

Mongolian Medicinal Formula Anshen-Buxin-Liuwei Pill Alleviate Cardiomyocyte Hypoxia/Reoxygenation Injury via Mitochondrion Pathway

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Abstract

Anshen Buxin Liuwei pill (ABLP), a Mongolian medicinal formula, composed of the six medicinal materials of Mongolian medicine *Bos taurus domesticus* Gmelin, *Choerospondias axillaris* (Roxb.) Burt et Hill, *Myristica fragrans* Houtt., *Eugenia caryophyllata* Thunb., *Aucklandia lappa* Decne., *Liqui dambar formosana* Hance, is considered to have a therapeutic effect on the symptoms such as coronary heart disease, angina pectoris, arrhythmia, depression and irritability, palpitation, and shortness of breath. Therefore, the present study was employed a network pharmacology approach to identify the potentially active ingredients and to evaluate the protective effect of ABLP on hypoxia/reoxygenation (HR)-induced H9c2 cardiomyocytes, and its influence on cell viability, apoptosis, oxidative stress. H9c2 cardiomyocytes were used to construct a HR injury model. CCK-8 assay and AnnexinV-FITC cell apoptosis assays were used for cell viability and cell apoptosis determination. The levels of LDH, SOD, MDA, CAT, CK, GSH-Px, $\text{Na}^+\text{-K}^+\text{-ATPase}$, and $\text{Ca}^{2+}\text{-ATPase}$ in the cells were determined to assess the effect of ABLP. the mRNA levels of Sirtuin3 (Sirt3) and Cytochrome C (CytC) in H9c2 cells were determined by quantitative real-time PCR. The finding of this study indicates that HR treatment cells began to shrink from the spindle in an irregular shape with some floated in the medium, well by increasing the therapeutic dose of ABLP (5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, and 50 $\mu\text{g/mL}$), the cells gradually reconverted in a concentration-dependent manner. The release of CK in HR-treated cells was significantly increased, indicating that ABLP exerts a protective effect in H9c2 cells against HR injury and can improve the mitochondrial energy metabolism and mitochondrial function integrity. The present study scrutinized the cardio-protective effect of ABLP against the HR-induced H9c2 cells injury through antioxidant and mitochondrion pathways. ABLP could be a promising therapeutic drug for the treatment of myocardial ischemic cardiovascular disease. The results will provide reasonable information for clinical use of the ABLP.

Introduction

Coronary heart disease (CHD) is the leading cause of death and disability worldwide [1]. hypoxia/reoxygenation(HR)-induced tissue injury progress stepwise involving the ischemia and reperfusion phases. The no-reflow phenomenon seriously endangers the therapeutic effect of reperfusion therapy [2]. Therefore, it is of great importance to find an effective and secure therapeutic solution to reduce the myocardial HR injury.

With the progress of medical treatment, an increasing number of patients with myocardial infarction are acceptable to receive therapy of traditional medicine. As one of the most important part of traditional Chinese medicine (TCM), traditional Mongolian medicine (TMM) system is also being practiced like other ethnic or traditional minority medicines in China. The origin of TMM belongs to the early 13th century [3]. TMM has been used to prevent and cure cardiovascular disease for many years using various herbal formulas and has lately been the subject of formal clinical studies [4]. Anshen Buxin Liuwei pill (ABLP), a Mongolian medicinal formula, was firstly recorded in the Compilation of Mongolian medicine prescriptions. TMM medical records consider that ABLP has a therapeutic effect on the symptoms such as coronary heart disease, angina pectoris, arrhythmia, depression and irritability, palpitation, and short

breath. It has consisted of 6 medicinal materials include *Bos taurus domesticus* Gmelin (named “Niu-Xin” in Chinese), *Myristica fragrans* Houtt (named “Rou-Dou-Kou” in Chinese), *Choerospondias axillaris* (Roxb.) Burt et Hill (named “Guang-Zao” in Chinese), *Eugenia caryophyllata* Thunb (named “Ding-Xiang” in Chinese), *Aucklandia lappa* Decne (named “Mu-Xiang” in Chinese), *Liquidambar formosana* Hance (named “Feng-Xiang-Zhi” in Chinese). The formula was prescribed based on TMM theory. It could inhibit disorder *Heyi*, a special substantive in TMM which means human spirit and consciousness. *Heyi* exists in all the organs of the body. Disturbance of *Heyi* leads to various diseases. *Bos taurus domesticus* Gmelin is the main constituent and contributes to the efficiency of tonifying the heart and relieving pain. *Choerospondias axillaris* (Roxb.) Burt et Hill is the assisted constituents to help patients keep cardiogenic and calm. *Myristica fragrans* Houtt and *Eugenia caryophyllata* Thunb could inhibit cardiac and venous *Heyi* disturbance. *Liquidambar formosana* Hance and *Aucklandia lappa* Decne could regulate the cardiovascular system and have the effect of analgesia. In a word, The compatibility of the above medicine could inhibit *Heyi* disturbance, easing pain, relieving depression, and tonifying the heart, thus leading to the major function of heart tinging and cardiopalmus. A clinical study revealed that ABLP could improve hemodynamics, thus relieve chest tightness, breathe hard, lacking of strength, repeated pain, etc in the patients [5]. Although plenty of clinical experience of ABLP on coronary heart disease has been accumulated, the mechanism remains uncertain.

The present study was thus conducted to evaluate the protective effect of ABLP on HR-induced H9c2 cardiomyocytes. The influence on cell viability and apoptosis, oxidative stress, the expression of Sirtuin 3 (Sirt3) and Cytochrome C (CytC), intercellular $\text{Na}^+\text{-K}^+\text{-ATPase}$, $\text{Ca}^{2+}\text{-ATPase}$ as well as mitochondrion function were detected. The results will provide reasonable information for the clinic's use of the TMM formula.

Material And Methods

Materials

ABLP was provided by (Wulan Haote Zhongmeng Pharmacy Co., Ltd, Inner Mongolia, China. No: 190722). According to the original composition of this formula, four fundamental components were detected according to 2020 China Pharmacopoeia by an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Thermo Fisher Scientific Dionex, Sunnyvale, CA, USA). The contents of compounds in ABLP were about 0.34mg/g (Dehydrodiisoeugenol), 1.85mg/g (Costunolide), 1.88mg/g (Dehydrocostuslactone) and 0.64mg/g (Gallic acid). Among the four compounds, Dehydrocostuslactone was the most abundant compound in the ABLP and were shown to possess various biological and pharmacological activities such as anti-inflammatory, antiulcer, antiviral, and hepatoprotective effects [6].

Cell culture and Hypoxia/reoxygenation treatment

Rat-derived H9c2 cardiomyocytes were purchased from the China center for type culture collection (CCTCC). Cells were cultured in DMEM supplemented with 15% FBS and 1% antibiotic (100 U/ml

Penicillin and 100 g/ml Streptomycin) at 37 °C in 5% CO₂. As for hypoxia, cells were transferred to the hypoxic chamber (Modular Incubator Chamber) maintained with 95% N₂ and 5% CO₂ at 37 °C and incubated for 4h. The reoxygenation was achieved by returning cell cultures to the ambient 5% CO₂ atmosphere and the medium was replaced with a normoxic complete medium for 4-24 h. The time of 24 h reoxygenation was selected according to the results. The H9c2 cells were subjected to 4 h of hypoxia and then incubated with or without ABLP for 24 h of reoxygenation (To seen in Fig. 1A).

Cell viability assessment

Cell viability was determined by CCK-8 assay according to the instruction of the commercial kit. The results were qualified by determining the absorbance at 450 nm using a microplate reader.

Real-time cellular analysis

H9c2 cardiomyocytes were seeded on E16-plate (ACEA Biosciences, San Diego, USA) at 1×10^5 cells/well for evaluation of proliferation. Real-time cell proliferation analysis was performed using the xCELLigence RTCA S16 system (Agilent Technologies, USA), and real-time changes in electrical impedance were measured and expressed as cell index (CI). Cell growth index at each time point after cell treatment was normalized.

Determination of LDH, CK, and oxidative stress indexes

The LDH and CK, as well as oxidative stress indexes, include SOD, CAT, and GSH-Px activities as well as MDA content in the cells were determined commercial kits (Jiancheng Biotechnology, Nanjing, China) according to the manufacturer's directions.

Detection of Na⁺-K⁺-ATP and Ca²⁺-ATP activity

The enzyme of Na⁺-K⁺-ATP and Ca²⁺-ATP were detected using commercial kits (Jiancheng Biotechnology, Nanjing, China) according to the manufacturer's directions. The results were qualified by determining the absorbance at 660 nm using a microplate reader (TECAN A-5082, Magellan, AUSTRIA).

Assessment of apoptosis and necrosis

After treatment, the H9c2 cells were collected after centrifugation at 2000 r·min⁻¹ for 5 min. The cells were treated according to the instructions of the manufacturer using Annexin V-FITC cell apoptosis assay kit. Then, a FACS Calibur flow cytometer (FACS Calibur™, BD Biosciences, San Jose, CA, USA) was used to evaluate cell apoptosis and necrosis.

Assessment of reactive oxygen species and mitochondrial membrane potential (ΔΨ_m)

Cells were seeded in confocal dish (Corning, USA) at a density of 5×10^4 and cultured under either hypoxic (0.5% O₂) or normoxic (20% O₂) conditions. Mitochondrial membrane potential (ΔΨ_m) of the

cells were measured following instructions by the manufacturers of respective assay kits. To determine $\Delta\Psi_m$, cells were stained with JC-1 staining solution (5 $\mu\text{g}/\text{ml}$) for 20 min at 37 °C. $\Delta\Psi_m$ was measured after washing the cells twice with JC- washing buffer [7]. All samples were analysed using the FACS Calibur flow cytometer (FACS Calibur™ BD Biosciences, San Jose, CA, USA) .

Real-time quantitative polymerase chain reaction (qPCR)

Quantitative real-time PCR was used to determine the mRNA levels of Sirt3 and Cyt c in H9c2 cells. Total RNA was extracted from the cells using Trizol Reagent according to the manufacturer's instructions. Then, total RNA was reverse-transcribed to cDNA using an SYBR®primeScript®RT-PCR reagent kit with gDNA Eraser (TaKaRa, Japan). The master mix (10 μl) included: PrimerScript RT Enzyme Mix I (1.0 μl), RT Primer Mix^{*4} (1.0 μl), 5×PrimeScript Buffer 2 (for real-time) (4.0 μl), and RNase Free ddH₂O (4.0 μl). PCR amplification was performed as follows: Stage 1, preheat denaturation at 95° C for 40 sec, Stage 2, circulatory system cycled 40 times at 95°C for 15 sec and 58°C for 1 min. Each sample was measured three times. The expression levels of the above genes were normalized to those of β -actin and measured by the comparative 2^{-DDCt} method. The forward and reverse sequences for the primers are listed in Table 1.

Statistical analysis

Data analysis and calculation of standard deviation were done by SPSS 22.0 (IBM, US). The results were presented as means $\pm$ SEM. The data comparison was performed by using a one-way analysis of variance (ANOVA) followed by Dunnett's test to detect inter-group differences. A probability (P) value of less than 0.05 was considered statistically significant.

Result

ABLP inhibited HR-induced cell death in H9c2 cardiomyocytes

As shown in Fig. 1B, time-dependent damage occurred in HR injury cells. 24 h of reoxygenation after 4 h of hypoxia led to a 27% decrease in cell viability. Based on this, we chose HR treatment at a time of 4 h hypoxia and 24 h of reoxygenation for further evaluation. To evaluate the cardioprotective effects of ABLP against HR-treatment cardiotoxicity, HR-stimulated H9c2 cells were treated with different concentrations of ABLP and cell viability was assessed using CCK-8 assay and RTCA. Stimulation with HR led to a significant decrease in H9c2 cell viability, which was significantly reversed by the treatment with ABLP in a dose-dependent manner. Under the microscope, the normal H9c2 cells were sleek and legible with a spindle shape. HR treatment cells started to shrink from spindle to **irregular** shape with some **floated** in the medium. With the increase in the treatment dose to 5 $\mu\text{g}/\text{mL}$, 25 $\mu\text{g}/\text{mL}$, and 50 $\mu\text{g}/\text{mL}$ of ABLP, the cells were **reconverted** gradually in concentration-dependent manners (To seen in Fig. 2A). LDH leakage in cells is a significant indicator of the damage to the cell membranes during HR injury. HR treatment could induce a significant decrease in cell viability and LDH content, indicating cell damage

of H9c2 cells. 1-200 µg/mL of ABLP did not influence H9c2 cell viability. However, ABLP (5-50 µg/mL) could dose-dependently protect the cells against HR-induced injury.

The release of CK in HR-treated cells was markedly increased. However, ABLP treatment significantly restored these effects, indicating that isoquercetin exerts a dose-dependent protective effect in H9c2 cells against HR injury. The results were presented in Fig. 2.

Table 1 Primers used in this study

Gene	Species	Forward(5'-3')	Reverse(5'-3')	Annealing Temperature
Sirt3	Rat	GCCTACTCAAGGAGGTCTG	TGTTTATGCCTGGTGCTG	57°C
Cytc	Rat	AGGCAAGCACAAGACTGGA	CTGACTATCACCAAGAACCACC	57°C
β-actin	Rat	ACCGTGAAAAGATGACCCAG	TCTCAGCTGTGGTGGTGAAG	57°C

ABLP alleviates HR induced oxidative stress in H9c2 cells

Oxidative stress is an important cause to induce H9c2 cell injury. As shown in Fig. 3, The SOD, CAT, and GSH-Px activities markedly decreased as well as MDA content increased in HR induced H9c2 cells, which could be attenuated by ABLP in a dose-dependent manner.

ABLP attenuates the apoptosis induced by HR injury in H9c2 cells

The result of the flow cytometer showed that HR treatment led to prominent cell apoptosis and necrosis in H9c2 cells (Fig. 4). ABLP (5-50 µg/ml) could dose-dependently prevent cell apoptosis and increase cell survival rates.

ABLP effectively protected mitochondria membrane potential from HR injury

As shown in Fig. 4, ΔΨm of H9c2 cells were decreased dramatically after HR treatment. ABLP (5-50 µg/ml) could dose-dependently attenuate the decrease of MMP, demonstrating the protective effects of ABLP against HR injury by maintaining ΔΨm (Fig. 4).

ABLP significantly up-regulate gene levels of Na⁺-K⁺-ATPase, Ca²⁺-ATPase, Sirt3 and inhibit the intracellular release of Cytc

As shown in Fig. 5, the gene levels of Na⁺-K⁺-ATPase, Ca²⁺-ATPase, andSirt3 decreased notably in HR induced model group compared with the control group. At the same time, the Cytc was released from mitochondrion into the cytosol, indicating the mitochondria damage induced by HR. ABLP

could significantly up-regulate gene levels of Na⁺-K⁺-ATPase, Ca²⁺-ATPase, Sirt3 and inhibit the intracellular release of Cyt c, thus protect the mitochondria in HR-induced cell injury.

Discussion

Myocardial ischemic heart disease is a common cardiovascular disease and it is having a higher incidence of mortality and morbidity worldwide. The specific mechanism of HR injury is complicated but mainly caused by calcium overload, inflammation, oxidative stress, and so on [8]. Various research has been explored to scrutinized the cardioprotective drug. It is well known that myocardial ischemia-reperfusion is an effective treatment for acute myocardial ischemia, which can decrease the size of myocardial infarction and release the symptoms of heart disease such as heart failure [9, 10]. Although previous research showed the cardio-protective effect of ABLP in a clinic [11, 12], the essential mechanism of the myocardial protection of ABLP has not yet been fully explained. In the current experimental study, we explored the preventive effects of ABLP against the HR-induced myocardial cell injury and the possible mechanism of action.

Apoptosis of cardiac myocardial injury induced via oxidative stress and inflammation plays an important role in the pathogenesis of ischemic heart disease, heart disease, and HR injury [13, 14]. Moreover, the preclusion of oxidative stress and inflammation-induced apoptosis may serve as a favorable effect for the treatment of myocardial injury [15]. Mitochondrial injury is associated with oxidative stress and loss of respiratory chain enzyme coupling. In the current investigation, we observed that the HR significantly enhanced oxidative indices and induced H9c2 cell damage and pathological changes, enhanced MDA production, and decreased the level of SOD, CAT, and GSH-Px. Dose-dependent treatment of ABLP significantly ($P < 0.05$) decreased the level of MDA and increased the level of SOD, CAT, and GSH-Px in the H9c2 cells. Our result revealed that the ABLP considerably reduced the oxidative stress in the H9c2 cells, suggesting that ABLP reduced the myocardial injury via inhibition of oxidative stress.

Cardiomyocytes are rich in mitochondria. The release of Cyt c from the mitochondria to the cytoplasm as a result of the increased mitochondrial membrane permeability can trigger apoptosis [16]. The Sirtuin (Sirt) family is a highly conserved and dependent nicotinamide adenine dinucleotide (NAD⁺) histone deacetylase comprising of seven members (Sirt1-Sirt7) in humans, among which three (Sirt3, Sirt4 and Sirt5) are exclusively localized to mitochondria [17]. As the most crucial one, Sirt3, a class III histone deacetylase in mitochondria [18], has been proved to be a versatile molecule in modulating mitochondrial function such as metabolic enzyme activity, oxidative phosphorylation efficacy, anti-oxidant machinery as well as mitochondrial dynamics [19].

Sirt3 can also maintain the redox balance and metabolism of mitochondria under physiological and pathological conditions plays a crucial role in the pathogenesis of cardiovascular diseases [20]. In most mammalian cells, sodium potassium Na⁺-K⁺-ATPase is an energy-transducing ion pump across the plasma membrane [21]. Na⁺ and K⁺ channels are closely related to electro cardio and are involved in maintaining and stabilizing electrical activity features of myocardial cells and myocardium [22]. Na⁺-K⁺-

ATPase is the key enzyme of membrane and participates in active transmembrane transport of $\text{Na}^+\text{-K}^+$ and maintains the concentration gradient of high intracellular sodium and low extracellular potassium. Intracellular Ca^{2+} homeostasis is maintained by the mitochondria through the Ca^{2+} -ATPase action on the endoplasmic reticulum and the intracellular Ca^{2+} pool. The free $[\text{Ca}^{2+}]$ in mitochondria is closely related to the proper functioning of mitochondria [23].

The study showed that HR treatment could decrease the MMP in H9c2 cells, which could be reversed by ABLP treatment via up-regulating $\text{Na}^+\text{-K}^+$ -ATPase, Ca^{2+} -ATPase, Sirt3, and inhibiting the intracellular release of Cytc, thus protect the mitochondria against HR induced cell injury.

In all, our work indicated that ABLP exhibited convincing pharmacological effects inhibiting the production of oxidative indices and protect mitochondrion pathways via multi-target agents. The present investigation was undertaken to further study the therapeutic potential of ABLP and further explore the underlying mechanisms involved.

Conclusion

The present study scrutinized the cardio-protective effect of ABLP against the HR-induced H9c2 cells injury via antioxidant and mitochondrion pathways. ABLP could be a promising therapeutic drug against myocardial ischemic cardiovascular disease. A further detailed investigation needs to be conducted to elucidate the mechanisms involved in the cardio-protective effect of ABLP in detail.

Declarations

Disclosure of conflict of interest

The authors declare that there are no conflicts of interest.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

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Author contributions

Hai-Ying Tong and Xian-Ju Huang contributed to the conception of the study;

Yu-Jia Huang performed the experiment;

Yu-Jia Huang and Xian-Ju Huang contributed significantly to analysis and manuscript preparation;

Yu-Jia Huang and Xian-Ju Huang performed the data analyses and wrote the manuscript;

Xin-Cai Xiao, Yue Dong and Muhammad Sarfaraz Iqbal helped perform the analysis with constructive discussions.

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Ethical Statement for Molecular and Cellular Biochemistry

I testify on behalf of all co-authors that our article submitted to Molecular Biology Reports.

Title: Mongolian medicinal formula Anshen-Buxin-Liuwei pill alleviate cardiomyocyte Hypoxia/reoxygenation injury via mitochondrion pathway.

All authors: .

- 1) This material has not been published in whole or in part elsewhere;
- 2) The manuscript is not currently being considered for publication in another journal;
- 3) All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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Figures

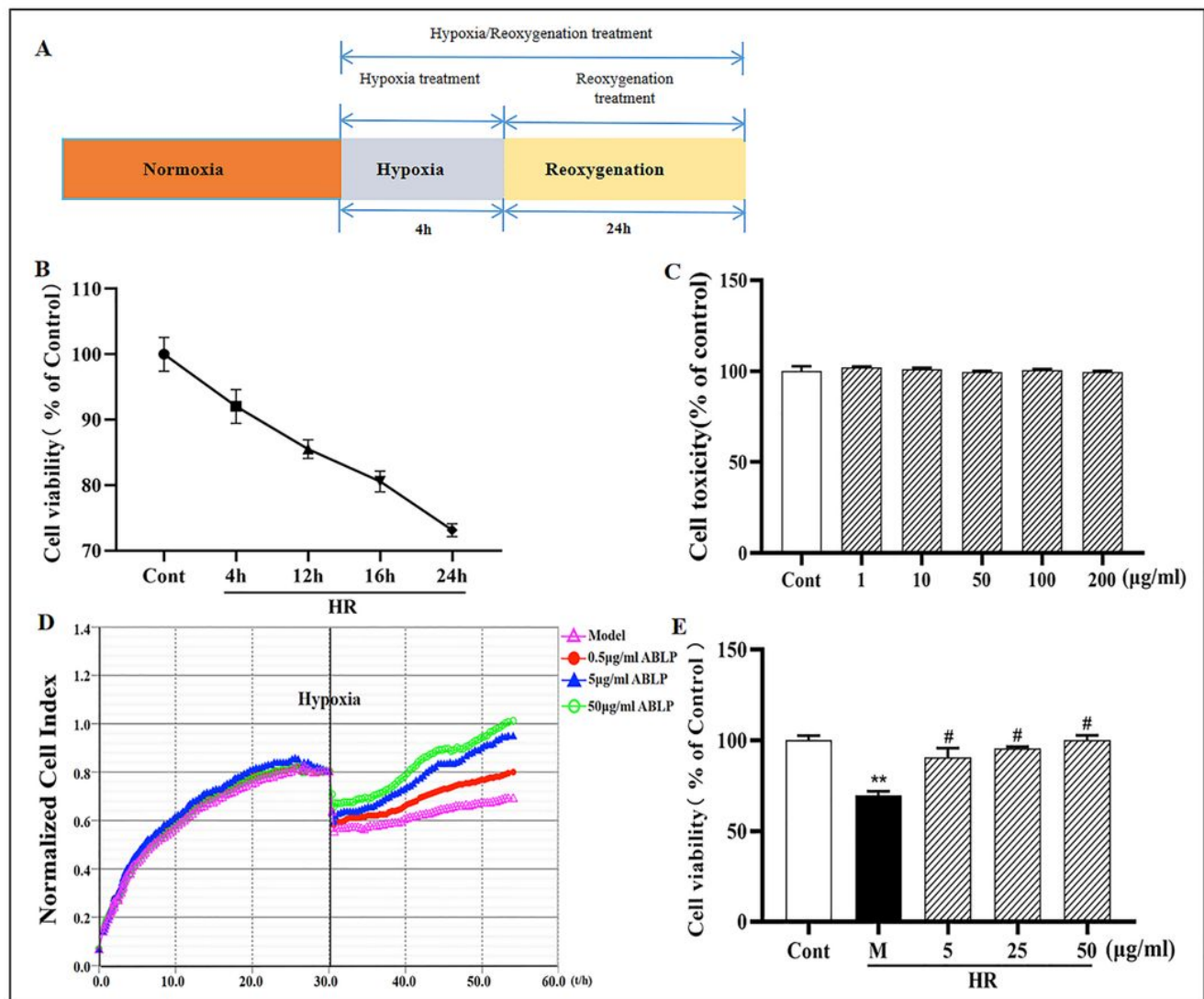


Figure 1

The effects of ABLP on the cell viability in the HR-injured H9c2 cells. "Cont" means Control group and "M" means Model group. (A) The experimental process of HR and drug treatment. (B) The influence of different reoxygenation times of HR on the cell viability. (C) The influence of different concentrations of ABLP on the cell viability. (D) RTCA technology analysis of proliferation of H9c2 cells using E-16 plates; (E) The effects of ABLP treatment on the cell viability after HR injury. n=6 (n, the number of experiments), *P< 0.05 versus the control group, **P< 0.01 versus the control group, #P< 0.05 versus the model group, ##P< 0.01 versus the model group.

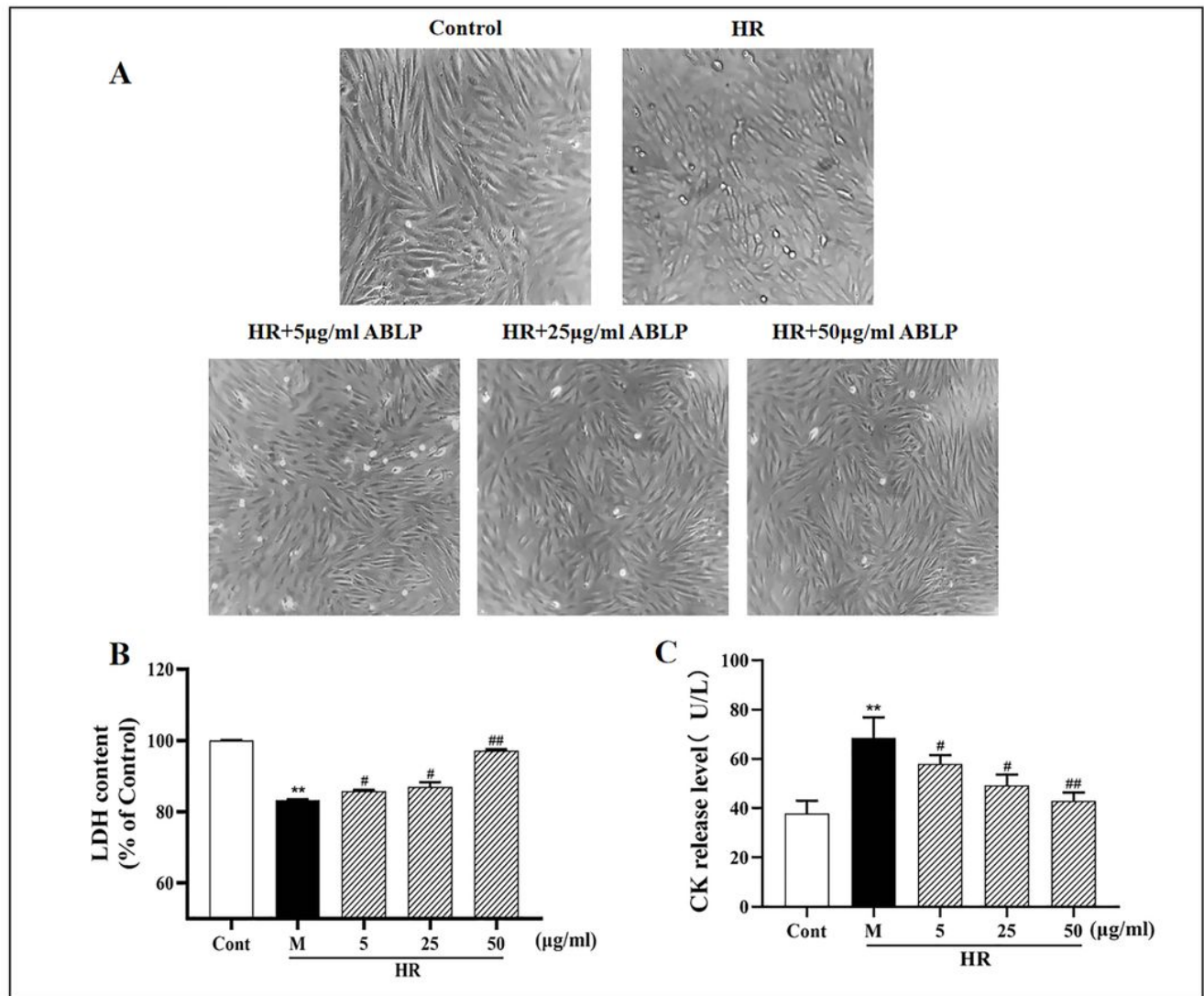


Figure 2

Results of electron microscopy and functional indexes of H9c2 cells. (A) Changes in cell morphology. (B) The effects of ABLP on the LDH activity after HR injury. (C) The effects of ABLP on the CK release after HR injury. $n=6$, * $P < 0.05$ versus the control group, ** $P < 0.01$ versus the control group, # $P < 0.05$ versus the model group, ## $P < 0.01$ versus the model group.

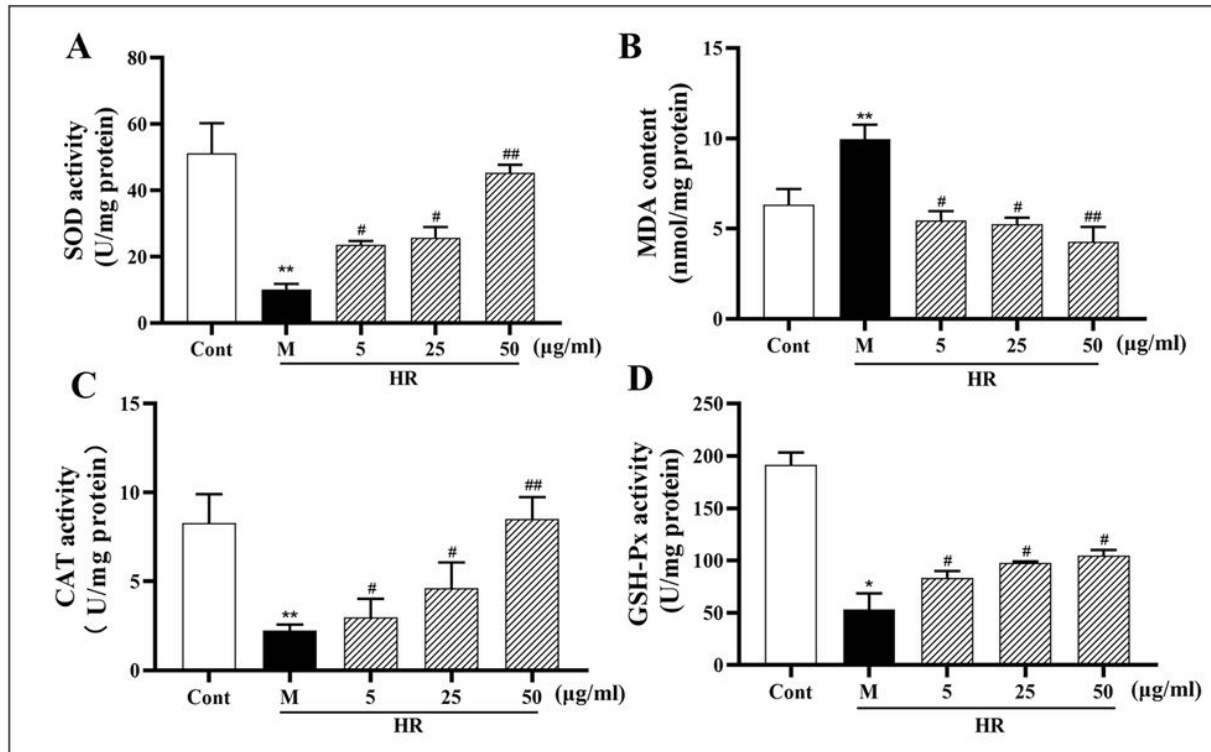


Figure 3

The effects of ABLP on the HR-induced oxidative indexes in H9c2 cells. (A) The effects of ABLP on the SOD activity after HR injury. (B) The effects of ABLP on the MDA content after HR injury. (C) The effects of ABLP on the CAT activity after HR injury. (D) The effects of ABLP on the GSH-Px activity after HR injury. $n=6$, * $P < 0.05$ versus the control group, ** $P < 0.01$ versus the control group, # $P < 0.05$ versus the model group, ## $P < 0.01$ versus the model group.

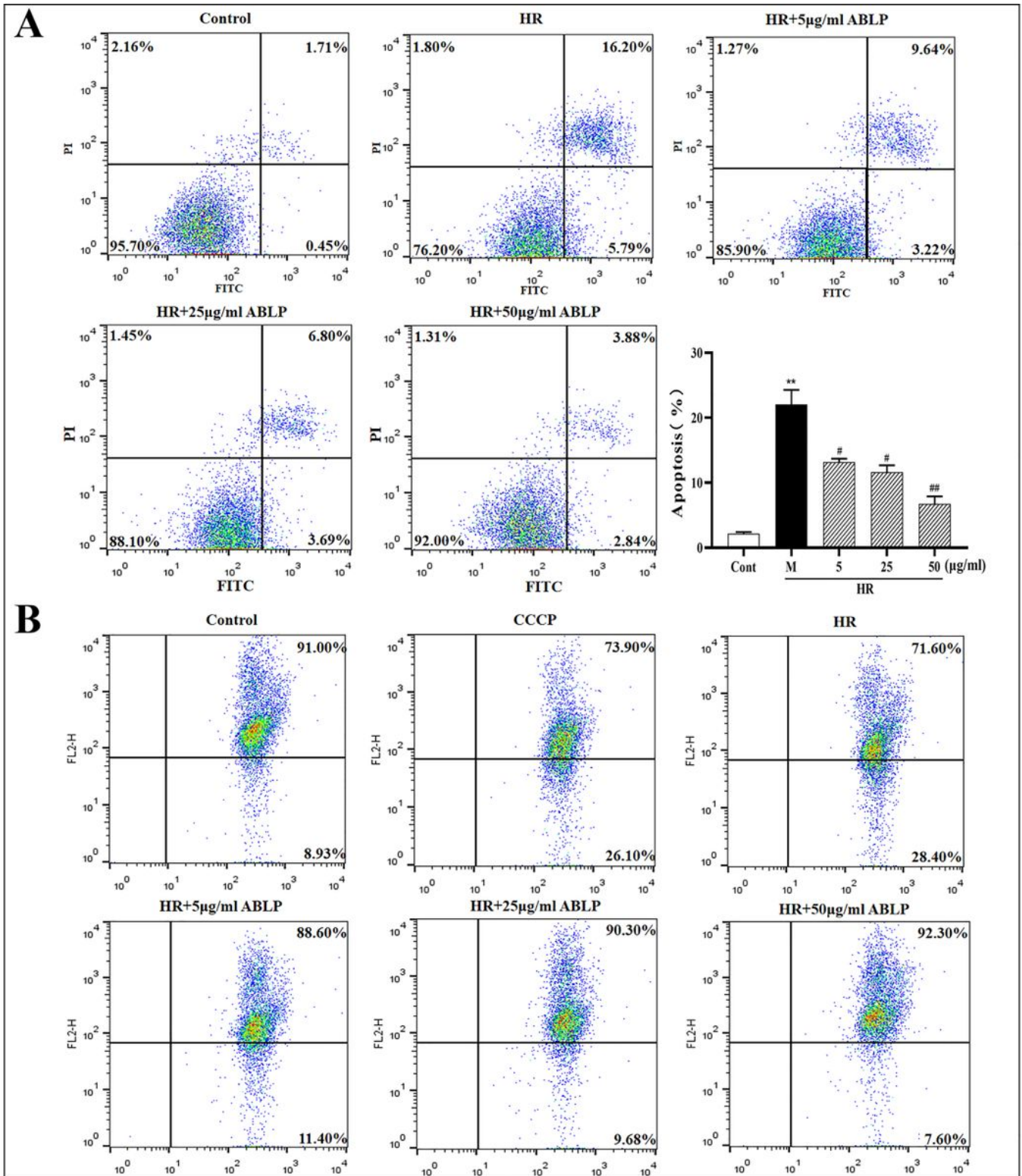


Figure 4

Detection of apoptosis and MMP levels in H9c2 cells by Flow Cytometry after HR injury. (A) The effects of the subtracted prescriptions of ABLP on the apoptosis induced by HR was determined by annexin V/PI double staining using flow cytometry. (B) The MMP levels are evaluated by the ratio of fluorescence in the upper right quadrant and lower right quadrant. $n=4$, $**P < 0.01$ versus the control group, $\#P < 0.05$ versus the model group, $\#\#P < 0.01$ versus the model group.

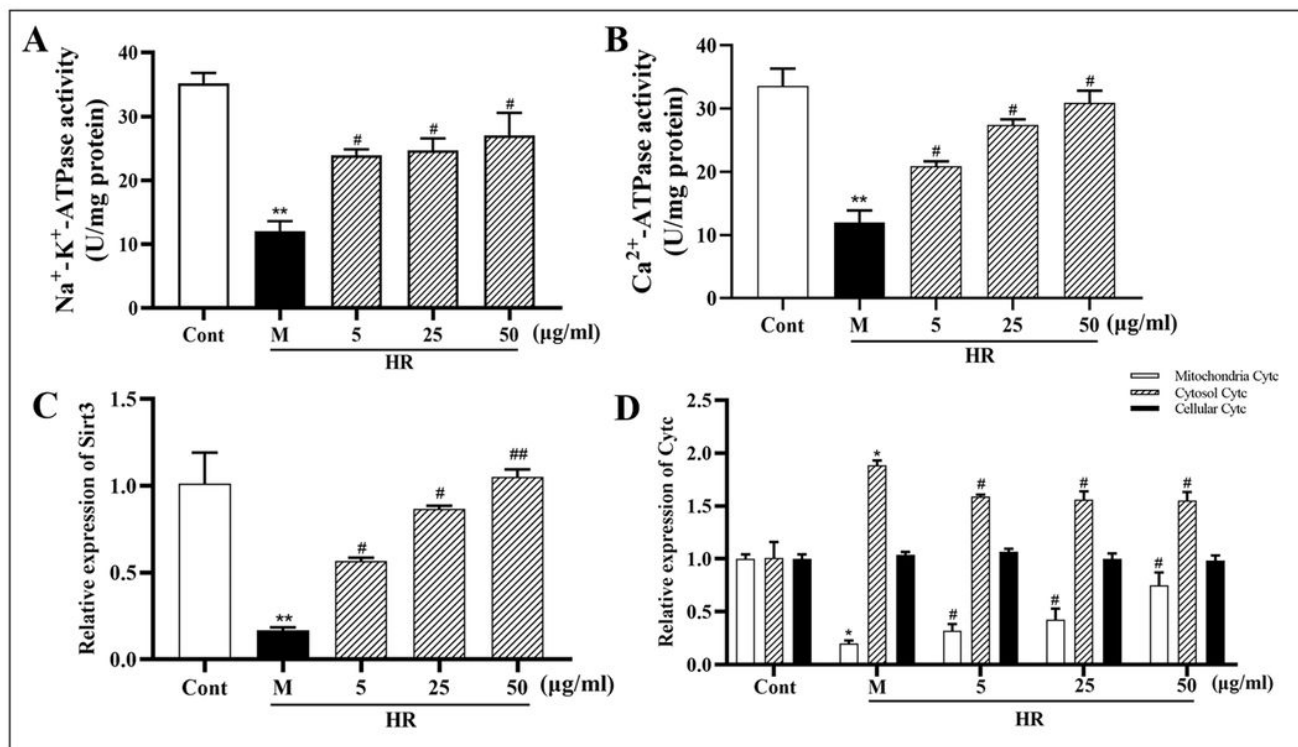


Figure 5

The effects of ABLP on the Na⁺-K⁺-ATPase activity, Ca²⁺-ATPase activity and the expressions of Sirt3 and Cytc after HR injury. (A) The effects of ABLP on the Na⁺-K⁺-ATPase activity after HR injury. (B) The effects of ABLP on the Ca²⁺-ATPase activity after HR injury. (C) Relative expression of Sirt3 in cells. (D) Relative expression of Cytc in Mitochondria and Cytosol of cells. n=6, *P< 0.05 versus the control group, **P< 0.01 versus the control group, #P< 0.05 versus the model group, ##P< 0.01 versus the model group.

Supplementary Files

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- [GraphicalA.png](#)