samples and 1.66×10^{-5} for urine samples). Excellent segregation was also achieved in the OPLS-DA score plots of the sham *vs.* model groups (Figure 1C and 1D). Discriminatory metabolites between subgroups were identified with OPLS-DA S-plots (Figure 1E and 1F) and are summarized in Figure 1G and Supplementary Table S1, <http://links.lww.com/AHM/A2>.

Compared with the sham group, the model group showed higher serum fumarate, citrate, pyruvate, lactate, glutamate, and succinate levels and lower serum tryptophan, glutamine, and methionine levels. The model group had higher urinary hypoxanthine, hippurate, glutamate, arginine, lactate, and citrate levels and lower urinary uridine, histidine, and glutamine levels. Except for urinary arginine and hippurate, all other MI-modified metabolites were significantly reversed after four consecutive weeks of BYD treatment. A network enrichment analysis revealed that these metabolic changes were associated mainly with abnormal mitochondrial energy metabolism and oxidative damage. These responses might have contributed to the apoptotic signals (Figure 2).

3.3 BYD improved cell viability following OGD/R-induced injury

The MTT assay was used to evaluate cell viability. BYD was not toxic to H9c2 cells (Figure 3A) (*P* > 0.05; data not shown). H9c2 cells were subjected to OGD for 4 h followed by reoxygenation for 24 h to simulate I/R injury. BYD at 50, 100, 200, 400, 600, 800 or 1,000 μ g/mL was added to the culture medium immediately before reoxygenation. Figure 3B shows that cell viability decreased after the OGD/R insult ($50.65\% \pm 13.19\%$ of control; *P* < 0.01 *vs.* control). In contrast, cell viability increased after treatment with >100 μ g/mL BYD. LDH assays confirmed the protection of H9c2 cells by BYD against OGD/R induction. BYD reduced LDH leakage in a dose-dependent manner (Figure 3C). Hence, 100, 200 and 400 μ g/mL BYD were selected for use in the subsequent studies.

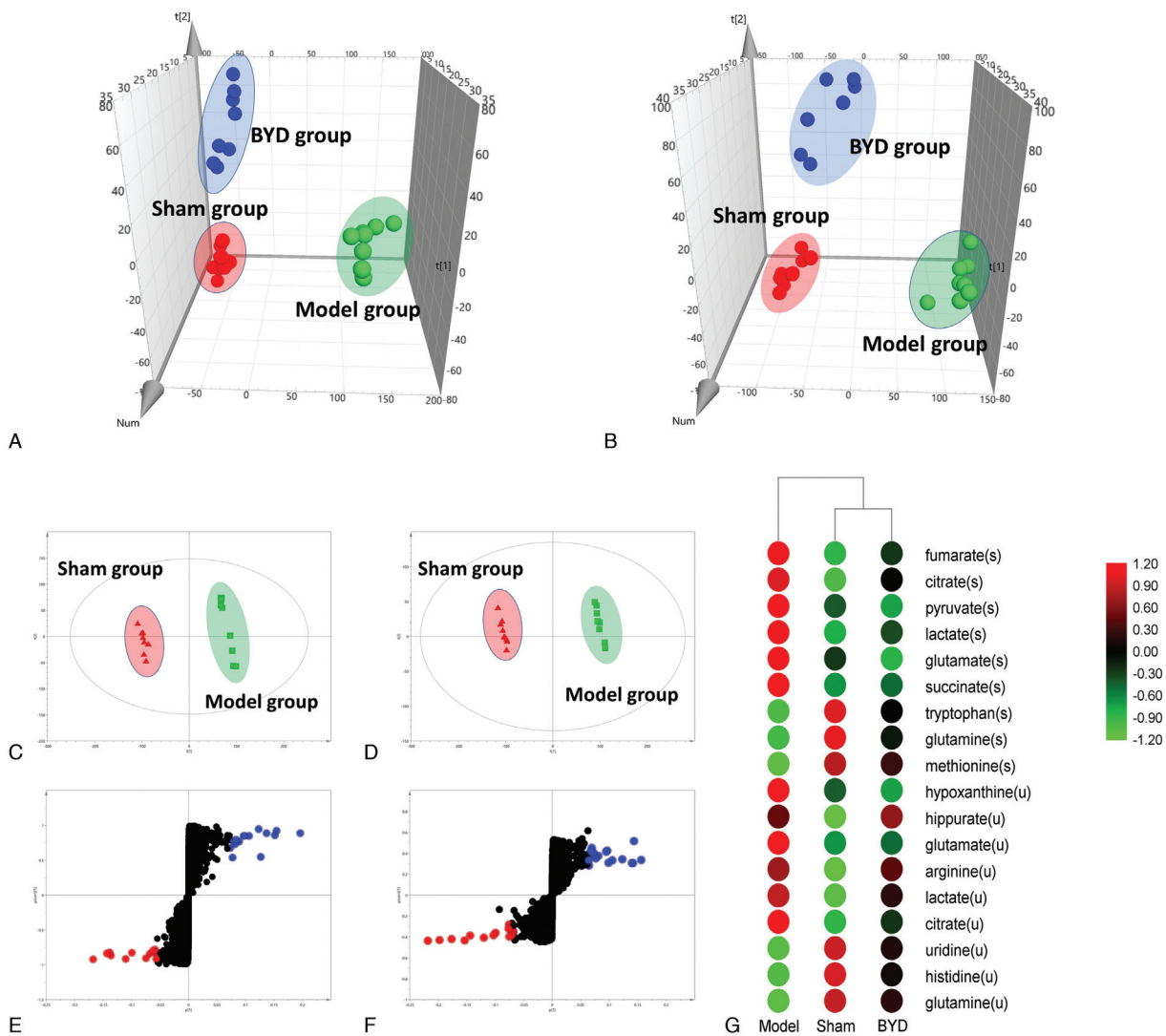


Figure 1. Multivariate statistical analysis discriminating metabolic signatures among various groups. (A) and (B) PCA score plots depicting effects of BYD treatment on serum (A) and urine (B) metabolome changes in MI rats. (C) and (D) OPLS-DA score scatterplot discriminating clusters of metabolome datasets between sham and model groups (C, serum; D, urine). (E) and (F) S-plots of OPLS-DA model identifying altered metabolites in the model vs. sham rats (E, serum; F, urine). Metabolite *P* (corr) value increases with distance from the origin. Red and blue points represent higher and lower levels in the model vs. sham group, respectively. (G) Heatmap cluster analysis with TBtools v. 0.6738 visualizing influences of BYD treatment on changes in key MI-induced metabolites. BYD: Baoyuan decoction; MI: myocardial infarction; OPLS-DA: orthogonal partial least squares discriminate analysis, PCA: principal component analysis.

3.4 BYD inhibited OGD/R-induced H9c2 cell apoptosis according to the Hoechst 33258 assay

Hoechst 33258 is a membrane-permeable blue fluorescent dye. It is sensitive to DNA conformations. Figure 4A shows that cytoplasmic condensation and cell shrinkage were markedly increased in the OGD/R-induced H9c2 cells. In a concentration-dependent manner, 100, 200, and 400 $\mu\text{g/mL}$ BYD decreased nuclear condensation and mitigated fragmentation induced by OGD/R. These results suggest that BYD inhibits OGD/R-induced cardiomyocyte apoptosis.

3.5 BYD inhibited OGD/R-induced apoptosis in H9c2 cells according to the AO/EB assay

An AO/EB assay was conducted to assess the nuclear morphology of apoptotic cells. Figure 4B shows uniform green fluorescence in the normal and large nuclei of the

control group. In contrast, the OGD/R group presented with orange/red fluorescence. Partial green and orange/red fluorescence were observed in the BYD groups. Green fluorescence gradually intensified with BYD concentration. In contrast, orange/red fluorescence gradually decreased with increasing BYD concentration. AO/EB staining confirmed that OGD/R treatment markedly induced apoptosis in H9c2 cells whereas BYD significantly inhibited OGD/R-induced cardiomyocyte apoptosis in a concentration-dependent manner.

3.6 BYD stabilized the MMP of H9c2 cells

To determine whether OGD/R affects mitochondrial apoptosis, we measured the expression levels of mitochondria-mediated apoptotic proteins, detected the MMP, and observed mitochondrial ultrastructures. Bcl-2 and Bax are major mitochondrial apoptosis-related proteins. Figure 5A shows that anti-apoptotic Bcl-2 was down-

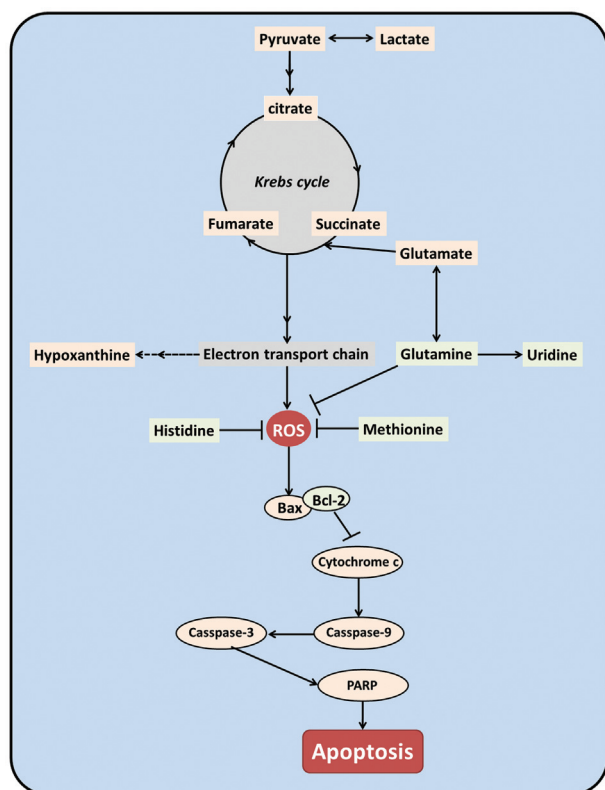


Figure 2. Cardioprotective effect and molecular mechanism of BYD against MI injury. Rectangle and oval represent MI-altered metabolites and proteins, respectively. Beige and green colors indicate a decrease and increase after BYD treatment, respectively. BYD: Baoyuan decoction; MI: myocardial infarction.

regulated and proapoptotic Bax was upregulated after OGD/R treatment. However, Bcl-2 was gradually upregulated and Bax was gradually downregulated with increasing BYD concentration. We used Rhodamine 123 staining to identify variations in MMP among H9c2 cells.

Figure 5B shows uniform green fluorescence in the control group. Nevertheless, OGD/R substantially attenuated it. Green fluorescence gradually increased with BYD concentration. TEM was employed to observe mitochondrial ultrastructure. Figure 5C shows that the mitochondria in the control group appeared normal whereas those in the OGD/R group were swollen and dissociated. The number of normal mitochondria gradually increased and the numbers of swollen mitochondria gradually decreased with increasing BYD concentration. The foregoing results indicated that BYD protected mitochondrial membrane integrity and mitigated mitochondrial injury.

3.7 BYD inhibited the mitochondria-dependent caspase apoptosis signal against OGD/R-induced injury

To elucidate the mechanisms by which OGD/R induces apoptosis in H9c2 cells, we measured the caspase-9, caspase-3, cleaved caspase-3, and PARP levels in the mitochondria-driven apoptosis pathway. Figure 6A and 6B shows that caspase-9 and caspase-3 were down-regulated in the OGD/R group. After BYD administration, however, both caspases were gradually upregulated in a manner dependent on BYD concentration. Moreover, the increase in cleaved caspase-3 level induced by OGD/R was reversed by the BYD treatment. PARP is a substrate for caspase cascades and a key signaling nuclear protein triggering DNA repair. Figure 6C shows that BYD increased OGD/R-induced PARP expression in a dose-dependent manner. The foregoing results suggested that BYD inhibits OGD/R-induced cardiomyocyte apoptosis by activating the mitochondria-dependent caspase-9/3 apoptosis pathway (Figure 2).

4 Discussion

BYD is a classic TCM formula that has been widely used in clinical trials for the treatment of coronary artery disease. Our findings demonstrated that BYD significantly regulates MI-induced metabolic changes associated with mitochondrial damage and apoptosis. Previous studies

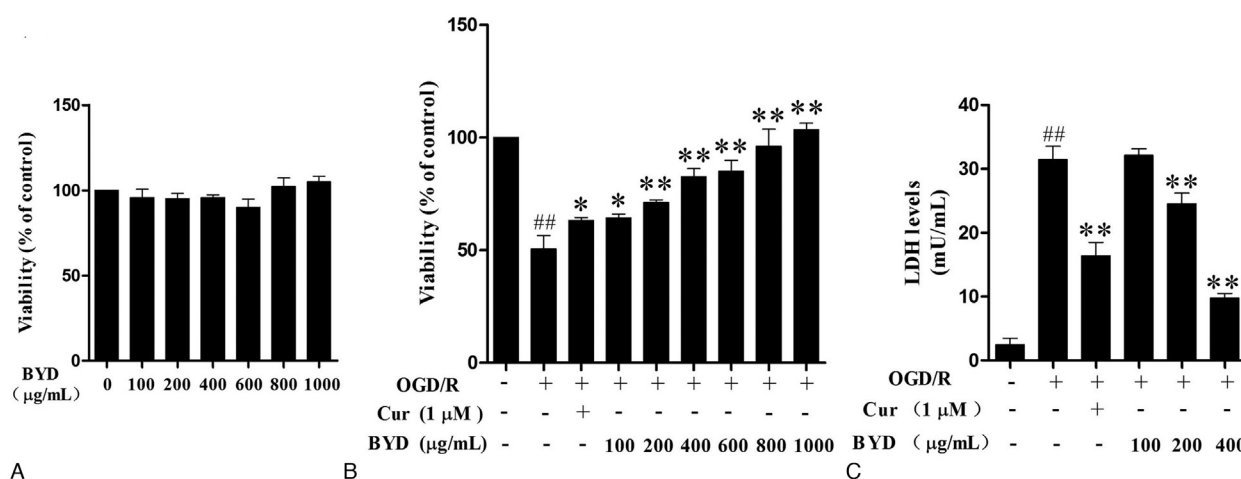


Figure 3. BYD enhances the viability of cells injured by OGD/R. (A) BYD does not induce cell toxicity. (B) BYD attenuates OGD/R-induced H9c2 cardiomyocyte injury. (C) BYD decreases LDH in OGD-induced H9c2 injury. Curcumin is a positive control. Values are mean $\pm$ SD of three independent experiments. $^{##}P < 0.01$ relative to the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the OGD/R treatment group. BYD: Baoyuan decoction; MI: myocardial infarction; LDH: lactate dehydrogenase; OGD/R: oxygen-glucose deprivation/recovery.

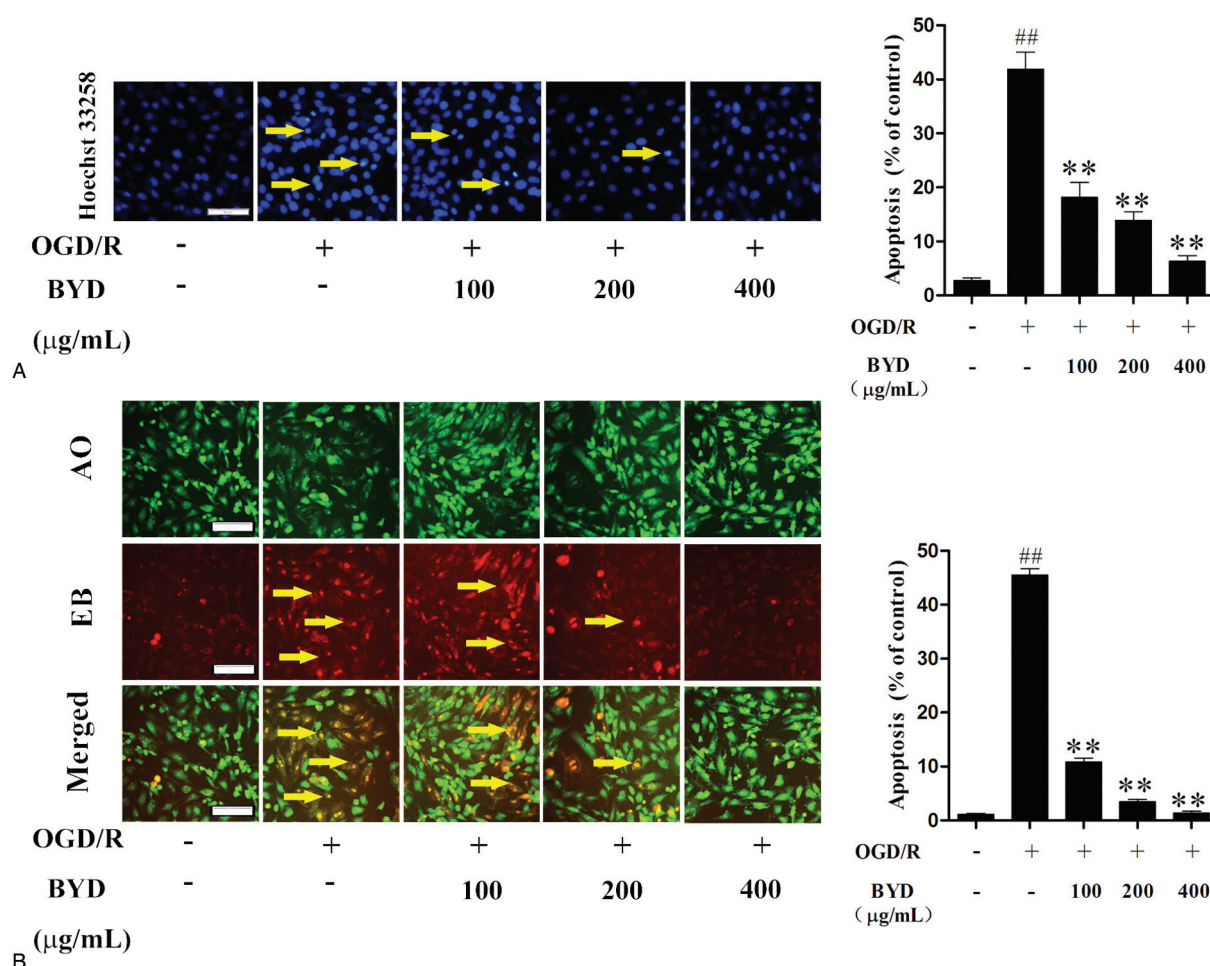


Figure 4. BYD attenuates OGD/R-induced H9c2 cardiomyocyte apoptosis. Cells with or without BYD treatment were subjected to OGD for 5 h followed by 24 h recovery. Apoptosis was determined with Hoechst 33258 (A) (bar=100 μm) and AO/EB staining assay (B) (bar=100 μm). Arrowheads in photographs indicate apoptotic nuclei. Apoptosis rates were calculated separately. Values are mean ± SD of three independent experiments. ^{##} $P < 0.01$ relative to control group. ^{*} $P < 0.05$, ^{**} $P < 0.01$ relative to OGD/R treatment group. AO/EB: acridine orange/ethidium bromide; BYD: Baoyuan decoction; OGD/R: oxygen-glucose deprivation/recovery.

revealed that MI dysregulated energy metabolism and overproduced reactive oxygen species (ROS) in mitochondria^[8,21]. Persistent dysfunction of mitochondrial energy also triggered cardiomyocyte apoptosis by reducing the energy supply, releasing Bax and cytochrome *c*, and activating caspases *via* massive ROS accumulation^[22–23]. The present study indicated that BYD ameliorates metabolic intermediates in the Krebs cycle and glycolysis such as citrate, succinate, pyruvate, and lactate. Hence, BYD might play a significant role in regulating mitochondrial energy metabolism^[24]. The present study also demonstrated that BYD increased endogenous ROS scavengers such as glutamine and methionine^[25]. Taken together, our results disclosed that BYD may functionally impede mitochondrial energy metabolism and oxidative stress and could have anti-apoptosis efficacy in MI treatment.

We established an I/R model to determine whether BYD protects against cardiomyocyte apoptosis. BYD significantly protected H9c2 cells against OGD/R-induced apoptosis possibly by increasing MMP and blocking the mitochondria-related caspase-9/3 apoptosis pathway. Numerous studies showed that various factors such as hyperlipidemia, myocardial I/R injury, diabetes, hypertension, inflammation, and aging could lead to CVD^[26].

MI is the major factor contributing to death and disability associated with CVD. Several studies demonstrated that prolonged cardiac ischemia could also culminate in various pathophysiological changes^[27]. The foregoing results clarified the potential mechanism of BYD in the treatment of the myocardial ischemic injury.

Our findings suggested that BYD was not cytotoxic and did not reduce H9c2 cell viability. However, the viability of H9c2 cells challenged with OGD/R was significantly increased in response to BYD treatment. Hoechst 33258 and AO/EB staining revealed fewer apoptotic cells in the BYD treatment groups than the OGD/R-induced model group. Hence, BYD might protect H9c2 cells against OGD/R-induced apoptosis. Numerous studies have reported that MI injury induces mitochondrial dysfunction and cellular apoptosis. Here, we investigated the effects of BYD on the mitochondrial-dependent apoptosis pathway.

It was previously shown that the proapoptotic protein Bax induces mitochondrial dysfunction and cellular apoptosis *via* a mitochondrial-dependent caspase apoptotic cascade pathway^[28]. In contrast, the anti-apoptotic protein Bcl-2 maintains mitochondrial integrity and prevents the release of cytochrome *c* from mitochondria. A WB assay showed that BYD significantly upregulated

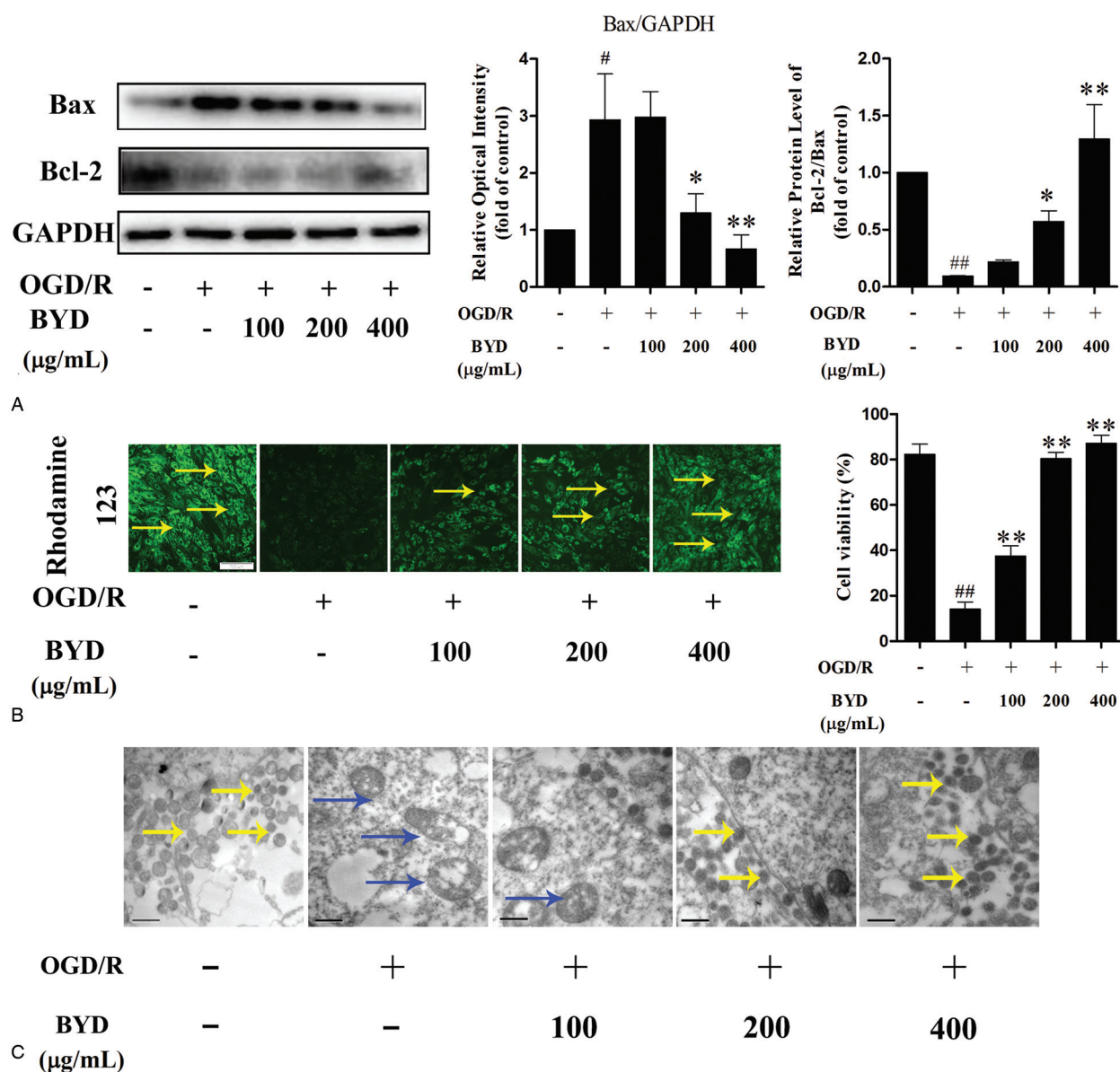


Figure 5. BYD stabilizes MMP against OGD/R injury. (A) BYD downregulates Bax protein but not Bcl-2. (B) BYD stabilizes MMP according to Rhodamine 123 staining assay (bar=100 μm). (C) TEM showing that BYD improves mitochondrial morphology in cells challenged by OGD/R. Image magnification=20 K. Mitochondrial cristae are grossly swollen and lysed in the model group. BYD treatment ameliorated crista swelling and lysis. ^{##} $P < 0.01$ relative to control group. ^{*} $P < 0.05$, ^{**} $P < 0.01$ relative to OGD/R treatment group. $n = 3$. BYD: Baoyuan decoction; MMP: mitochondrial membrane potential; OGD/R: oxygen-glucose deprivation/recovery; TEM: transmission electron microscopy.

Bcl-2 and downregulated Bax. Rhodamine 123 staining indicated that BYD markedly decreased apoptosis and increased the MMP in H9c2 cells challenged with OGD/R. TEM revealed that BYD stabilized the MMP against OGD/R injury. We also explored the roles of BYD in the mitochondrial-mediated caspase apoptosis pathway involving caspase-9, caspase-3, and PARP. A WB assay disclosed that BYD treatment suppressed the caspase apoptotic cascade by inactivating caspase-9/-3 and PARP.

Our study established that the putative cardioprotective mechanisms of BYD were associated with mitochondrial protection *via* the mitochondria-dependent metabolic pathway and caspase-9/3 apoptosis signals. The present study clarified the cardioprotective mechanism of BYD and may facilitate the discovery of alternative TCM-based approaches toward MI therapy.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Yong Jiang, Kewu Zeng, and Pengfei Tu designed the research. Zhiyong Du, Zeliu Shu, and Chun Li performed animal experiments and conducted metabolomics and molecular mechanism studies. Xiaoli Ma and Jun Li

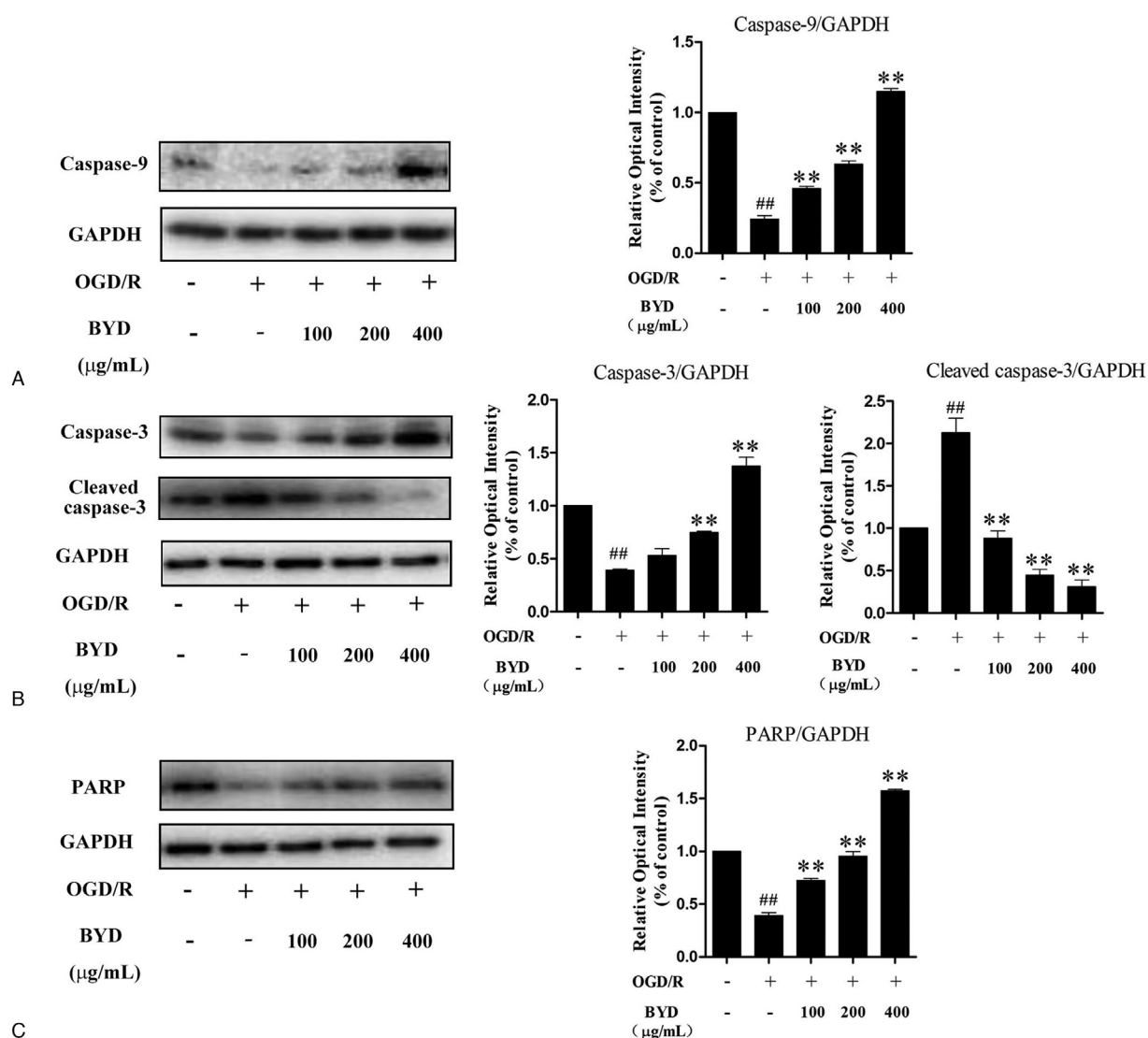


Figure 6. BYD protects against OGD/R injury by inhibiting the mitochondria-dependent caspase apoptosis pathway. WB assay was performed on H9c2 cells to measure expression levels of mitochondria-dependent caspase apoptosis proteins including caspase-9, caspase-3, cleaved-caspase-3, and PARP. (A) BYD upregulates caspase-9. (B) BYD upregulates caspase-3 and downregulates cleaved caspase-3. (C) BYD upregulates PARP. GAPDH was the loading control. Neither cleaved caspase-9 nor cleaved PARP was detected. Values are mean $\pm$ SD of three independent experiments. ^{##} $P < 0.01$ relative to control group. ^{*} $P < 0.05$, ^{**} $P < 0.01$ relative to OGD/R treatment group. BYD: Baoyuan decoction; OGD/R: oxygen-glucose deprivation/recovery; PARP: poly ADP-ribose polymerase; WB: Western Blot.

performed the chemical analysis of BYD. Xiaomin Song and Lixi Liao assisted with the molecular mechanism study. Zhiyong Du and Zeliu Shu wrote the article. Yong Jiang and Kewu Zeng revised and finalized the article. All authors read and approved the final manuscript. All data were generated in-house and no paper mill was used. All authors agreed to be accountable for all aspects of the work and ensure its integrity and accuracy.

Ethical approval of studies and informed consent

All animal studies followed the relevant national legislation and were approved by the Animal Care Committee of Beijing University of Chinese Medicine (BUCM-4-2018001201-1014).

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