

Mitochondrial autophagy promotion contributes to the protective effects of BYHWD glycoside-enriched fraction against OGD/R-induced injury in H9C2 cells

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Abstract. Myocardial ischemia-reperfusion injury is a major cause of mortality among patients with cardiovascular diseases and critically ill individuals. Its pathogenesis is complex and effective preventive or therapeutic strategies remain limited. Buyang Huanwu Decoction (BYHWD) is a classical Traditional Chinese Medicine formula used to tonify deficiency and promote blood circulation and is widely applied in the treatment of cardiovascular and cerebrovascular disorders. The total glycosides extracted from BYHWD, which mainly comprise astragaloside IV, paeoniflorin and amygdalin, represent its key bioactive components. Notably, the extract used is a glycoside-enriched fraction, in which astragaloside IV, paeoniflorin and amygdalin are the three major quantified components, rather than a pure mixture of these three compounds. In the present study, an H9C2 cell model of ischemia-reperfusion injury was established by 21 h of hypoxia followed by 6 h of reoxygenation to evaluate the cardioprotective effects of BYHWD glycosides. Autophagy and reactive oxygen species levels

were analyzed by flow cytometry, while malondialdehyde and superoxide dismutase activities were measured using appropriate assay kits. Mitochondrial membrane potential changes were observed by fluorescence microscopy. The expression of PINK1 and Parkin pathway-associated proteins and genes was examined by western blotting and reverse transcription-quantitative PCR. Overall, BYHWD glycosides significantly reduced oxidative damage in H9C2 cells and protected cardiomyocytes from reperfusion injury, potentially by promoting mitochondrial autophagy. These protective effects may therefore be associated with regulation of the PINK1/Parkin-mediated mitochondrial autophagy pathway.

Introduction

Ischemic heart disease, including myocardial infarction, has remained the leading cause of mortality worldwide. Driven by population aging and increasing cardiovascular risk factors, the absolute global incidence and mortality of myocardial infarction continue to rise, and this upward trend is particularly pronounced in China (1-3). Thrombolysis or percutaneous coronary intervention is an effective treatment for myocardial infarction, but it often results in myocardial ischemia-reperfusion injury (MIRI), which can markedly hinder patient recovery (1,2). At present, the main strategies for the prevention and treatment of MIRI include ischemic preconditioning, ischemic post-adaptation, drug preconditioning and drug post-processing. Commonly used clinical agents, such as vitamin E, cariporide and allopurinol, often yield suboptimal therapeutic outcomes due to the complex pathophysiology of MIRI (3). Unlike single-target pharmacological agents, traditional Chinese medicine (TCM) compound prescriptions exert their therapeutic effects through a synergistic 'multi-component, multi-target, and multi-pathway' mechanism. This enables the various active ingredients within a single formula to simultaneously modulate interconnected pathological networks, such as oxidative stress, inflammation and apoptosis, thereby offering comprehensive protection against MIRI (4).

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Abbreviations: BYHWD, Buyang Huanwu decoction; OGD/R, oxygen-glucose deprivation/reoxygenation; MDC, monodansylcadaverine; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; SIRT1, silent mating type information regulation 2 homolog-1; PINK1, PTEN induced kinase 1; MMP, mitochondrial membrane potential; MIRI, myocardial ischemia reperfusion injury

Key words: Buyang Huanwu Decoction, glycosides, mitochondrial autophagy, myocardial ischemia-reperfusion injury, PTEN-induced kinase 1/Parkin

Buyang Huanwu Decoction (BYHWD) is a representative prescription of the traditional Yiqi Huoxue formula and a classical treatment for cardio-cerebrovascular ischemic diseases in TCM(4-8).BYHWD originates from 'Yi Lin Gai Cuo' (meaning 'Correcting the Errors of Medical Works' in English), authored by Qingren (9) during the Qing Dynasty, and is composed of *Radix Astragali*, *Radix Angelicae Sinensis*, *Radix Paeoniae Rubra*, *Lumbricus*, *Rhizoma Chuanxiong*, *Flos Carthami* and *Semen Persicae*. Modern studies have shown that BYHWD can effectively improve myocardial function in patients with MIRI by reducing the level of serum inflammatory cytokines, including TNF- α , IL-6 and IL-1 β . In addition, it can scavenge oxygen free radicals, inhibit calcium overload and improve vascular microcirculation, with beneficial effects having been demonstrated in numerous cardiovascular diseases, including myocardial infarction, MIRI, angina pectoris, heart failure and arrhythmia (5,6). In previous years, active-component extracts of TCM prescriptions have gradually become a key direction in compound research, establishing an experimental foundation for clarifying the material basis of TCM compound prescriptions (10). Previous studies have also shown that glycosides are among the main active components of BYHWD in the treatment of cardio-cerebrovascular ischemia-associated diseases (4,7). Fu *et al* (8) found that the glycosides of BYHWD can alleviate inflammatory reactions *in vivo* and *in vitro* by downregulating the JAK/STAT signaling pathway, thereby exerting an anti-atherosclerotic effect. This is similar to the overall effect of BYHWD, suggesting that glycosides are important active components of BYHWD. The main active glycoside-associated components are astragaloside IV, paeoniflorin and amygdalin (8). However, there is still a lack of studies specifically investigating the effects of the total glycosides of BYHWD on MIRI. This is primarily as previous pharmacological research has predominantly focused either on the crude aqueous decoction to reflect holistic properties, or on single isolated monomers (such as pure astragaloside IV). Furthermore, BYHWD has traditionally been studied more extensively in the context of cerebrovascular ischemic diseases. Consequently, the synergistic protective potential of its glycoside-enriched fraction in myocardial ischemia-reperfusion injury remains relatively underexplored.

Mitochondria, as organelles that generate energy, serve an important role in cardiomyocytes, participating in a number of cellular functions, such as the synthesis of metabolites, calcium storage, reactive oxygen species (ROS) production and inflammation. When reperfusion occurs, more damaged mitochondria are produced and an increase in ROS, released after mitochondrial damage, is observed. Higher levels of ROS can cause cell homeostasis imbalance, resulting in severe oxidative stress, which subsequently triggers lipid peroxidation, protein oxidation, DNA damage, and ultimately apoptotic or necrotic cell death (1). Mitochondrial autophagy can clear the damaged mitochondria in time, thus maintaining the normal function and number of cardiomyocytes (11). Therefore, the maintenance of normal mitochondrial function by regulating mitochondrial autophagy is markedly important for the maintenance of myocardial homeostasis during reperfusion injury (12,13). The PTEN-induced kinase 1 (PINK1)/Parkin pathway is one of the classical mitochondrial autophagy pathways, capable

of rapidly responding to mitochondrial damage and serving a key role in maintaining mitochondrial network integrity and promoting cell survival (14).

Overall, prescriptions for replenishing qi and activating blood circulation have been widely used in the clinical treatment of MIRI in TCM. In recent years, research regarding TCM compound prescriptions has increasingly focused on their active components and the total glycosides in BYHWD have been indicated to be major active components involved in improving ischemic myocardial injury. It should be noted that the extract used is a glycoside-enriched fraction, in which astragaloside IV, paeoniflorin and amygdalin are the three major quantified components, rather than a pure mixture of these three compounds. However, the efficacy and mechanism of BYHWD glycosides against MIRI remain unclear. Mitochondrial autophagy is an important component of MIRI. Therefore, investigating the regulation of mitochondrial autophagy by TCM compound prescriptions is of marked importance. Accordingly, the present study focused on investigating the effects of BYHWD glycosides on MIRI, potentially through the regulation of mitochondrial autophagy.

Materials and methods

Reagents. Standard paeoniflorin (batch no. R002155; purity, 98%) was obtained from RHAWN; Shanghai Saihan Technology Co., Ltd. Amygdalin reference compound (batch no. BMMI20687; purity $\geq 98\%$) was supplied by Shanghai Yuanye Bio-Technology Co., Ltd. Astragaloside IV standard (batch no. A800922; purity $\geq 98.5\%$) was purchased from Shanghai Macklin Biochemical Co., Ltd. Primary antibodies against PTEN-induced kinase 1 (PINK1; cat. no. YN2037), Parkin (cat. no. YT3593), p62 (cat. no. YT7058) and silent mating type information regulation 2 homolog-1 (SIRT1; cat. no. YT4302) were obtained from ImmunoWay Biotechnology Company. Antibodies against LC3 (cat. no. 14600-1) and Beclin-1 (cat. no. PD017) were purchased from Proteintech Group, Inc. and MBL International Co., respectively. Anti- β -actin antibody (cat. no. DW9562), HRP-conjugated anti-rabbit secondary antibody (cat. no. WD-GAR007) and anti-mouse secondary antibody (cat. no. WD0990) were provided by Hangzhou Dawen Biotech Co., Ltd.

RNA extraction was performed using an RNA Quick Purification Kit (cat. no. RN001; Shanghai Yishan Biotechnology Co., Ltd.) and complementary DNA (cDNA) synthesis was conducted with the BeyoRT™ II First Strand cDNA Synthesis Kit (cat. no. D7170M; Beyotime Biotechnology). Quantitative PCR (qPCR) reactions employed PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.). Assay kits for reactive oxygen species (ROS; cat. no. S0033), JC-1 mitochondrial membrane potential (MMP) detection (cat. no. M8650), malondialdehyde (MDA; cat. no. S0131M), monodansylcadaverine (MDC; cat. no. C3018S) and superoxide dismutase (SOD; cat. no. S0101S) were obtained from Beyotime Biotechnology or Beijing Solarbio Science & Technology Co., Ltd., through local suppliers. High-performance liquid chromatography (HPLC)-grade acetonitrile (cat. no. 75-05-8) was purchased from Merck KGaA.

Table I. Composition and source of BYHWD.

Source	Chinese herbal name	Latin name	Species name	Weight ratio
BYHWD prepared	Huang Qi	Radix Astragali	<i>Astragalus membranaceus</i> (Fisch.) Bge. var. <i>Mongholicus</i> (Bge.) Hsiao	20
by the affiliated	Tao Ren	Semen Persicae	<i>Amygdalus persica</i> L.	3
Hospital of Zhejiang	Hong Hua	Flos Carthami	<i>Carthamus tinctorius</i> L.	3
Chinese Medical	Chi Shao	Radix Paeoniae Rubra	<i>Paeonia lactiflora</i> Pall.	3
University	Dang Gui	Radix Angelicae Sinensis	<i>Angelica sinensis</i> (Oliv.) Diels	3
(batch no. 2001621)	Chuan Xiong	Rhizoma Chuanxiong	<i>Ligusticum chuanxiong</i> .	2
in 10g/bags	Di Long	Lumbricus	Hort <i>Pheretima aspergillum</i> (E. Perrier)	3

BYHWD, Buyang Huanwu Decoction.

Preparation of BYHWD glycosides. BYHWD, which is composed of *Radix Astragali* (Huang Qi), *Semen Persicae* (Tao Ren), *Flos Carthami* (Hong Hua), *Radix Paeoniae Rubra* (Chi Shao), *Radix Angelicae Sinensis* (Dang Gui), *Rhizoma Chuanxiong* (Chuan Xiong) and *Lumbricus* (Di Long) were supplied by The Affiliated Hospital of Zhejiang Chinese Medical University (Hangzhou, China) and identified by the co-author, Professor Ding Zhishan from Zhejiang Chinese Medicine University (Hangzhou, China). The proportions of each aforementioned medicinal material were 20:3:3:3:3:2:3. The detailed composition and sources of BYHWD are summarized in Table I. A packet of proportionally compatible compound samples was taken, powdered and sifted through 60 mesh. The compound crude powder was accurately weighed (50 g) and 11 volumes of distilled water were added. Next, the sample was boiled at 82°C for 75 min and filtered. The process was repeated three times, combining the filtrate each time, followed by centrifugation at 3,000 x g for 10 min at room temperature to remove the precipitate. The filtrate was concentrated by rotary evaporation. Finally, the concentrated liquid reached ~150 ml. Then, 95% ethanol was gradually added to the aqueous extract until the final ethanol concentration reached 85% (v/v) and the mixture was sealed and kept at 4°C overnight (≥12 h) for alcohol precipitation. The supernatant was then concentrated to ~20 ml. Extraction with petroleum ether was performed three times and the aqueous layer was retained, followed by extraction with ethyl acetate three times. Next, the ethyl acetate layer was reserved and the aqueous layer was extracted with n-butanol solution three times. Finally, the n-butanol and ethyl acetate layers were combined in an evaporation dish and dried under vacuum to obtain crude glycosides. Notably, the extract used was a glycoside-enriched fraction, in which astragaloside IV, paeoniflorin and amygdalin were the three major quantified components, rather than a pure mixture of these three compounds.

Qualitative and quantitative analyses of glycosides. Chromatographic separation was carried out using a reversed-phase C18 analytical column (250 mm in length) maintained at 30°C. The mobile phase consisted of acetonitrile (solvent A) and purified water (solvent B), delivered at a constant flow rate of 0.8 ml/min. Sample injection volume was set at 10 µl. Detection wavelengths were adjusted to 230 nm for paeoniflorin and 210 nm for amygdalin. A gradient elution program was applied as follows: 8-10% solvent A for 0-15 min, 10-15% solvent A for 15-25 min, 15-18% solvent A for 25-35 min and 18-21% solvent A for 35-40 min. The system operated under a column pressure of 0.71±0.41 MPa. Quantification of astragaloside IV in BYHWD total glycosides was performed using a vanillin-sulfuric acid colorimetric assay. Briefly, the colorimetric reaction was conducted at 60°C for 15 min. The standard calibration curve was constructed based on the modified protocol described by Le *et al* (15) and the astragaloside IV content was calculated accordingly. Preparation of the astragaloside IV standard curve, calculation formula and determination of the results are provided in Fig. S1 and Tables SI and SII.

Cell culture and model establishment. H9C2, the rat cardiomyoblast cell line was obtained from the China Center for Type Culture Collection. Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (CellMax Technologies AB) and 1% penicillin-streptomycin (Northrend) at 37°C in a humidified incubator containing 5% CO₂. Cells were passaged and used for subsequent experiments upon reaching 80-90% confluence.

H9C2 cells were randomly assigned to three groups: A control group, an oxygen-glucose deprivation/reoxygenation (OGD/R) group and a BYHWD total glycosides pre-treatment group. The OGD/R model was established (as detailed below) to simulate ischemia-reperfusion injury *in vitro*. For the pre-treatment, cells in the BYHWD group were incubated with the BYHWD glycoside-enriched fraction at 37°C for

12 h prior to OGD/R induction. Specifically, the glycosides were prepared in complete culture medium and added to the cells, while the model and control groups received an equal volume (100 μ l/well) of normal complete medium. Next, with the exception of the control group, complete culture medium was replaced with minimum essential medium without FBS in the other groups, followed by exposure to an airtight chamber (cat. no. MIC-101; Embriant, Inc.) containing a mixture of 95% N₂ and 5% CO₂ anoxic culture at 37°C for 21 h. The supernatant was replaced with complete medium and cultured in a normal cell incubator for 6 h to induce reperfusion injury in H9C2 cells.

Cell viability assay. H9C2 cells were seeded into 96-well culture plates at a density of 5,000 cells per well and allowed to adhere prior to experimental treatment. Following the indicated interventions, cellular viability was assessed using an MTT assay (BioFroxx; neoFroxx GmbH). Briefly, 10 μ l MTT working solution was added to each well and the plates were incubated at 37°C for 4 h to allow for formazan crystal formation. The culture supernatant was subsequently removed and the resulting formazan precipitate was solubilized by adding 200 μ l dimethyl sulfoxide to each well. Absorbance was recorded at 560 nm using the BioTek Epoch 2 microplate reader (Agilent Technologies, Inc.).

Detection of oxidative stress in H9C2 cells. After treatment, the cells in each group were collected. Subsequently, ROS (cat. no. S0033), MDA (cat. no. S0131M) and SOD (cat. no. S0101S) assay kits obtained from Beyotime Biotechnology or Beijing Solarbio Science & Technology Co., Ltd., through local suppliers were used to detect the changes in cellular ROS levels, MDA content and SOD activity, respectively, strictly according to the manufacturers' protocols.

Detection of autophagy by the MDC method. Upon treatment, the culture medium was removed and the cells were rinsed three times with PBS. Cells were then exposed to 200 μ l trypsin per well for ~2 min at 37°C to achieve detachment. Enzymatic digestion was terminated by adding 1 ml complete culture medium. Subsequently, the cell suspension was gently pipetted and transferred into 1.5 ml centrifuge tubes. Samples were centrifuged at 250 x g for 5 min at room temperature and the supernatant was discarded. The cell pellets were washed three times with assay buffer. Subsequently, the cells were incubated with 1 ml MDC staining solution for 30 min at room temperature in the dark. After staining, cells were centrifuged again at 250 x g for 5 min at room temperature, washed three times with assay buffer and finally subjected to flow cytometric analysis to evaluate autophagic activity.

Flow cytometric analysis. As MDC is a live-cell tracking dye, no fixation, blocking or antibodies were used in this assay. Fluorescence signals of the MDC-stained autophagic vacuoles were acquired using an Accuri C6 flow cytometer (Becton, Dickinson and Company). Data analysis was subsequently performed using FlowJo v10 (BD Biosciences).

Detection of MMP by JC-1. MMP was assessed using the JC-1 fluorescent probe according to the manufacturer's protocol.

Briefly, JC-1 staining solution and working buffer were freshly prepared prior to use. Treated cells were incubated with the JC-1 dye at 37°C for 20 min, followed by removal of the staining solution. Cells were then washed with JC-1 buffer to eliminate excess dye. Fluorescence signals were observed using a fluorescence microscope. JC-1 monomers were detected with excitation at 490 nm and emission at 530 nm, exhibiting green fluorescence, whereas JC-1 aggregates were visualized at an excitation wavelength of 530 nm and emission at 590 nm, producing red fluorescence. MMP is expressed as the ratio of red to green fluorescence intensity and was calculated as follows: MMP (%)=(red fluorescence intensity/xgreen fluorescence intensity) x100%.

Reverse transcription (RT)-qPCR. Total RNA was isolated from treated cells using the RNA Quick Purification Kit and stored at -80°C until further analysis. cDNA was synthesized using the BeyoRT™ II First Strand cDNA Synthesis Kit according to the manufacturer's instructions. RT-qPCR was performed using the PowerUp™ SYBR™ Green Master Mix. Amplification and fluorescence signal acquisition were conducted on a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial UDG activation at 50°C for 2 min, initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Relative gene expression levels were normalized to GAPDH and calculated using the 2^{- $\Delta\Delta C_q$} method (16). The primer sequences used in the present study are listed in Table II.

Western blotting. After the experimental treatment, cells were lysed in RIPA buffer (Beyotime Biotechnology) supplemented with a protease inhibitor cocktail (including pepstatin, leupeptin and aprotinin). Lysates were centrifuged at 14,000 x g for 15 min at 4°C and the supernatants were collected for protein analysis. Protein concentrations were determined using a BCA assay. Equal amounts of protein (20 μ g per lane) were mixed with loading buffer and denatured by heating at 95°C for 10 min, followed by separation on 10% SDS-PAGE. Proteins were transferred onto PVDF membranes, which were subsequently blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) in TBS-Tween-20 for 1 h at room temperature. Membranes were then incubated overnight at 4°C with the appropriate primary antibodies (diluted at 1:1,000). After washing with TBS-T, membranes were incubated with HRP-conjugated secondary antibodies (diluted at 1:5,000) for 2 h at 37°C. Protein bands were visualized using an ECL detection system (Shenyang Wanli Biotechnology Co., Ltd.). Band intensities were quantified using a C-DiGit Blot Scanner (LI-COR Biotech, LLC) and normalized to β -actin expression.

Statistical analysis. Statistical analyses were conducted using GraphPad Prism software (version 6.02; GraphPad; Dotmatics). Experimental data are presented as the mean \pm SD from three independent experiments. Differences among multiple groups were analyzed using one-way ANOVA tests followed by Tukey's post hoc test for multiple comparisons between two specific groups. P<0.05 was considered to indicate a statistically significant difference.

Table II. Sequences of primers used in reverse transcriptase-quantitative PCR.

Gene name	Sequence (5'-3')	Product size, bp
SIRT1	Forward: CCAGATCCTCAAGCCATG	18
	Reverse: TTGGATTCTGCAACCTG	18
Beclin-1	Forward: GAATGGAGGGGTCTAAGGCG	20
	Reverse: CTCCTCCTGGCTCTCTCCT	20
PINK1	Forward: ACTACCTATGCCCATCCATCTA	22
	Reverse: CTCGGTGACAGCTAAGTCATC	21
Parkin	Forward: CAGCTAAACCCACCTACCACAG	22
	Reverse: CATCCGGTTTGAATTAAGACA	22
p62	Forward: CTGCTCCATCAGAGGATCCCAA	22
	Reverse: GGTCTAGAGAGCTTGGCCCT	20
GAPDH	Forward: AGGTCGGTGTGAACGGATTTG	21
	Reverse: TGTAGACCATGTAGTTGAGGTCA	23

SIRT1, silent mating type information regulation 2 homolog- 1; PINK1, PTEN induced putative kinase 1.

Table III. Quantitative analysis of main active components of BYHWD glycosides

Component	BYHWD glycosides
Astragaloside IV	12.61%
Paeoniflorin	6.61%
Amygdalin	1.04%

BYHWD, Buyang Huanwu decoction.

Results

Quantitative analysis of the main effective components of BYHWD glycosides. Paeoniflorin and amygdalin in BYHWD glycosides were detected by HPLC and the two main components were quantitatively analyzed by the external standard method as previously described (17). The results are presented in Fig. 1A and B. In addition, since astragaloside IV could not be well separated by HPLC, a standard curve of astragaloside IV was established by improving the vanillin-sulfuric acid colorimetric method previously described (17) and the content of astragaloside IV in three types of compound was determined. The chemical structure formula of astragaloside IV is shown in Fig. 1C. Astragaloside IV exhibited the highest content, accounting for 12.61% of the total glycosides, followed by paeoniflorin (6.61%) and amygdalin (1.04%). The quantitative data of the main glycosides is summarized in Table III.

Screening of safe and effective concentrations of BYHWD glycosides. As shown in Fig. 2A, BYHWD glycosides showed significant dose-dependent toxicity on H9C2 cells and significantly inhibited the viability of H9C2 cells when the concentration was $>9.765 \mu\text{g/ml}$. In addition, the protective effects of BYHWD glycosides on the survival rate of H9C2

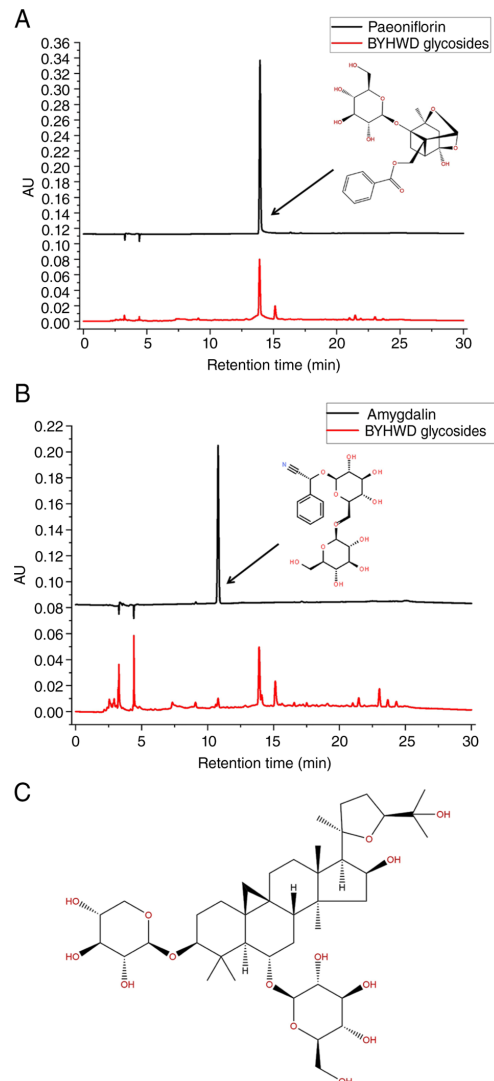


Figure 1. Chemical structures and high-performance liquid chromatography chromatogram of glycosides. Characteristic peaks of (A) paeoniflorin and (B) amygdalin. (C) Chemical structure of astragaloside IV. AU, absorbance units; BYHWD, Buyang Huanwu Decoction.

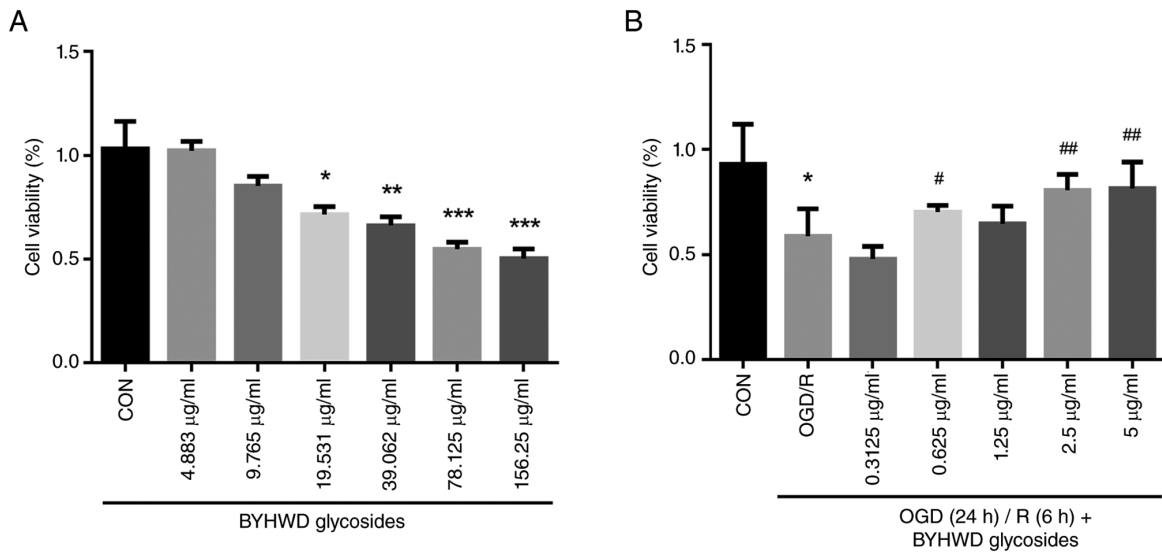


Figure 2. MTT assay was used to detect the effects of BYHWD glycosides on H9C2 cell viability. (A) Screening of safe concentration of BYHWD glycosides on H9C2 cells. (B) Screening of effective concentration of BYHWD glycosides on OGD/R damage of H9C2 cells. Data are expressed as the mean±SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control, #P<0.05 and ##P<0.01 vs. OGD/R group. CON, control; BYHWD, Buyang Huanwu Decoction; OGD/R, oxygen-glucose deprivation/reoxygenation.

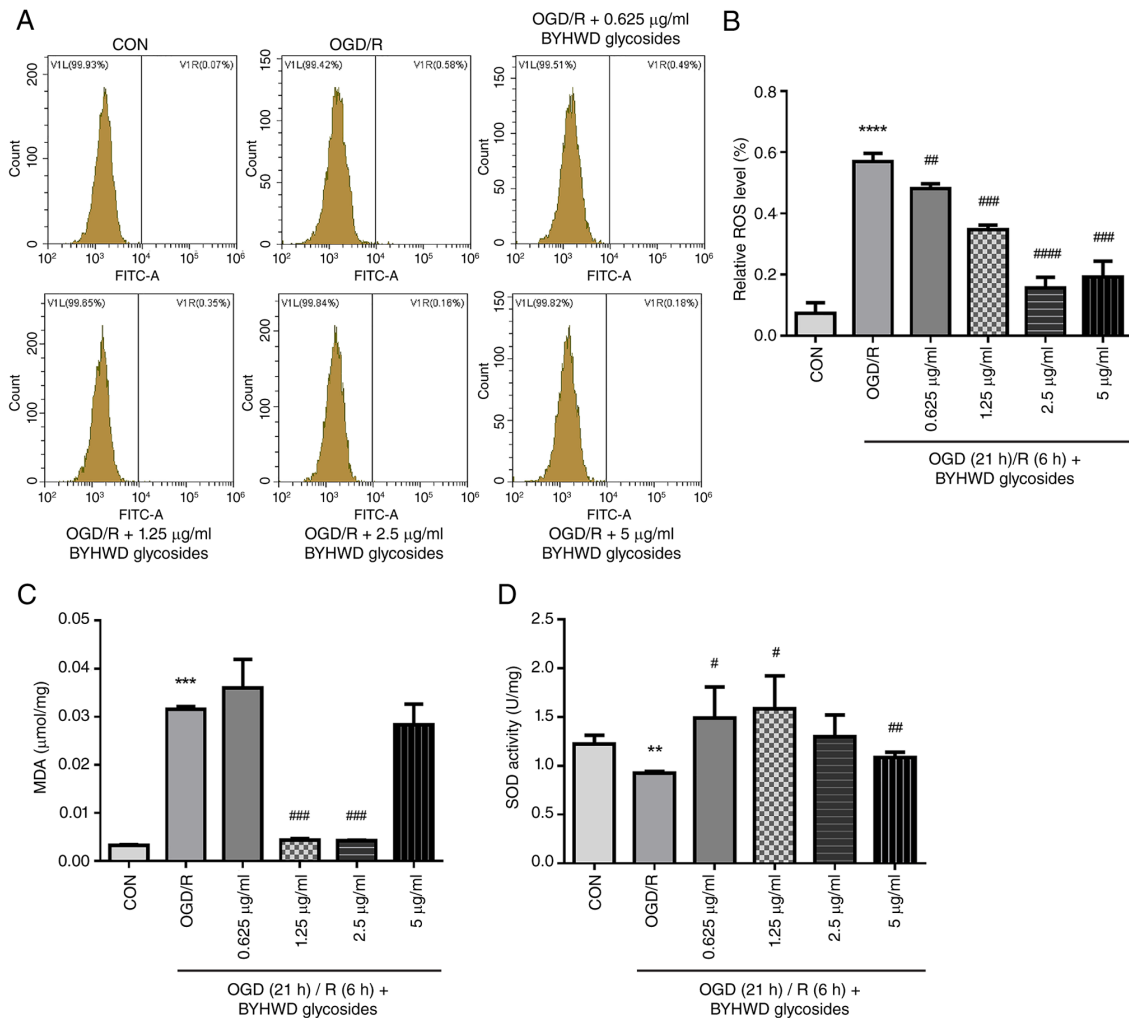


Figure 3. Effects of BYHWD glycosides on OGD/R induced oxidative damage of H9C2 cells. (A) The level of ROS in each group was detected by flow cytometry. (B) Quantitative analysis of ROS by flow detection. (C) Detection of the MDA levels in each group. (D) Detection results of the SOD levels in each group. Data are expressed as the mean±SD (n=3). **P<0.01, ***P<0.001, ****P<0.0001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 and ####P<0.0001 vs. OGD/R group. OGD/R, oxygen-glucose deprivation/reoxygenation; ROS, reactive oxygen species; BYHWD, Buyang Huanwu Decoction; MDA, malondialdehyde; SOD, superoxide dismutase; CON, control.

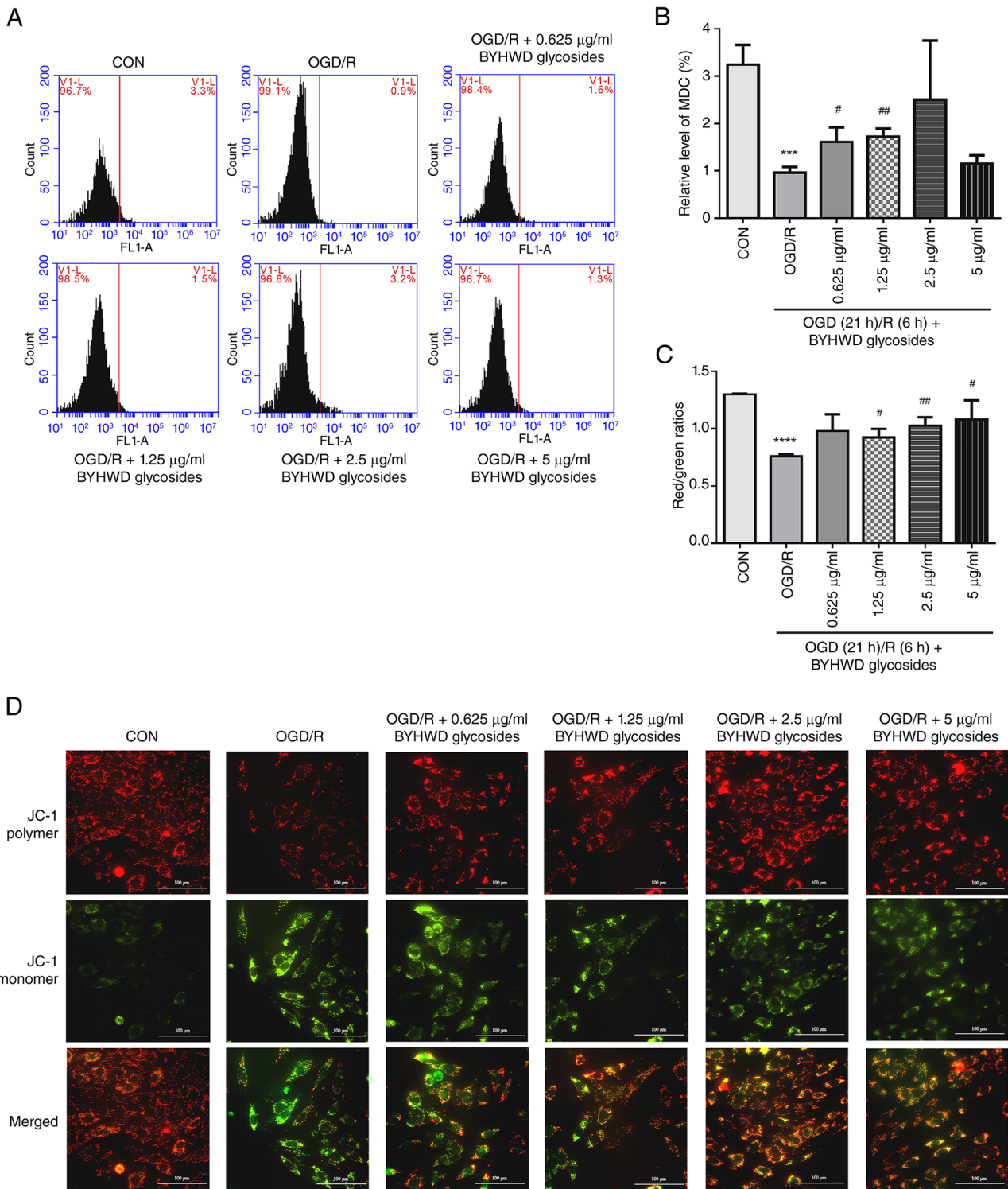


Figure 4. Effects of BYHWD glycosides on autophagy and mitochondrial membrane potential. (A) Detection of MDC autophagy fluorescence level by flow cytometry. (B) Quantitative analysis of MDC fluorescence level. (C) Quantitative analysis of MMP fluorescence expression. (D) Observation of MMP changes under fluorescence microscopy. Scale bar, 100 μ m. Data are expressed as the mean \pm SD (n=3). ***P<0.001, ****P<0.0001 vs. control, #P<0.05 and ##P<0.01 vs. OGD/R group. MDC, monodansylcadaverine; MMP, mitochondrial membrane potential; BYHWD, Buyang Huanwu Decoction; CON, control; OGD/R, oxygen-glucose deprivation/reoxygenation.

induced by OGD/R were evaluated. As shown in Fig. 2B, the survival rate of H9C2 cells subjected to OGD/R was significantly lower compared with that of the untreated group. However, BYHWD glycosides (0.625-5 μ g/ml) significantly increased the activity of H9C2 cells. These results suggest that, in a specific concentration range, BYHWD glycosides can

promote the viability of H9C2 cells after OGD/R injury. This provided a concentration reference for subsequent experiments.

Effects of BYHWD glycosides on the oxidative stress level of H9C2 cells induced by OGD/R. Cardiomyocytes injured by ischemia-reperfusion are regulated by processes such

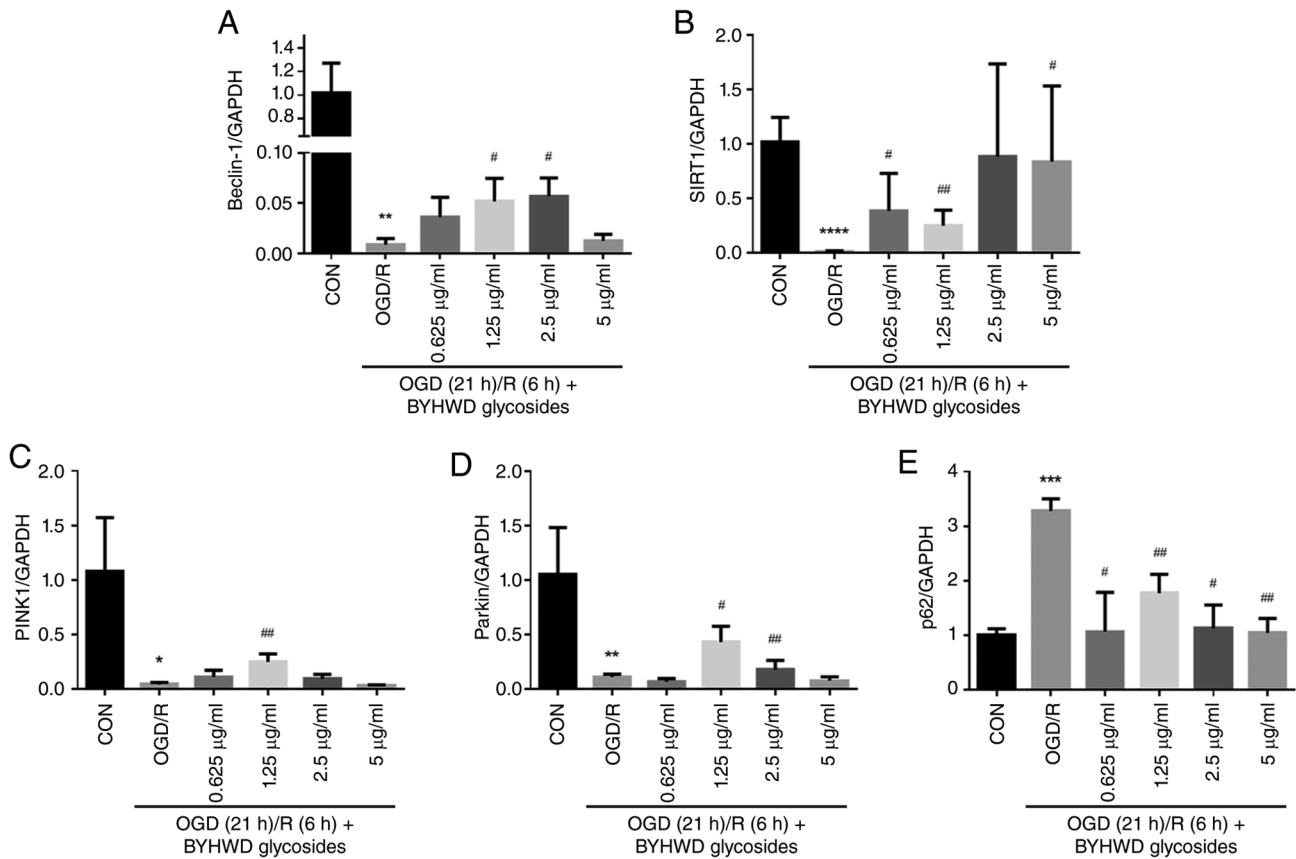


Figure 5. Effect of BYHWD glycosides on PINK1/Parkin pathway-associated mRNA. (A) Beclin-1 mRNA expression. (B) SIRT1 mRNA expression. (C) PINK1 mRNA expression. (D) Parkin mRNA expression. (E) p62 mRNA expression in H9C2 cells stimulated by OGD/R after BYHWD glycosides treatment. Data are expressed as the mean \pm SD (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control, #P<0.05 and ##P<0.01 vs. OGD/R group. BYHWD, Buyang Huanwu Decoction; PINK1, PTEN-induced kinase 1; OGD/R, oxygen-glucose deprivation/reoxygenation; SIRT1, silent mating type information regulation 2 homolog-1; CON, control.

as autophagy and oxidative stress. ROS, MDA and SOD are all important indicators to assess the level of oxidative stress. Thus, these three indicators were measured in the present study to assess the effects of BYHWD glycosides on OGD/R-induced oxidative stress in H9C2 cells. The results are shown in Fig. 3A-D. The contents of ROS and MDA in the model group were significantly higher compared with those in the untreated group, while the level of SOD in the model group was significantly lower compared with that in the untreated group. However, pre-administration of BYHWD glycosides significantly inhibited the increase in ROS levels after modeling, exhibiting dose-dependent effects. Compared with the model group, BYHWD glycosides pre-treatment significantly reduced the elevated MDA levels and significantly restored the depleted SOD activity (Fig. 3).

Effects of BYHWD glycosides on the autophagy of H9C2 cells induced by OGD/R. To detect the effects of BYHWD glycosides on autophagy in H9C2 cells induced by OGD/R, MDC autophagy fluorescence labeling was used and the autophagy levels in cardiomyocytes were analyzed by flow cytometry. As shown in Fig. 4A and B, the relative level of MDC in the OGD/R model group was significantly lower compared with that in the control group. This finding suggests that OGD/R-induced reperfusion injury in H9C2 cardiomyocytes can inhibit the level of autophagy (11,18). Pretreatment with BYHWD glycosides

significantly increased the relative levels of MDC-labeled autophagic vacuoles specifically at doses of 0.625 and 1.25 μ g/ml compared with the OGD/R model group, indicating a potent promotion of autophagic activity at these concentrations.

Effects of BYHWD glycosides on the MMP of H9C2 cells induced by OGD/R. ROS production and mitochondrial dysfunction are associated with mitophagy during reperfusion injury (11,13,18). A normal membrane potential is key in maintaining proper mitochondrial function. JC-1 is a widely used fluorescent probe (19,20) for detecting MMP. In the present study, MMP was observed using a fluorescence inverted microscope. The results of the JC-1 assay are shown in Fig. 4C and D. In the control group, the cells maintained a highly polarized MMP, indicating that JC-1 accumulated in the matrix of mitochondria to form polymers, thus producing strong red fluorescence. However, it appeared difficult for JC-1 to accumulate in mitochondrial matrix in the OGD/R group; thus, the MMP was observed to be lower, demonstrating a stronger green fluorescence signal. Compared with the findings in the model group, BYHWD glycosides were shown to promote the aggregation of JC-1 in mitochondrial matrix and thus resulted in an increase of red/green fluorescence intensity.

Effects of BYHWD glycosides on mitochondrial autophagy-associated mRNA in H9C2 cells induced by OGD/R.

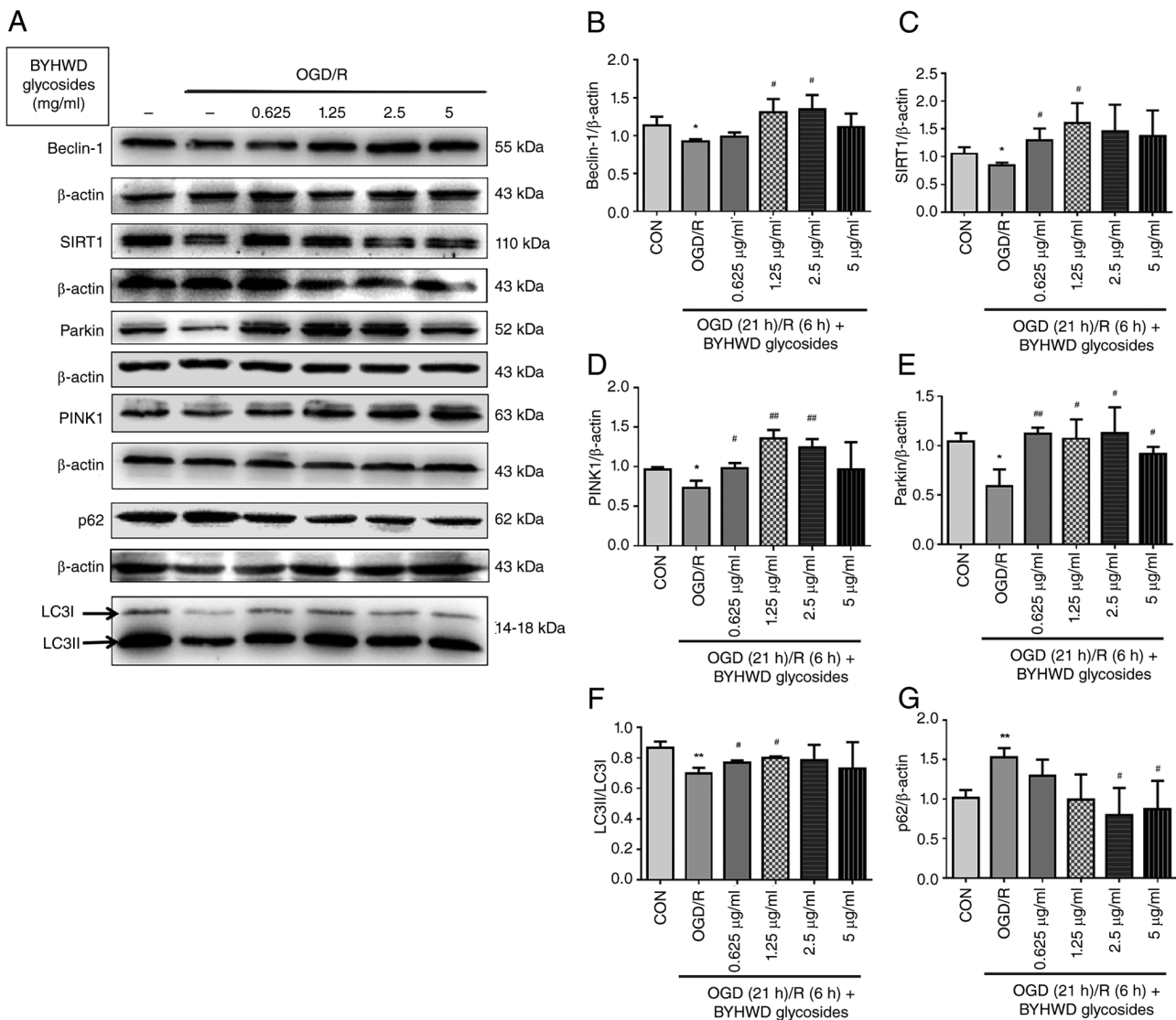


Figure 6. Effect of BYHWD glycosides on the expression levels of Beclin-1, SIRT1, PINK1, Parkin, LC3 and p62 in OGD/R-treated H9C2 cells detected by western blotting. (A-G) Beclin-1, SIRT1, PINK1, Parkin, LC3 and p62 protein expression levels. Data are expressed as the mean ± SD (n=3). *P<0.05, **P<0.01 vs. control, #P<0.05 and ##P<0.01 vs. OGD/R group. SIRT1, silent mating type information regulation 2 homolog-1; PINK1, PTEN-induced kinase 1; OGD/R, oxygen-glucose deprivation/reoxygenation; BYHWD, Buyang Huanwu Decoction; CON, control.

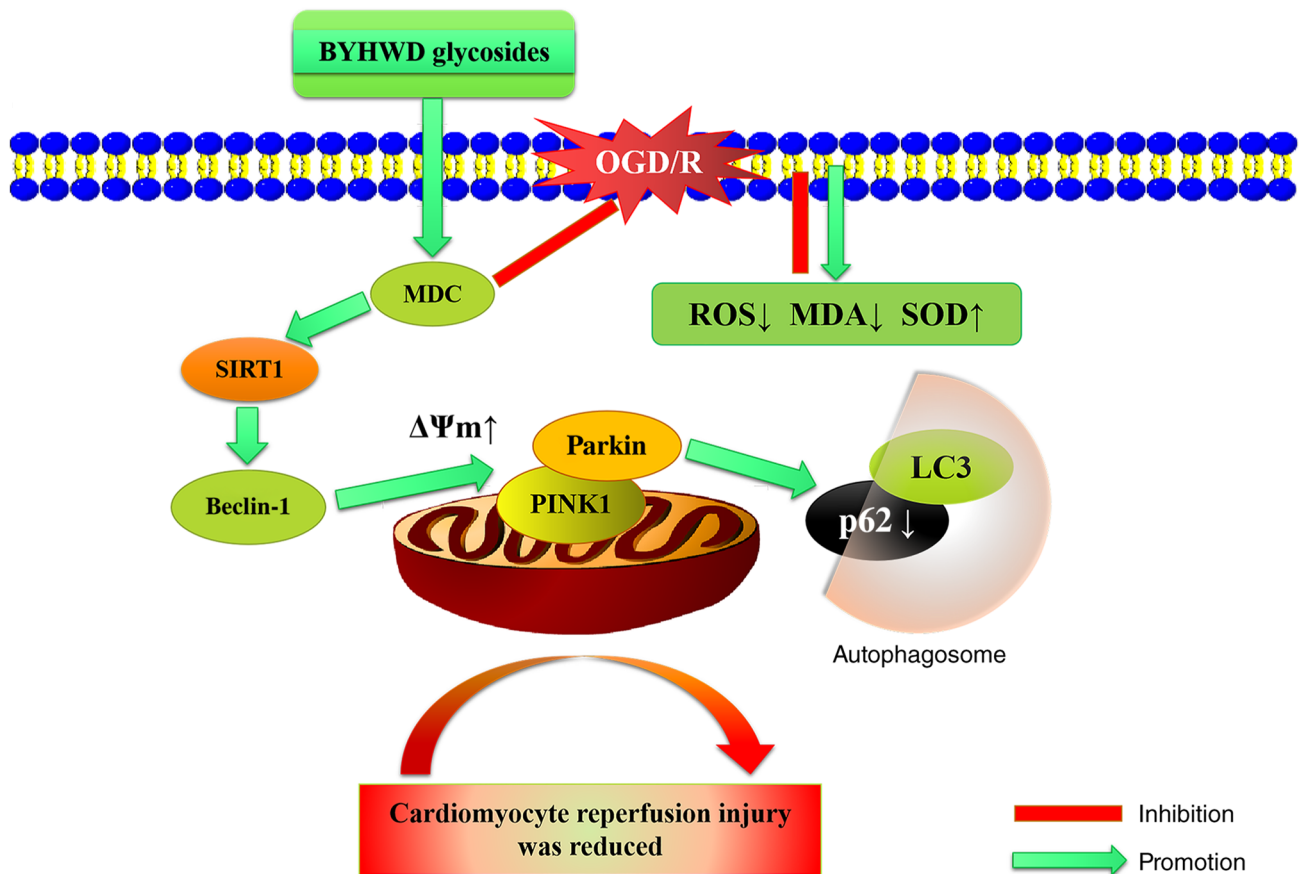
PINK1/Parkin-mediated activation of mitochondrial autophagy helps to protect mitochondria from hypoxia-induced decreases in MMP. As shown in Fig. 5A-E, the level of p62 mRNA in the model group was significantly higher compared with that in the control group. The expression levels of Beclin-1, SIRT1, PINK1 and Parkin were significantly decreased in the model group, compared with the control group. The majority of concentrations of BYHWD glycosides were shown to significantly reverse the abnormal expression of autophagy-associated mRNA after OGD/R injury. Among them, the regulatory effect of 1.25 μg/ml BYHWD glycosides was found to be the most notable.

Effects of BYHWD glycosides on mitochondrial autophagy-associated proteins induced by OGD/R in H9C2 cells. To further determine the effects of BYHWD glycosides on the aforementioned mRNA levels, proteins associated with the PINK1/Parkin mitochondrial autophagy pathway were detected using western blotting analysis. As

shown in Fig. 6A-G, compared with the untreated group, the expression of Beclin-1, SIRT1, PINK1, Parkin and LC3II/I protein in the OGD/R group decreased significantly, while the expression of p62 protein increased significantly. In addition, compared with the model group, the majority of BYHWD glycosides were shown to significantly reverse the abnormal expression of these proteins. The trends observed in these western blotting results was found to be consistent with that of the present RT-qPCR results, further demonstrating that OGD/R inhibited mitochondrial autophagy in H9C2 cells. This suggested that treatment with BYHWD glycosides may promote the autophagy level of H9C2 cells to varying degrees and its mechanism of action may involve the PINK1/Parkin mitochondrial autophagy pathway.

Discussion

Myocardial ischemia is a common pathological condition, with its prevalence in China increasing annually. During myocardial



BYHWD glycosides inhibit OGD/R-induced H9C2 cells injury by regulating PINK1/Parkin mitochondrial autophagy signal pathway

Figure 7. BYHWD glycosides inhibit OGD/R-induced H9C2 cell injury by regulating the PINK1/Parkin mitochondrial autophagy signaling pathway. $\Delta\Psi_m \uparrow$ represents the increase or recovery of mitochondrial membrane potential. BYHWD, Buyang Huanwu Decoction; OGD/R, oxygen-glucose deprivation/reoxygenation; PINK1, PTEN-induced kinase 1; ROS, reactive oxygen species; MDC, monodansylcadaverine; SIRT1, silent mating type information regulation 2 homolog-1; MDA, malondialdehyde; SOD, superoxide dismutase; OGD/R, oxygen-glucose deprivation/reoxygenation.

ischemia, a marked number of cardiomyocytes undergo necrosis and the affected area cannot effectively regenerate due to its limited proliferative capacity. Currently, the clinical application of revascularization therapy can effectively mitigate or delay disease progression. However, accumulating evidence suggests that, once blood flow is restored to the vessels and myocardial tissue, the extent of myocardial damage may further worsen, leading to the phenomenon of MIRI (1,15,21). MIRI is one of the primary reasons for the poor prognosis of ischemic heart disease and myocardial ischemia. Reducing MIRI is a primary focus of cardiovascular disease research (14). At present, the mechanism of MIRI is not completely clear. Previous studies (22,23) have shown that it is associated with the accumulation of oxygen free radicals, abnormal myocardial energy metabolism, mitochondrial dysfunction, calcium overload, inflammation and autophagy. Mitochondrial autophagy serves an important role in myocardial reperfusion injury. Moderate and well-regulated mitochondrial autophagy can enhance energy supply to cardiomyocytes, reduce the production of ROS and protect myocardial function (24).

The method of replenishing qi and activating blood circulation is widely used (25,26) in the clinical treatment of MIRI. Compared with qi-tonifying drugs or blood-activating

agents used alone, Yiqi Huoxue compound prescriptions may exert broader protective effects through multi-component, multi-targeted actions. Previous studies (27,28) have shown that BYHWD glycosides are the main effective components against ischemic brain injury (29). However, the role of this component in the protection of ischemic myocardial injury is not clear. In the present study, BYHWD glycosides were separated and extracted by water extraction, alcohol precipitation and organic solvent extraction. The main effective components of BYHWD include astragaloside IV, paeoniflorin and amygdalin, which were determined by HPLC. However, astragaloside IV could not be well separated by HPLC. Thus, an ultraviolet spectrophotometer was used to detect the content of astragaloside IV in accordance with the literature (17). The results indicated that astragaloside IV exhibited the highest content, followed by paeoniflorin, with amygdalin being the lowest among the three main active components.

Oxidative stress has been shown to mediate the pathological process of MIRI injury (30). Myocardial necrosis is initially caused by ischemic injury, while further damage during reperfusion results from metabolic alterations upon the restoration of ischemic oxygen (31). The levels of SOD, MDA and ROS

can indirectly reflect antioxidant capacity and the severity of oxidative damage in cells. Therefore, increasing the activity of the antioxidant enzyme SOD while reducing MDA and ROS can effectively protect cardiomyocytes from reperfusion injury (32,33). The findings of the present study thus align with previous studies (34). The injury model of H9C2 cells cultured *in vitro* under OGD/R conditions was mitigated by BYHWD glycosides. Findings suggested that BYHWD glycosides could significantly promote the rate of cell viability, inhibit the levels of MDA and ROS and increase the level of SOD. This indicated that BYHWD glycosides may have enhanced the viability of H9C2 cells under OGD/R conditions, inhibited oxidative stress and alleviated myocardial injury.

Mitochondrial autophagy is a specific autophagy process for damaged mitochondria that helps maintain normal intracellular mitochondrial function (35). Previous studies have shown that adaptive mitochondrial autophagy occurs during reperfusion injury and protects cardiomyocytes by eliminating dysfunctional mitochondria (11,18). Acidic autophagy vesicles are released during mitochondrial autophagy (10). As a fluorescent probe, MDC can bind to such autophagy vesicles and emit strong green fluorescence. In the present study, the fluorescence level of MDC in the cells of each group was detected by flow cytometry. The results suggested that normal H9C2 cardiomyocytes exhibit a certain level of autophagic activity. After OGD/R modeling, the number of autophagy vesicles decreased and thus the level of autophagy decreased significantly. However, pretreatment with BYHWD glycosides restored this autophagic activity to varying degrees.

Furthermore, the stability of MMP is important in maintaining normal mitochondrial function. Previous research has demonstrated that effective mitochondrial protection can be achieved by reducing excessive ROS secretion and enhancing MMP (36). Therefore, by detecting the changes in MMP within the present study, whether mitochondrial autophagy alleviated mitochondrial dysfunction in the model of reperfusion injury, was investigated.

The results appeared to be consistent with the literature (37). The present study demonstrated that the red-to-green fluorescence ratio in the OGD/R group was significantly lower compared with that in the normal control group, indicating mitochondrial damage and decreased MMP. However, BYHWD glycosides significantly promoted MMP. These findings suggest that BYHWD glycosides may promote autophagy, prevent the accumulation of damaged mitochondria and further inhibit cardiomyocyte injury induced by OGD/R. It has been established that autophagy exhibits a dual mechanism of action during IRI (38). While excessive autophagic flux can lead to self-digestion and autophagic cell death, moderate mitophagy is highly protective. In the specific experimental setting of severe stress (oxygen glucose deprivation for 21 h followed by 6 h of reoxygenation), the moderate upregulation of mitophagy facilitated by the BYHWD glycoside-enriched fraction is considered beneficial (39). It promotes the timely clearance of markedly damaged mitochondria, thereby preventing the release of pro-apoptotic factors (specifically cytochrome *c*) (40) and ROS, ultimately conferring a survival advantage to cardiomyocytes.

The PINK1/Parkin pathway is a classical mitochondrial autophagy pathway. Previous research has shown that in response to a decrease in mitochondrial membrane potential,

PINK1 accumulates on the outer mitochondrial membrane and subsequently recruits and activates Parkin. Activated Parkin then ubiquitinates mitochondrial surface proteins, which tags the damaged mitochondria for recognition and engulfment by autophagosomes (41). Therefore, the present study further explored the expression of signaling molecules associated with the PINK1/Parkin pathway.

SIRT1, a NAD⁺-dependent deacetylase, is widely distributed in mammals. Firstly, SIRT1 can directly act on Beclin-1, enhancing its function through deacetylation, thereby promoting autophagy (42). Recent evidence has highlighted that the deacetylase activity of SIRT1 is important in stabilizing the PINK1/Parkin axis. Specifically, SIRT1-mediated deacetylation events can enhance the stability of PINK1 on the outer mitochondrial membrane, which subsequently facilitates the recruitment and activation of Parkin (43). Furthermore, recent research regarding MIR models have demonstrated that SIRT1 acts as a key upstream regulator and its silencing directly suppresses PINK1/Parkin-mediated cardioprotective mechanisms, such as the clearance of dysfunctional mitochondria, attenuation of oxidative stress and inhibition of mitochondria-dependent apoptosis (44). The present results aligned with this mechanism, suggesting that the BYHWD glycoside-enriched fraction upregulates SIRT1, which may consequently drive PINK1/Parkin-dependent mitophagic clearance to protect cardiomyocytes against OGD/R injury. In addition, SIRT1 regulates the expression and activity of numerous transcription factors and coactivators (such as FOXO3a and PGC-1 α) associated with mitochondrial proliferation and autophagy, serving an important role in maintaining mitochondrial quality and quantity (45). Beclin-1 is a key regulator of the autophagy pathway, recognized as a marker of autophagy initiation and positively associated with autophagy levels (46). SIRT1 can promote autophagy by deacetylation of the K430 and K437 sites of Beclin-1, which facilitates its binding to VPS34 to assemble the active Class III phosphatidylinositol 3-kinase (PI3K-III) complex, thereby directly driving the initiation and maturation of autophagosomes (47). The present experimental results demonstrated that BYHWD glycosides significantly reversed the decreased expression of SIRT1 and Beclin-1 in cardiomyocytes following reperfusion injury.

Secondly, the PINK1/Parkin pathway is one of the most important pathways for mitochondrial autophagy (48). SIRT1 promotes the deacetylation of PINK1, thereby promoting PINK1 accumulation and recruitment of Parkin protein to the mitochondrial outer membrane. PINK1 accumulates and recruits Parkin protein to the outer mitochondrial membrane, initiating mitochondrial autophagy to clear damaged mitochondria and maintain cellular homeostasis (49). In the present study, the expression levels of PINK1 and Parkin in the BYHWD glycosides-treated group were significantly higher compared with those in the model group.

LC3 is not only an important marker of autophagy but also an important downstream signal of PINK1/Parkin-mediated mitophagy and it has two forms: LC3-I and LC3-II. At the beginning of autophagy, LC3-I is converted into LC3-II; therefore, an increased LC3-II/LC3-I ratio indicates the initiation of autophagy (50). p62 binds to ubiquitin substrates and LC3, targeting autophagosomes and promoting the

clearance of ubiquitinated proteins and its levels are negatively associated with autophagy activity. With the increase in LC3-II/LC3-I protein ratio, the expression of p62 decreases, indicating an increase in autophagy flux. The present results demonstrated a significant decrease in the LC3-II/LC3-I protein ratio and a significant increase in p62 after OGD/R in H9C2 cardiomyocytes. However, BYHWD glycosides effectively reversed the OGD/R-induced abnormal changes in these markers (specifically, restoring the LC3-II/LC3-I ratio and reducing p62 accumulation). These findings thus suggest that BYHWD glycosides can enhance autophagy flux and promote mitochondrial autophagy.

Notably, the biological mechanism of mitophagy conceptually reflects the TCM principles of BYHWD. In TCM theory, BYHWD is prescribed as is it believed to tonify qi, remove blood stasis and clear pathological products (51). At the subcellular level, these traditional concepts can be translated into precise biological equivalents: The macroscopic concept of ‘blood stasis’ corresponds microscopically to the physical accumulation of undegraded cellular debris and dysfunctional organelles (such as damaged mitochondria), while ‘pathological products’ represent toxic metabolic byproducts (such as excessive ROS). By promoting mitophagy, the BYHWD glycoside-enriched fraction effectively clears these damaged organelles, thereby restoring cellular homeostasis. This represents a notable intersection between modern molecular clearance mechanisms and traditional macroscopic TCM theories.

Despite these promising findings, the present study exhibits a number of limitations. First, without the use of specific autophagy inhibitors (such as 3-methyladenine) or PINK1/Parkin genetic knockdown models, the present data remain primarily observational. Therefore, the present results demonstrate an association rather than a definitive causal relationship between mitophagy promotion, ROS reduction and the protective effects of the BYHWD fraction. Second, the present study was conducted exclusively using the H9C2 cardiomyoblast cell line. It must therefore be acknowledged that there are physiological and metabolic differences between these cells and primary adult cardiomyocytes. Unlike terminally differentiated adult cardiomyocytes that depend on fatty acid oxidation, H9C2 cells are proliferating embryonic myoblasts that lack mature sarcomeres and rely heavily on glycolysis (52). As a result, their autophagic flux and response to oxidative stress may not fully replicate the behavior of mature heart tissue. Therefore, the cardioprotective mechanisms observed in the present *in vitro* model should be interpreted carefully when considering their translational potential. To address these limitations, future research should aim to incorporate rigorous loss-of-function experiments with specific pharmacological inhibitors and small interfering RNA knockdown techniques to establish direct causality. In addition, there are plans to validate the present findings using primary adult rat ventricular myocytes and *in vivo* MIRI models, such as left anterior descending coronary artery ligation, to further evaluate the systemic effects of the BYHWD fraction.

In conclusion, to the best of our knowledge, the present findings indicated for the first time that BYHWD glycosides can effectively inhibit OGD/R-induced oxidative damage in

H9C2 cells, promote mitochondrial autophagy and stabilize mitochondrial function. This mechanism may be associated with the classical mitochondrial autophagy pathway of PINK1/Parkin (Fig. 7). The present results are therefore important in the clinical application and validation of BYHWD glycosides in treating MIRI.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XJH was responsible for study conceptualization, project administration, providing supervision and writing, reviewing and editing the manuscript. JJF was responsible for study conceptualization, data visualization and figure preparation, methodology and protocol design, conducting the investigation, curating data, performing formal analysis and writing the original manuscript draft. TS was responsible for methodology development, conducting the investigation, performing formal analysis, validation of experimental reproducibility, and writing the original manuscript draft. JHF was responsible for methodology design and conducting the investigation. FMZ contributed to study conceptualization and data interpretation, provided supervision and wrote, reviewed and edited the manuscript. MYZ conducted the investigation by performing molecular and cellular assays, contributed to data interpretation, provided supervision, and wrote, reviewed and edited the manuscript. ZSD was responsible for study conceptualization and the provision of essential resources, acquired the funding and wrote, reviewed and edited the manuscript. JJF, TS, JHF, MYZ and XJH confirm the authenticity of all the raw data. All authors have read and approved the final version of the 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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