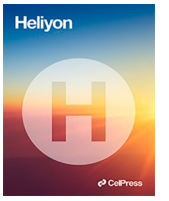
Heliyon 10 (2024) e33371

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Queen bee acid pretreatment attenuates myocardial ischemia/reperfusion injury by enhancing autophagic flux

Changhai Chen [a,b,d,e,1], Wen Ou [a,d,e,1], Chaobo Yang [a,d,e], Haiqiong Liu [c,d,e], Tao Yang [a,d,e,f], Huaqiang Mo [a,d,e], Weizhe Lu [a,d,e], Jing Yan [a,d,e,**], Aihua Chen [c,d,e,*]

[a] Department of Cardiology, Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China
[b] Department of Cardiology, The Affiliated Suqian First People's Hospital of Nanjing Medical University, Suqian, Jiangsu, People's Republic of China
[c] Department of Health Management, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China
[d] Laboratory of Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China
[e] Guangdong Provincial Key Laboratory of Shock and Microcirculation, Southern Medical University, Guangzhou, Guangdong, People's Republic of China
[f] Department of Cardiovascular Medicine, The Affiliated Changsha Central Hospital, Hengyang Medical School, University of South China, Changsha, People's Republic of China

ARTICLE INFO

Keywords:
Queen bee acid
Myocardial ischemia/reperfusion
Autophagic flux
Apoptosis

ABSTRACT

Queen bee acid (QBA), which is exclusively found in royal jelly, has anti-inflammatory, anti-hypercholesterolemic, and antiangiogenic effects. A recent study demonstrated that QBA enhances autophagic flux in the heart. Considering the significant role of autophagy in the development of myocardial ischemia/reperfusion (I/R) injury, we investigated the effect of pretreatment with QBA on myocardial damage. In an in vivo model, left coronary artery blockage for 30 min and reperfusion for 2 h were used to induce myocardial I/R. In an in vitro model, neonatal rat cardiomyocytes (NRCs) were exposed to 3 h of hypoxia and 3 h of reoxygenation (H/R). Our results showed that pretreatment with QBA increased the cell viability of cardiomyocytes exposed to H/R in a dose-dependent manner, and the best protective concentration of QBA was 100 μM. Next, we noted that QBA pretreatment (24h before H/R) enhanced autophagic flux and attenuated mitochondrial damage, cardiac oxidative stress and apoptosis in NRCs exposed to H/R injury, and these effects were weakened by cotreatment with the autophagy inhibitor bafilomycin A1 (Baf). In addition, similar results were observed when QBA (10 mg/kg) was injected intraperitoneally into I/R mice 30 min before ischemia. Compared to mice subjected to I/R alone, those treated with QBA had decreased myocardial infarct area and increased cardiac function, whereas, these effects were partly reversed by Baf. Notably, in NRCs exposed to H/R, tandem fluorescent mRFP-GFP-LC3 assays indicated increased autophagosome degradation due to the increase in autophagic flux upon QBA treatment, but coinjection of Baf blocked autophagic flux. In this investigation, no notable adverse effects of QBA were detected in either cellular or animal models. Our findings suggest that QBA pretreatment mitigates myocardial I/R injury by

* Corresponding author. Department of Health Management, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China.
** Corresponding author. Department of Cardiology, Heart Center, Zhujiang Hospital, Southern Medical University, Guangzhou, Guangdong, People's Republic of China.
E-mail addresses: 530305557@qq.com (J. Yan), chenaih@smu.edu.cn (A. Chen).
[1] These authors contributed equally.

https://doi.org/10.1016/j.heliyon.2024.e33371
Received 20 January 2024; Received in revised form 19 June 2024; Accepted 20 June 2024
Available online 21 June 2024

eliminating dysfunctional mitochondria and reducing reactive oxygen species via promoting autophagic flux.

1. Introduction

At present, acute myocardial infarction (AMI) is the greatest cause of illness and mortality in the human population [1,2]. To mitigate acute myocardial ischemia injury and reduce infarct size (IS), patients with AMI are treated with prompt and efficient myocardial reperfusion using primary percutaneous coronary intervention [3]. However, reperfusion after myocardial ischemia can induce further cardiomyocyte injury, which is called myocardial ischemia–reperfusion injury (MIRI) [4]. Unfortunately, there are limited effective treatments for ischemia/reperfusion (I/R)-induced cardiac cell damage [5]. Therefore, effective treatments are needed to alleviate the complications of myocardial reperfusion injury.

Under stressful circumstances, autophagy—a well-known conserved degradative pathway—is quickly accelerated to maintain cellular homeostasis [6]. Autophagy has been suggested to be an important mechanism involved in myocardial I/R. In fact, accumulating data have shown that autophagy activation protects the heart against I/R injury [7–9]. However, some studies have shown that the inhibition of autophagy may attenuate cell death and preserve cardiomyocyte viability during I/R injury [10,11]. Some studies suggest that the role of autophagy in I/R injury is highly context-dependent. During the early phase of I/R injury, autophagy appears to be protective by removing damaged organelles and proteins, thus preventing cell death. However, in the later stages, excessive autophagy may lead to autophagic cell death, contributing to further tissue damage [12,13]. Therefore, the timing and extent of autophagy modulation are crucial factors that determine the overall impact of autophagy on cardiomyocyte survival. Further investigation is required to improve our understanding of the role of autophagy in myocardial ischemia-reperfusion.

The therapeutic properties of royal jelly (RJ), which is used for larval and adult queen bee nutrition, have attracted increasing interest. Previous studies have suggested that RJ or its specific components possess various pharmacological effects, including antilipidemic, antioxidant, anti-inflammatory, neuroprotective, anti-aging and immunomodulatory effects [14,15]. Hassan Malekinejad et al. demonstrated that RJ administration protects against paclitaxel-induced cardiotoxicity by suppressing oxidative and nitrosative stress [16]. Moreover, recent research has indicated that RJ administration might have an antiatherogenic effect by improving vascular endothelial function [17]. However, it is still unknown which compounds and mechanisms exert cardioprotective effects. Yang et al. showed that the lipid composition of RJ may be responsible for its biological activity [18]. Queen bee acid (QBA), which is exclusively found in RJ, accounts for up to 40 % of all fatty acids in this substance and is frequently utilized as a quality indicator of RJ [19]. Investigations have indicated that QBA possesses anti-inflammatory, neuroprotective, and anticancer properties [20,21]. Prior research has shown that QBA can reduce neuroinflammation by activating the AMPK pathway and subsequently the PI3K/AKT pathway [20]. Moreover, a recent study indicated that the induction of autophagy is the critical mechanism underlying the beneficial health effects of QBA [22]. However, it is still unknown whether QBA therapy can offer protection against myocardial I/R damage. This study aimed to investigate the potential cardioprotective effects of QBA and elucidate the underlying mechanisms, particularly focusing on the role of autophagy in myocardial I/R injury.

2. Materials and methods

2.1. Mouse I/R model

The experimental procedures at Southern Medical University were approved by the Bioethics Committee and followed the Guidelines for the Care and Use of Laboratory Animals (Approval No. LAEC-2022-114). Each animal used in the study was kept in a standard laboratory environment with a 12-h light/dark cycle. We obtained male C57BL/6 mice aged 6–8 weeks and weighing 20–25 g from the Guangdong Province Laboratory Animal Center. We administered a 50 mg/kg intraperitoneal dose of sodium pentobarbital to anesthetize the mice. I/R surgery was performed on ventilated mice using an air-powered miniventilator, as detailed in our earlier study [23]. A power analysis was conducted in the experimental setup to determine the lowest sample size needed, hence reducing the number of animals used. Subsequently, after the experiments, all animals were euthanized in a humane manner through the administration of CO_2. Based on previous studies of QBA [22] and our preliminary experiments, we administered 10 mg/kg QBA (HY–N1363, MedChemExpress, USA) intraperitoneally 30 min prior to ischemia; in the sham group, 10 mg/kg NS was injected intraperitoneally into the mice. Bafilomycin A1(Baf)(10 mg/kg; HY-100558, MedChemExpress, USA) was administered to the indicated group 1 h prior to surgery. The following six groups were evaluated (each group consisted of five animals): Sham; Sham + QBA; I/R + NS; I/R + QBA; I/R + Baf; and I/R + QBA + Baf.

2.2. Cell isolation and culture

For the rat hypoxia/reoxygenation (H/R) model, newborn rat cardiomyocytes (NRCs) were extracted from Sprague–Dawley rats aged 1–3 days, as described in our previous study [24]. Cardiomyocytes were randomly divided into six groups as follows (each condition was replicated five times): (1) Control group: cells were grown under normoxic conditions (at 37 °C in a 21 % O_2 and 5 % CO_2 environment); (2) Control + QBA group: cells were cultured under normoxic conditions and pretreated for 24 h with QBA; (3) H/R group: cells were exposed to hypoxia (at 37 °C in a 1 % O_2 and 5 % CO_2 environment) for 3 h and then cultured under standard

(normoxic) conditions for 3 h; (4) H/R + QBA group: cells were pretreated with QBA (25, 50, 100 or 200 μM) for 24 h before H/R; (5) H/R + Baf group: cells were treated with Baf (100 nM) for 1 h before H/R; (6) H/R + QBA + Baf group: cells were pretreated with Baf and QBA before H/R.

2.3. Cell survival assay

A Cell Counting Kit-8 (CCK-8) assay kit (HY–K0301, MedChemExpress, USA) was used to assess cell viability following the manufacturer's instructions. In summary, neonatal rat cardiomyocytes were seeded in 96-well plates and then subjected to different treatments for specific periods. Each well was treated with DMEM supplemented with a 10 % solution of CCK-8 and incubated for 4 h at 37 °C in the absence of light. Subsequently, the absorbance was measured at a wavelength of 450 nm utilizing a microplate reader produced by Thermo Scientific (New York, USA). The obtained readings were then adjusted by comparison to those of the control group treated with the vehicle.

2.4. Propidium iodide staining

Propidium iodide (PI) staining (PH0532, Phygene, Fuzhou, China) was carried out. Briefly, the NRCs were cultured on laser confocal dishes. After collection, the cells were washed three times with PBS. The cells were treated with PI staining solution for 15 min, and then Hoechst 33342 solution was used for counterstaining. Afterward, images were acquired using a fluorescence microscope (Olympus, Japan).

2.5. cTnT and CKMB measurements

At the designated intervals, serum samples were taken from each of the mouse groups. Following the manufacturer's instructions, an enzyme-linked immunosorbent assay kit was used to measure the levels of cardiac damage indicators, such as cardiac troponin T (cTnT, YX-E032014 M, R&D Systems, USA) and creatine kinase-MB (CK-MB, YX-E032223 M, R&D Systems, USA). A microplate reader (Thermo Scientific, NY, USA) was used to analyze the absorbance at 450 nm.

2.6. Apoptosis assay

Using TUNEL assays (C1090, Beyotime, Shanghai, China), cardiac apoptosis was quantitatively examined in accordance with the manufacturer's instructions. Fluorescence microscopy (Olympus, Japan) was utilized to visualize apoptotic nuclei. The percentage of TUNEL-stained cardiomyocytes was determined using the obtained images.

2.7. Western blot

After treatment, proteins were isolated from NRCs or heart tissue using a protein assay kit. Western blot analysis was conducted in accordance with our previously established procedures [24]. Proteins were collected from NRCs or cardiac tissues and extracted. The proteins were separated by electrophoresis and subsequently transferred onto a PVDF membrane. Next, the membrane was blocked using a 5 % nonfat milk solution at room temperature for 2 h. The membrane was then subjected to overnight incubation at 4 °C with the designated primary antibody, followed by a 2-h incubation with an HRP-conjugated secondary antibody at room temperature. Finally, a chemiluminescence solution (Englin Biosystems, Beijing, China) was used for color development. The main antibodies used in the experiment were as follows: anti-LC3 I/II (1:1000; 12741S, Cell Signaling Technology), anti-p62 (1:1000; 23214S, Cell Signaling Technology), anti-cleaved caspase-3 (1:1000; 9664S, Cell Signaling Technology), and anti-β-actin (1:10000, 4970S, Cell Signaling Technology).

2.8. Intracellular reactive oxygen species (ROS)

Using 2,7-dichlorofluorescein diacetate (DCFH-DA; D5470, Solarbio, Beijing, China), intracellular ROS were measured in accordance with the manufacturer's recommendations. In brief, the cells were seeded into laser-coated Petri plates, treated for 30 min at 37 °C with 10 nM DCFH-DA, and then counterstained using a solution that included Hoechst 33342. The cells were then washed with PBS, and images were captured using fluorescence microscopy (Olympus, Japan).

2.9. Myocardial infarct size (IS)

The infarct size (IS) was measured using Evans blue dye (E2129, Sigma–Aldrich, USA) and 2,3,5-triphenyltetrazolium chloride (TTC, 17779, Sigma Aldrich, USA), as previously described [23]. The normal myocardium is indicated by Evans blue staining, while the area at risk (AAR) is indicated by red and white staining. Notably, the red region represents viable myocardium exposed to I/R injury, and the infarct appears white. After staining, images were acquired with a camera and then examined using ImageJ. The IS was determined by dividing the IS by the AAR and expressing the result as a percentage.

2.10. Echocardiography

The mouse echocardiograms were taken using a Vevo 2100 System with a 30 MHz transducer (Fujifilm Visual Sonics, Inc., Toronto, Canada) seven days after I/R. Each mouse had its left chest denuded after being given mild 2 % isoflurane anesthesia. M-mode images of the mid-ventricular short axis were acquired [23]. After the data were collected, the ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were calculated.

2.11. Evaluation of fluorescent LC3 puncta

Morphological evidence of autophagy was obtained using mRFP-GFP-LC3 (Hanyi Bio, China). NRCs cultivated in confocal dishes were exposed to adenovirus carrying the tandem fluorescent vector mRFP-GFP-LC3 at a multiplicity of infection (MOI) of 15. After being washed with PBS, the cells were fixed with 4 % paraformaldehyde, and DAPI was used as a counterstain. The distinctive distribution of LC3 on autophagosomes was observed by the initial display of red and green fluorescence, with the appearance of yellow in merged images indicating overlapping signals. The disappearance of the green fluorescent signal from merged autophagosome-lysosomes is attributed to the instability of GFP in the acidic environment of lysosomes. Consequently, in merged images, autolysosome formation is represented by red dots that do not overlap with green dots. Then, images were captured using a Carl Zeiss confocal fluorescence microscope.

2.12. Measurement of mitochondrial membrane potential (ΔΨm)

In compliance with the manufacturer's instructions, the mitochondrial membrane potential was determined using

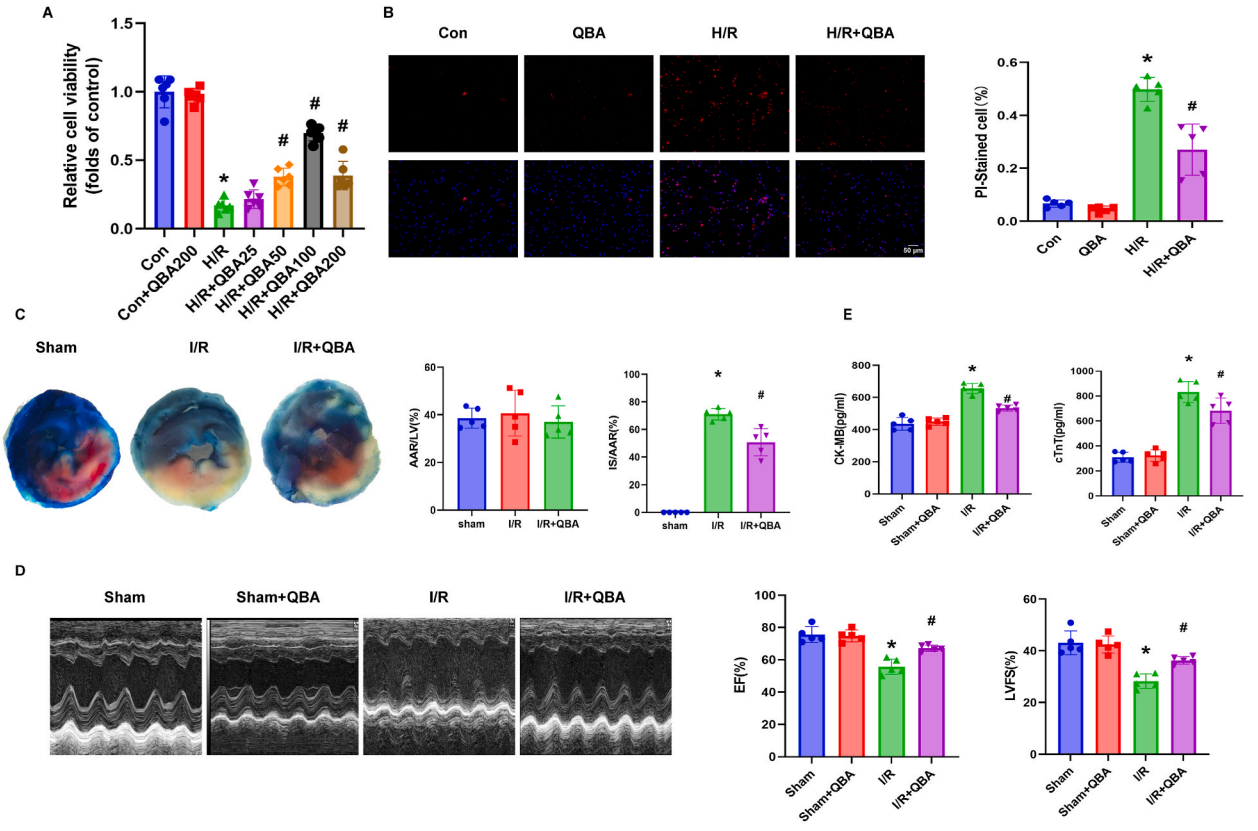


Fig. 1. QBA alleviates myocardial injury in mice.
(A) Effects of different doses of QBA (25, 50, 100 and 200 μM) on cell viability in cultured NRCs, as determined by the MTS assay. (B) Necrotic cells were visualized using PI staining (red nuclei) and quantitatively analyzed. (C) Illustrative photographs of heart sections stained with TTC-Evans blue were obtained for each experimental group. Brick red staining indicated viable myocardial tissue, while the unstained white areas corresponded to infarcted myocardium. The ratios of the area at risk (AAR) to the left ventricle (LV) and infarct size (IS) to the AAR are presented for analysis. (D) M-mode echocardiography was utilized to assess the left ventricular ejection fraction (EF) and fractional shortening (FS) across the various experimental groups, with illustrative echocardiograms presented for reference. (E) Following treatment, ELISA was utilized to measure the plasma levels of CK-MB and cTnT in the various groups. *P < 0.05, significantly different from the control or sham group; #P < 0.05, significantly different from the H/R or I/R group.

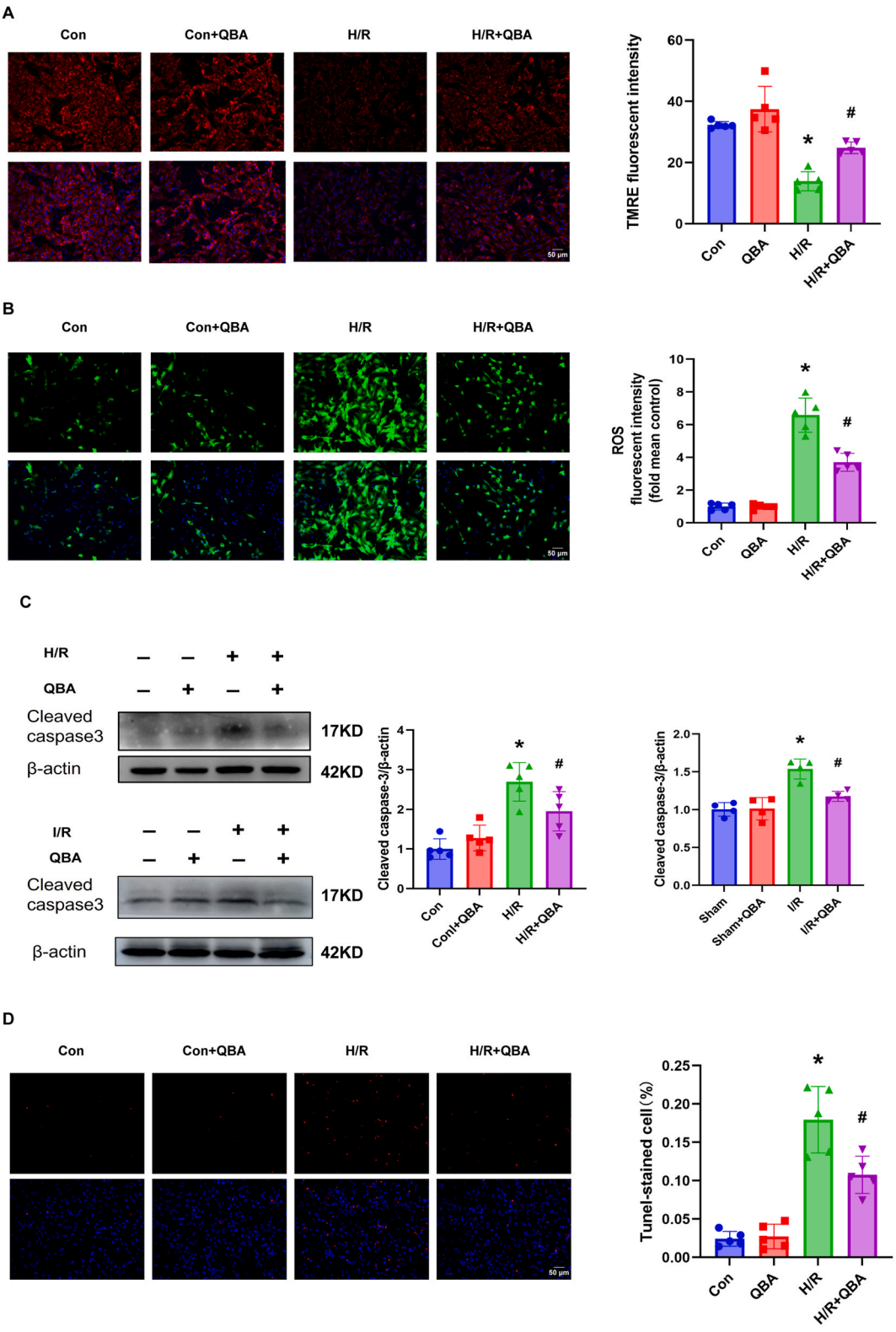


Fig. 2. QBA attenuates cardiac oxidative stress, mitochondrial damage, and apoptosis.
(A) The mitochondrial membrane potential was assessed using TMRE staining and visualized under a microscope, where the presence of red fluorescence indicates intact mitochondrial membrane potential. (B) Cellular reactive oxygen species (ROS) generation was visualized by staining with DCFH-DA and overlaid with the total nuclei stained with Hoechst 33342. Representative images and quantification of DCFH-DA are presented.

(C) Western blot analysis was conducted to assess the levels of cleaved caspase-3 in neonatal rat cardiomyocytes (NRCs) exposed to hypoxia/reoxygenation (H/R) and in murine hearts subjected to ischemia/reperfusion (I/R) injury. The cleaved caspase-3/β-actin ratio was quantified to determine the relative expression levels of cleaved caspase-3 in the samples. (D) Representative photomicrographs and quantification of TUNEL staining (red nuclei) in NRCs. *P < 0.05, significantly different from the control or sham group; #P < 0.05, significantly different from the H/R or I/R group.

tetramethylrhodamine, ethyl ester (TMRE) (C2001S, Beyotime, Shanghai, China). In brief, NRCs were treated with TMRE staining solution for 20 min at 37 °C. Next, three rounds of washing with cell culture media were performed on the NRCs. An Olympus fluorescence microscope (Japan) was used to obtain images.

2.13. Transmission electron microscopy

Transmission electron microscopy was used in accordance with a previously published protocol [24]. Briefly, small mouse heart pieces ~1 mm^3 in size were fixed for one night at 4 °C in 2.5 % glutaraldehyde, submerged in 1 % osmium tetroxide for 1 h, and then incubated for 2 h in 2 % aqueous uranyl acetate. The samples underwent dehydration using a sequence of ethanol solutions with different concentrations (50 %, 70 %, 90 %, and 100 %). Subsequently, they were treated with a mixture consisting of equal parts propylene oxide and EMbed 812 (Electron Microscopy Sciences). An ultramicrotome was used to cut ultrathin pieces measuring 75–80 nm, which were then placed on 200-mesh copper grids. Grids were analyzed using an 80 kV Philips CM 10 electron microscope.

2.14. Dihydroethidium (DHE) staining

As directed by the manufacturer, the concentration of reactive oxygen species (ROS) in cardiac tissue was measured using DHE fluorescent probes (S0063, Beyotime, Shanghai, China). Briefly, heart cryosections were incubated for 30 min at 37 °C with DHE (10 μM) and then washed three times with PBS. Using an Olympus, Japan, confocal laser scanning microscope, the slices were examined, and pictures were taken.

2.15. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). The data were statistically analyzed using SPSS version 20.0 software. To evaluate statistical variances among multiple groups following a normal distribution, we employed one-way ANOVA for comparative analysis. Bonferroni's test was chosen when heterogeneous, and Dunnett's T3 test was used when heterogeneous variances. For non-normally distributed variables, the Kruskal-Wallis post hoc test was applied. Additionally, the significance was defined as $p < 0.05$.

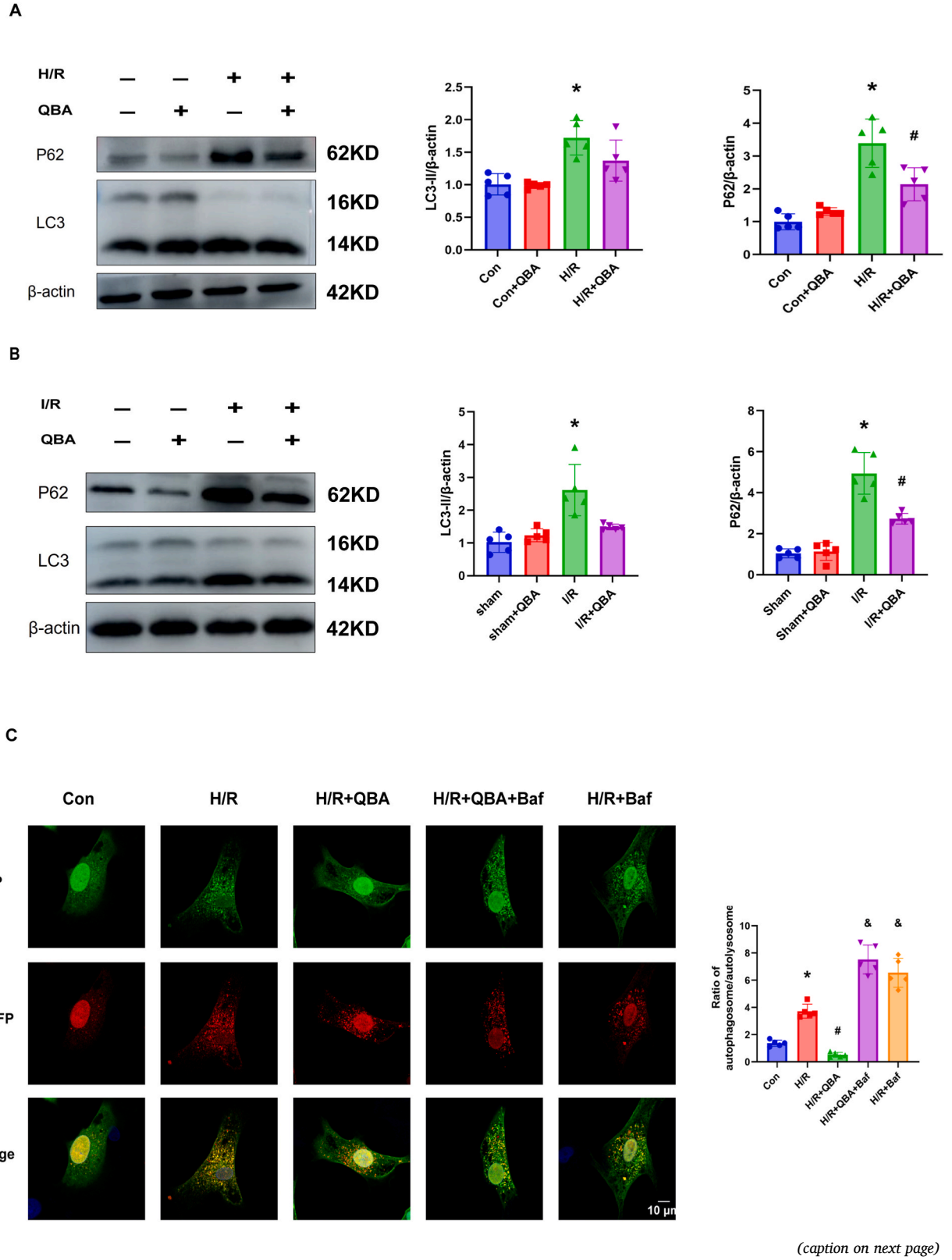
3. Results

3.1. QBA alleviates myocardial injury in mice

Different concentrations of QBA (25, 50, 100, and 200 μM) were applied to cardiomyocytes exposed to H/R to determine the effects of QBA on H/R-induced damage. In a dose-dependent manner, the CCK-8 assay results revealed that pretreatment with QBA increased the viability of cardiomyocytes exposed to H/R. The protective effects of QBA decreased at a concentration of 200 μM, and 100 μM was the best concentration for protection ($P = 0.001$) (Fig. 1A). As a result, 100 μM QBA was chosen for the subsequent in vitro investigations. Furthermore, PI staining revealed that QBA significantly reduced H/R-induced necrosis in NRCs (Fig. 1B). Then, we examined the effect of QBA on mice exposed to I/R. There were no significant differences in the AAR/LV between the groups ($P = 0.73$), showing that the animal model was stable. The IS/AAR was significantly greater in the I/R group than in the sham group ($P < 0.05$); however, this increase was partly reversed by QBA ($P < 0.05$) (Fig. 1C). Echocardiography was used to assess heart function one week following I/R. Compared with those in the I/R group, the left ventricular ejection fraction (LVEF) in the I/R + QBA group was 67.2 ± 1.9 %, whereas it was 55.7 ± 4.8 % ($p < 0.01$), and the left ventricular fractional shortening (LVFS) was 36.3 ± 1.5 %, which was greater than the 28.2 ± 1.3 % ($p < 0.01$) observed in the I/R + QBA group (Fig. 1D). Furthermore, whereas I/R damage resulted in a significant increase in the serum CK-MB and cTnT levels compared to those after the sham operation (CK-MB: 655.7 ± 32.2 vs. 435.1 ± 39.6 pg/ml; cTnT: 831.8 ± 83.8 vs. 308.9 ± 40.1 pg/ml; $P < 0.01$, respectively), QBA pretreatment successfully lowered the serum CK-MB and cTnT levels compared to those after I/R alone (CK-MB: 533.3 ± 19.2 vs. 655.7 ± 32.2 pg/ml; cTnT: 681.6 ± 100.9 vs. 831.8 ± 83.8 pg/ml; $P < 0.01$ and $P < 0.05$, respectively) (Fig. 1E).

3.2. QBA attenuates cardiac oxidative stress, mitochondrial damage, and apoptosis

Previous research has shown that mitochondrial function plays an important role in maintaining cell survival during I/R injury [25]. Thus, we investigated the effect of QBA on the mitochondrial membrane potential (ΔΨm) in NRCs by using TMRE. When myocardial cells were subjected to H/R in vitro, the ΔΨm decreased ($P < 0.05$). However, the ΔΨm was enhanced by QBA pretreatment($P < 0.05$) (Fig. 2A). Furthermore, ROS production in NRCs subjected to H/R increased significantly and was likewise



(caption on next page)

Fig. 3. QBA restores autophagic flux in cardiomyocytes in vivo and in vitro.
Western blots of LC3 and p62 in NRCs subjected to H/R (A) and murine hearts subjected to I/R (B) and quantification of LC3 II/β-actin and p62/β-actin are shown. (C) NRCs were transfected with adenovirus containing tandem fluorescent mRFP-GFP-LC3 and incubated for 24 h. Various treatments were applied to assess autophagic flux in the cells. Images of immunofluorescent NRCs expressing mRFP-GFP-LC3 are displayed, with DAPI staining indicating nuclei in blue, GFP dots in green, and mRFP dots in red. The ratio of autophagosomes to autolysosomes was determined and quantitatively evaluated. *$P < 0.05$, significantly different from the control or sham group; #$P < 0.05$, significantly different from the H/R or I/R group; & $P < 0.05$, significantly different from the H/R + QBA group.

reduced by QBA pretreatment ($P < 0.05$) (Fig. 2B). Next, we analyzed the effect of QBA on both in vivo and in vitro myocardial apoptosis. Western blot analysis revealed that in NRCs exposed to H/R or in mice after myocardial I/R injury, QBA dramatically decreased the protein levels of cleaved caspase-3, a marker of apoptosis ($P < 0.05$) (Fig. 2C). Additionally, QBA prevented H/R-induced NRC apoptosis, as determined by TUNEL staining ($P < 0.05$) (Fig. 2D). These data suggest that QBA can attenuate oxidative stress and myocardial apoptosis in I/R mice.

3.3. QBA restores autophagic flux in cardiomyocytes in vivo and in vitro

According to recent research, QBA may improve autophagy in a variety of cell types [22]. To ascertain whether QBA stimulates autophagic flux in NRCs exposed to H/R, Western blot analysis was used to measure the levels of p62 and LC3-II. Compared to control cells, H/R-exposed NRCs had higher levels of p62 and LC3-II, and the accumulation of these proteins suggested that autophagic flux was inhibited ($P < 0.05$). In NRCs subjected to H/R, QBA pretreatment reduced the levels of p62 and LC3-II ($P < 0.05$) (Fig. 3A). Similarly, the LC3-II and p62 levels in the I/R + QBA group were significantly lower than those in the I/R group ($P < 0.05$) (Fig. 3B). In another approach to measuring autophagic flux, mRFP-GFP-LC3 was introduced into NRCs, and tandem fluorescence was detected. Few autolysosomes or autophagosomes were present in the NRCs in the control group, indicating a low baseline level of autophagy. In contrast, NRCs exposed to H/R showed autophagosome accumulation but few autolysosomes, suggesting that autophagic flow was impeded and that autophagosome destruction was hindered. Nevertheless, compared to cells exposed to H/R, those pretreated with QBA produced more autolysosomes and fewer autophagosomes ($P < 0.05$) (Fig. 3C), indicating that autophagic flux was blocked by H/R therapy but restored by QBA pretreatment.

3.4. QBA-induced autophagic flux attenuates mitochondrial damage and reduces cellular ROS levels

To clarify whether QBA exerts a cardioprotective effect through autophagic flux, the lysosomal inhibitor bafilomycin A1 (Baf) was utilized to block autophagic flux [26]. Compared to the H/R group, the H/R + QBA group had considerably lower LC3-II and p62 levels ($P < 0.05$), suggesting that QBA potently increased autophagic flux in NRCs exposed to H/R. However, LC3-II and p62 levels were significantly increased in the H/R + QBA + Baf group and the H/R + Baf group ($P < 0.05$), suggesting that Baf hindered autophagosome breakdown, a process in which QBA was unable to change (Fig. 4A). Similarly, compared to those in the I/R group, the LC3-II and p62 levels in the I/R + QBA group were significantly lower ($P < 0.05$); however, this decrease was counteracted by the coadministration of Baf (Fig. 4B). Next, the cardiomyocyte ultrastructure was examined by transmission electron microscopy. Numerous tightly packed, compact mitochondria were observed in the cytoplasm of control NRCs. Conversely, the myocardium from the I/R group had fewer cristae, enlarged mitochondria, and an uneven arrangement of mitochondria. Treatment with QBA reduced I/R-induced mitochondrial damage, but the protective effects of QBA were eliminated when Baf was added ($P < 0.05$) (Fig. 4C). Furthermore, $\Delta\Psi m$ detection by TMRE revealed that QBA ameliorated the reduction in $\Delta\Psi m$ caused by H/R, but this protective effect was abolished by Baf ($P < 0.05$) (Fig. 4D). Additionally, compared to the I/R group, the I/R + QBA group showed reduced DHE staining in heart tissue, indicating a reduction in ROS production; this effect was blunted by Baf ($P < 0.05$) (Fig. 4E). By coadministering Baf with QBA, we were able to observe the reversal of the protective effects of QBA on myocardial cells, thereby supporting our hypothesis that the cardioprotective effects of QBA are mediated by enhancing autophagic flux.

3.5. QBA-enhanced autophagic flux attenuates myocardial apoptosis in vivo and in vitro

Next, we determined the level of apoptosis in mice and cells exposed to I/R and H/R, respectively. First, TUNEL assays demonstrated that QBA pretreatment significantly reduced the apoptosis of NRCs exposed to H/R, and this effect was partially inhibited by Baf ($P < 0.05$) (Fig. 5A), suggesting that the antiapoptotic activity of QBA requires the augmentation of autophagic flux. Mice exposed to I/R and pretreated with QBA likewise showed comparable outcomes ($P < 0.05$) (Fig. 5B). Additionally, Western blot analysis confirmed that QBA reduced cleaved caspase-3 levels both in vivo and in vitro, and this protective effect was reversed by cotreatment with Baf ($P < 0.05$) (Fig. 5C and D).

3.6. QBA-enhanced autophagic flux reduces myocardial IS and improves cardiac function

Previous research has shown that increasing autophagic flux reduces myocardial I/R damage [23,27]. Thus, we aimed to determine whether the protective effect of QBA requires an increase in autophagic flux. In vivo, QBA pretreatment clearly decreased the IS in mice subjected to I/R; however, Baf cotreatment partially reversed this protective effect($P < 0.05$) (Fig. 6A and B). Heart function was then assessed using M-mode echocardiography. Although QBA pretreatment enhanced the LVFS and LVEF in the I/R group compared to

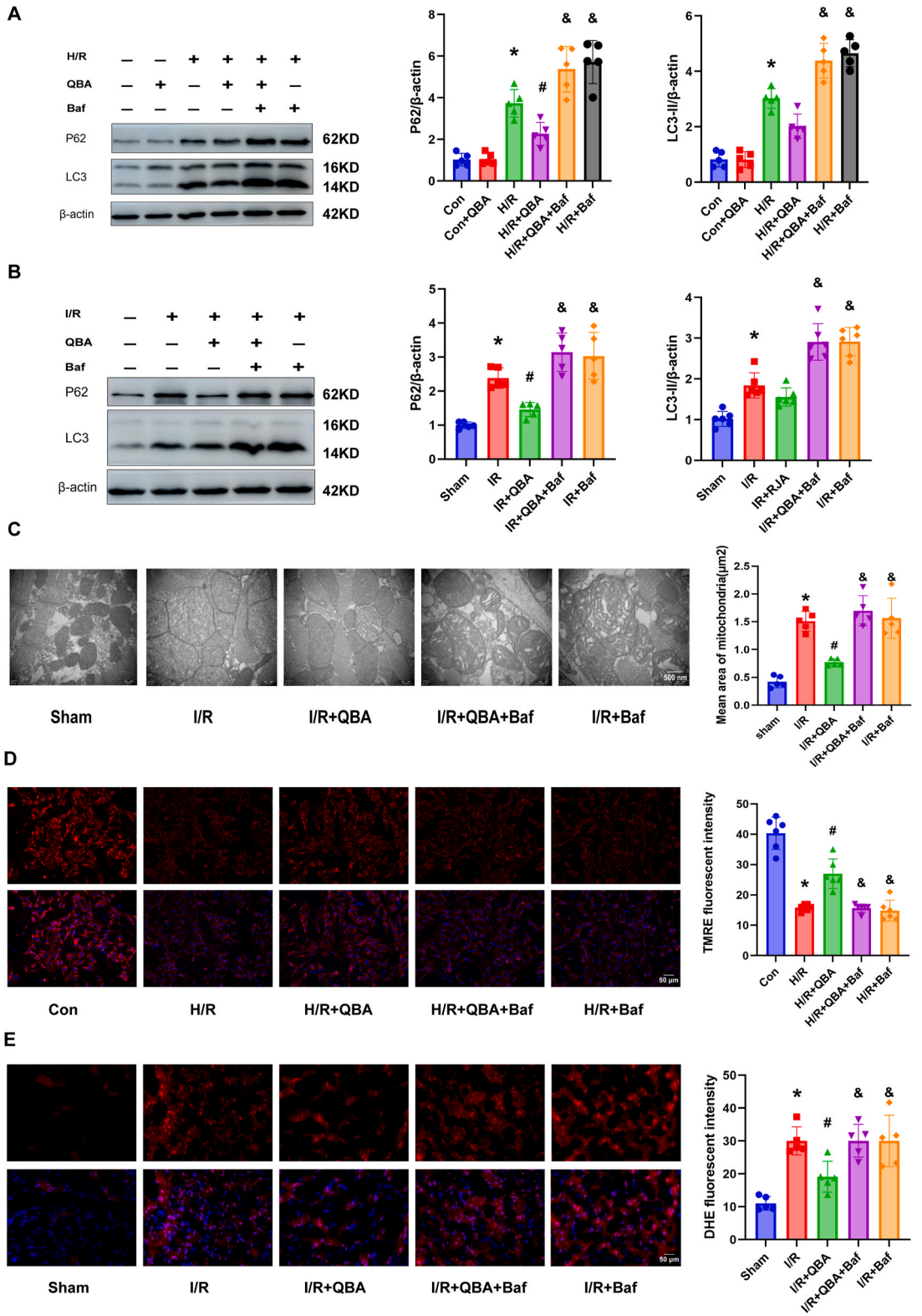


Fig. 4. QBA-induced autophagic flux attenuates mitochondrial damage and reduces cellular ROS levels.
(A) Representative Western blotting images and quantitative analysis of LC3 and p62 in NRCs subjected to H/R and subjected to different treatments. (B) Representative Western blotting images and quantitative analysis of LC3 and p62 in murine hearts subjected to I/R and subjected to different treatments. (C) The ultrastructure of the myocardium is shown by TEM. Representative images of cardiomyocyte mitochondria and

myofibrils in different groups. Scale bar = 500 nm. (D) Mitochondrial membrane potential was estimated with TMRE staining and captured by a microscope. Scale bar = 50 μm. (E) Representative images of DHE-stained mouse heart tissue from each group. *P < 0.05, significantly different from the control or sham group; #P < 0.05, significantly different from the H/R or I/R group; & P < 0.05, significantly different from the H/R or I/R + QBA group.

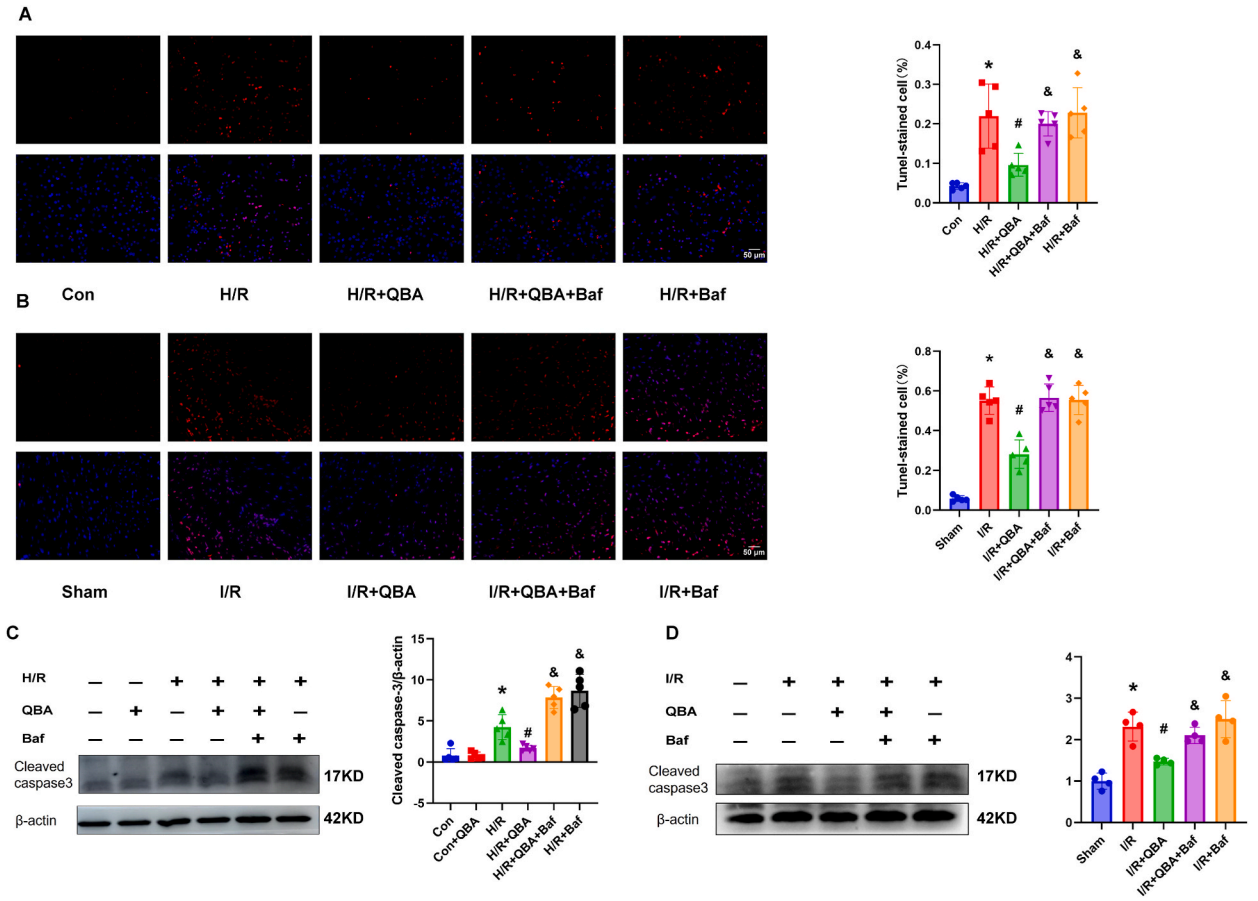


Fig. 5. QBA-enhanced autophagic flux attenuates myocardial apoptosis in vivo and in vitro.
(A) Representative images and TUNEL staining images of neonatal rat cardiomyocytes (NRCs) subjected to various treatments are presented. TUNEL-positive cells were identified by the presence of red dots. (B) Apoptotic cardiomyocytes were detected using TUNEL staining. Red dots indicate TUNEL-positive cardiomyocytes. (C) Representative Western blotting images and quantitative analysis of cleaved caspase-3 in NRCs under H/R. (D) Representative Western blotting images and quantitative analysis of cleaved caspase-3 in murine hearts subjected to I/R. *P < 0.05, significantly different from the control or sham group; #P < 0.05, significantly different from the H/R or I/R group; & P < 0.05, significantly different from the H/R or I/R + QBA group.

those in the sham group (LVFS: 35.7 ± 1.2 % vs. 27.5 ± 3.8 %; LVEF: 66.6 ± 1.2 % vs. 54.5 ± 5.9 %; P < 0.01, respectively), this effect was somewhat attenuated by Baf cotreatment (LVFS: 29.3 ± 1.9 % vs. 35.7 ± 1.2 %; LVEF: 57.3 ± 2.9 % vs. 66.6 ± 1.2 %; P < 0.01 and P < 0.05, respectively) (Fig. 6C and D). Furthermore, Baf partially hindered the ability of QBA to decrease CK-MB and cTnT activity (CK-MB: 689.1 ± 39.6 vs. 553.3 ± 20.3 pg/ml; cTnT: 877.0 ± 93.9 vs. 596.4 ± 35.4 pg/ml; P < 0.01, respectively) (Fig. 6E). Collectively, our results indicate that QBA attenuates I/R-mediated myocardial injury and cardiac dysfunction at least partly by enhancing autophagic flux.

4. Discussion

We demonstrated in the present work that QBA protects against myocardial I/R damage both in vivo and in vitro, potentially through the enhancement of autophagic flux. We present the main conclusions in Fig. 7.

RJ has drawn considerable attention because of its many health benefits, including its ability to fight inflammation, aging, and hypertension, as well as its immunomodulatory activity [14,15,28]. Previous preclinical research has demonstrated its potential for a cardioprotective impact. Notably, a recent clinical study showed that RJ might have an antiatherogenic effect by improving vascular

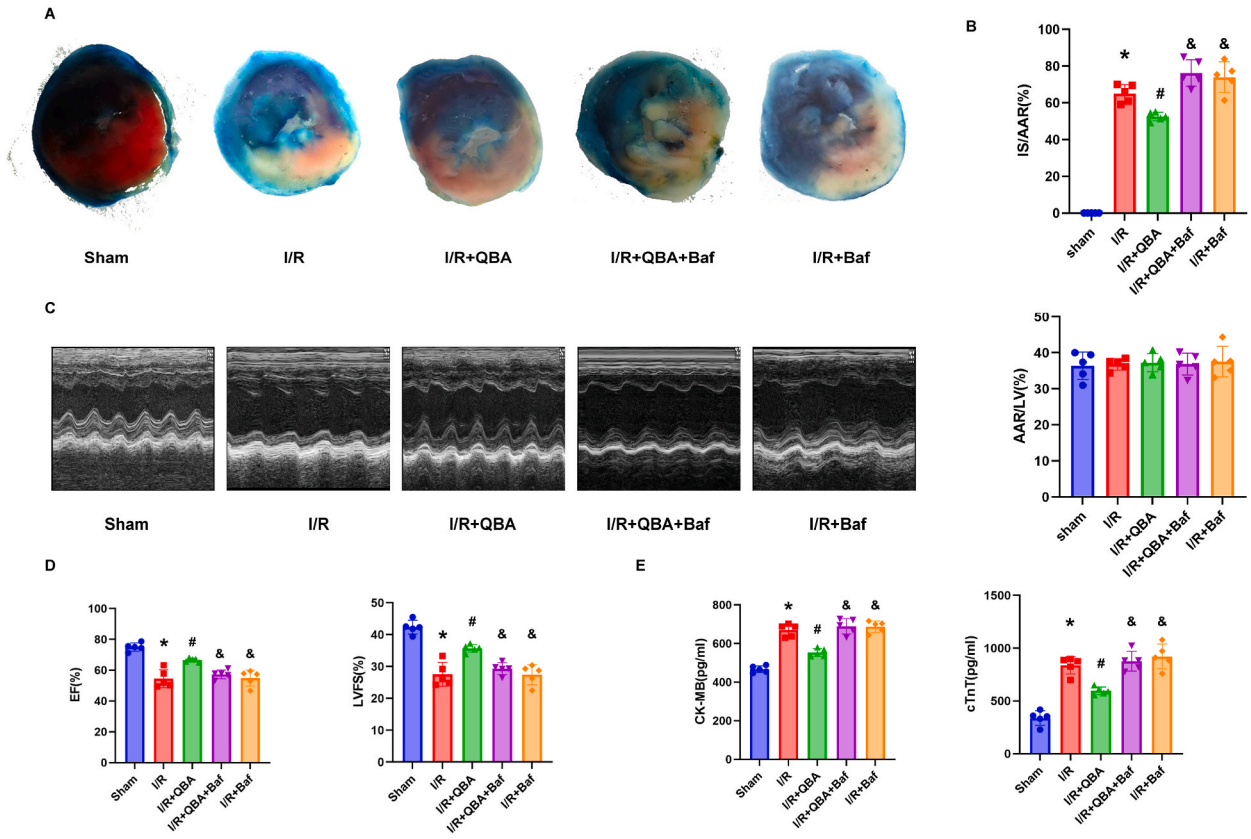


Fig. 6. QBA-enhanced autophagic flux reduces myocardial IS and improves cardiac function.
(A, B) Representative images of heart sections stained with TTC-Evans blue were obtained for each experimental group. Brick red staining indicated viable myocardial tissue, while the unstained white areas corresponded to infarcted myocardium. The AAR/LV and IS/AAR ratios are shown. (C, D) Representative echocardiograms from mice in different groups. LVEF and LVFS were measured and calculated. (E) After treatment, the plasma concentrations of CK-MB (G) and cTnT (H) in the different groups were measured by ELISA. *P < 0.05, significantly different from the control or sham group; #P < 0.05, significantly different from the H/R or I/R group; & P < 0.05, significantly different from the H/R or I/R + QBA group.

endothelial function [17]. However, it is still unclear which of the constituents provides protection, which somewhat limits its continued development into clinical medications. As a result, we sought to investigate the cardioprotective effects of QBA (a fatty acid exclusive to RJ) in myocardial I/R. In this study, the CCK-8 results showed that QBA, depending on the dose, decreased the myocardial damage caused by H/R. It also reduced cardiac dysfunction and myocardial injury induced by I/R in mice. These findings provide a better understanding of the protective effect of RJ on cardiovascular disease. Previous studies have shown that treatment with 10-HDA markedly attenuated the LPS-induced increase in cleaved caspase-3 levels in the mouse hippocampus [29]. Similarly, the protective effects of QBA found in our study may partly depend on inhibiting apoptosis. In contrast, Abdullah Aslan's group reported that RJ may exert cardioprotective effects on heart tissue by increasing apoptosis [30]. This discrepancy might be explained by differences in the experimental conditions and models used. Moreover, a previous study suggested that RJ may increase the expression of extracellular superoxide dismutase through histone acetylation and has potential as a candidate agent for treating atherosclerosis [31]. Other studies have also explored whether QBA has the strongest anti-inflammatory effect [32]. In our experiments, we found that QBA pretreatment inhibited ROS generation and attenuated mitochondrial damage. Nevertheless, there is still a need for more research to determine whether QBA decreases myocardial harm from I/R.

Autophagy is an evolutionarily conserved process that maintains cellular homeostasis. Several investigations have shown that autophagy is increased in cardiac tissue during both ischemia and reperfusion [6,33]. However, there is still enormous controversy regarding whether autophagy is detrimental or beneficial to myocardial I/R injury [12]. Evidence suggests that the activation of autophagy minimizes I/R damage to the heart [7–9,26,34–36], and our previous research also suggested that the induction of autophagy mitigates myocardial I/R injury [23,24]. Moreover, other research has shown that excessive autophagy may contribute to cardiomyocyte death [10,11,37–40]. The protective effects of QBA against myocardial I/R damage in this study may be associated with increased autophagic flux. According to Xiucui Ma et al., an undigested autophagosome may produce harmful substances such as ROS that exacerbate cell death [41]. Similarly, we found that QBA reduced ROS production and inhibited apoptosis in cardiomyocytes subjected to H/R. However, cotreatment of these cells with Baf led to increases in autophagosome abundance and apoptosis. Our subsequent experiments using tandem fluorescence (mRFP-GFP-LC3) revealed an increase in the number of autophagosomes during

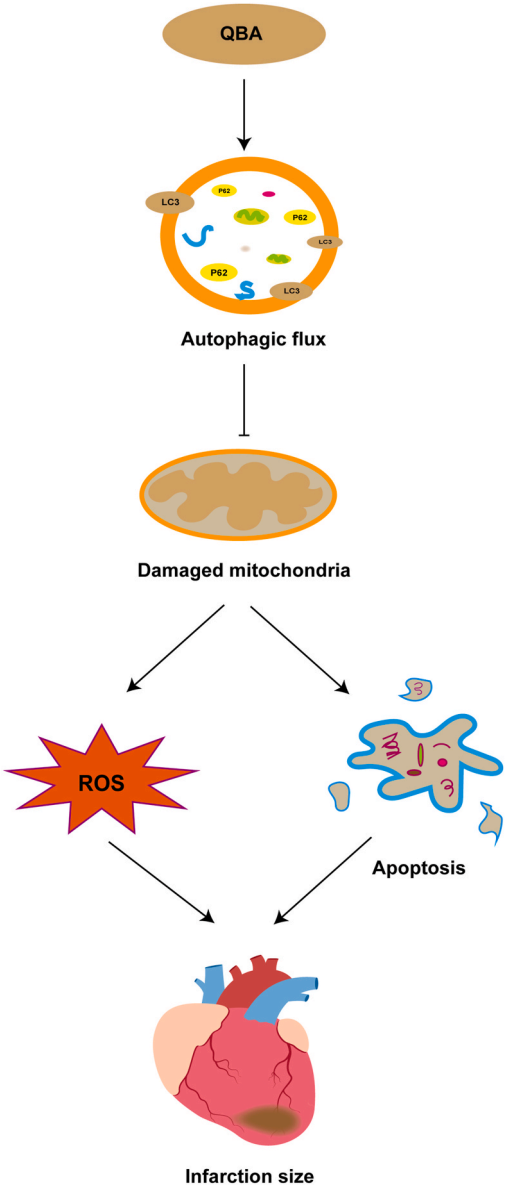


Fig. 7. Illustration of the effects and mechanisms of QBA on myocardial I/R injury.

H/R, but QBA accelerated autophagosome clearance. Impaired autophagosome clearance might induce excessive apoptosis. Guadalupe Martnez-Chacón et al. reported that QBA treatment significantly increased the conversion of LC3-I to LC3-II in cardiac tissue [22]. However, similar data were not obtained in our investigation. This discrepancy might be explained by the degree of autophagic flow: increasing autophagic flux may stimulate LC3-II clearance. Additionally, the I/R context might also account for the difference. Therefore, we postulate that in the event of myocardial I/R damage, increasing autophagic flux would be advantageous for myocardial survival.

For constant beating and cardiac homeostasis, maintaining mitochondrial metabolism and functional stability is essential. Notably, many studies have shown a strong correlation between mitochondrial dysfunction and myocardial I/R injury, as evidenced by reduced fusion, increased fission and impaired mitophagy [25,42,43]. Previous studies have shown that damaged mitochondria may leak cytochrome C, ROS, and apoptosis-inducing factor (AIF) into the cytoplasm, which will cause the cell to die [44]. Prompt removal of aberrant mitochondria prevents additional damage to nearby mitochondria. An increasing body of research indicates that mitophagy is crucial for preserving healthy mitochondria [45]. In the present study, QBA enhanced the H/R-induced inhibition of autophagy flux, potentially improving the removal of damaged mitochondria. Our results showed that pretreatment with QBA lessens the decrease in the mitochondrial membrane potential caused by H/R in myocardial cells. Furthermore, transmission electron microscopy demonstrated that QBA treatment reversed the pathological changes in mitochondria caused by I/R. However, these effects were partly

blocked by Baf (autophagy inhibitor) cotreatment. Overall, we hypothesize that QBA may increase cardiac cell viability in part due to the decrease in damaged mitochondria resulting from increased autophagic flux.

Moreover, in the context of myocardial ischemia–reperfusion, inflammation is a key factor, and existing research has indicated that QBA possesses anti-inflammatory properties [20,21]. Additional investigations are required to determine whether QBA can provide myocardial protection by modulating the inflammatory response. Autophagy could represent a single pathway through which QBA exerts its cardioprotective effects, and additional mechanisms, including the potential inhibition of ferroptosis, inflammation, calcium overload, and oxidative stress, warrant further investigation.

These results indicate that QBA has potential for development as a therapeutic agent for myocardial I/R injury. QBA, being a food-sourced compound, exhibits a commendable safety profile, which is a significant advantage for its potential therapeutic applications. Its monomeric composition allows for easier conversion into medications compared to that of royal jelly, indicating its significant potential for use in therapeutic applications. However, we did not establish a concentration gradient in our in vivo research, and the long-term effects and safety profile of QBA have not been extensively studied. Second, our findings are based on in vivo and in vitro models, which may not fully replicate the complexities of human myocardial I/R injury. The translational value of these results to clinical scenarios needs to be carefully evaluated.

In summary, we noted that QBA demonstrated a mitigating effect on myocardial I/R injury. This effect is mediated by the elimination of impaired mitochondria, attenuation of reactive oxygen species (ROS) levels, and suppression of apoptosis through the enhancement of autophagic flux. These results may provide a new approach for the treatment of myocardial I/R injury.

Ethics statement

This study was reviewed and approved by the Ethics Committee of Southern Medical University with the approval number LAEC-2022-114, dated August 11, 2022. The animal experiments were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

CRediT authorship contribution statement

Changhai Chen: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition. **Wen Ou:** Visualization, Methodology, Investigation. **Chaobo Yang:** Investigation, Data curation. **Haiqiong Liu:** Writing – review & editing, Resources, Data curation. **Tao Yang:** Resources, Data curation. **Huaqiang Mo:** Resources, Data curation. **Weizhe Lu:** Resources, Data curation. **Jing Yan:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Aihua Chen:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 82000249 to Jing Yan, No. 81873460 to Aihua Chen). and the Suqian Sci&Tech Program (No. KY202210).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33371.

References

[1] V.L. Aengevaeren, et al., Exercise and coronary atherosclerosis, Circulation 141 (16) (2020) 1338–1350.
[2] S.M. Davidson, et al., Multitarget Strategies to reduce myocardial ischemia/reperfusion injury, J. Am. Coll. Cardiol. 73 (1) (2019) 89–99.
[3] D.L. Bhatt, R.D. Lopes, R.A. Harrington, Diagnosis and treatment of acute coronary syndromes: a review, JAMA 327 (7) (2022) 662–675.
[4] M. Algoet, et al., Myocardial ischemia-reperfusion injury and the influence of inflammation, Trends Cardiovasc. Med. 33 (6) (2023) 357–366.
[5] G. Heusch, Myocardial ischaemia–reperfusion injury and cardioprotection in perspective, Nat. Rev. Cardiol. 17 (12) (2020) 773–789.
[6] D.J. Klionsky, et al., Autophagy in major human diseases, EMBO J. 40 (19) (2021) e108863 n/a.

[7] Y. Xing, et al., Blunting TRPML1 channels protects myocardial ischemia/reperfusion injury by restoring impaired cardiomyocyte autophagy, Basic Res. Cardiol. 117 (1) (2022).
[8] T. Khuanjing, et al., Donepezil attenuated cardiac ischemia/reperfusion injury through balancing mitochondrial dynamics, mitophagy, and autophagy, Transl. Res. 230 (2021) 82–97.
[9] Z. Sui, et al., Targeting MCOLN1/TRPML1 channels to protect against ischemia-reperfusion injury by restoring the inhibited autophagic flux in cardiomyocytes, Autophagy 18 (12) (2022) 3053–3055.
[10] Z. Lu, et al., Propofol upregulates MicroRNA-30b to inhibit excessive autophagy and apoptosis and attenuates ischemia/reperfusion injury in vitro and in patients, Oxid. Med. Cell. Longev. (2022. 2022) 1–17.
[11] K. Huang, et al., Antithrombin III alleviates myocardial ischemia/reperfusion injury by inhibiting excessive autophagy in a phosphoinositide 3-kinase/akt-dependent manner, Front. Pharmacol. 10 (2019), 516-516.
[12] M. Aghaei, et al., Targeting autophagy in cardiac ischemia/reperfusion injury: a novel therapeutic strategy, J. Cell. Physiol. 234 (10) (2019) 16768–16778.
[13] S. Lv, H. Liu, H. Wang, The interplay between autophagy and NLRP3 inflammasome in ischemia/reperfusion injury, Int. J. Mol. Sci. 22 (16) (2021).
[14] N. Collazo, et al., Health promoting properties of bee royal jelly: food of the queens, Nutrients 13 (2) (2021) 543.
[15] S. Ahmad, et al., New insights into the biological and pharmaceutical properties of royal jelly, Int. J. Mol. Sci. 21 (2) (2020) 382.
[16] H. Malekinejad, et al., Cardioprotective effect of royal jelly on paclitaxel-induced cardio-toxicity in rats, Iran J Basic Med Sci 19 (2) (2016) 221–227.
[17] K. Fujisue, et al., A randomized, double-blind comparison study of royal jelly to augment vascular endothelial function in healthy volunteers, J. Atherosclerosis Thromb. 29 (9) (2022) 1285–1294.
[18] X.Y. Yang, et al., 10-Hydroxy-2-decenoic acid from Royal jelly: a potential medicine for RA, J. Ethnopharmacol. 128 (2) (2010) 314–321.
[19] X. Li, C. Huang, Y. Xue, Contribution of lipids in honeybee (Apis mellifera) royal jelly to health, J. Med. Food 16 (2) (2013) 96–102.
[20] M. You, et al., Trans-10-hydroxy-2-decenoic acid alleviates LPS-induced blood-brain barrier dysfunction by activating the AMPK/PI3K/AKT pathway, Eur. J. Pharmacol. 865 (2019) 172736.
[21] Y. Yang, et al., 10-hydroxy-2-decenoic acid of royal jelly exhibits bactericide and anti-inflammatory activity in human colon cancer cells, BMC Compl. Alternative Med. 18 (1) (2018) 202.
[22] G. Martínez-Chacón, et al., Neuroprotective properties of queen bee acid by autophagy induction, Cell Biol. Toxicol. 39 (3) (2023) 751–770.
[23] Z. Tan, et al., Honokiol post-treatment ameliorates myocardial ischemia/reperfusion injury by enhancing autophagic flux and reducing intracellular ROS production, Chem. Biol. Interact. 307 (2019) 82–90.
[24] J. Yan, et al., Spermidine-enhanced autophagic flux improves cardiac dysfunction following myocardial infarction by targeting the AMPK/mTOR signalling pathway, Br. J. Pharmacol. 176 (17) (2019) 3126–3142.
[25] M. Yang, et al., Mitophagy and mitochondrial integrity in cardiac ischemia-reperfusion injury, Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1865 (9) (2019) 2293–2302.
[26] Y. Li, et al., CARD9 promotes autophagy in cardiomyocytes in myocardial ischemia/reperfusion injury via interacting with Rubicon directly, Basic Res. Cardiol. 115 (3) (2020).
[27] Y. Ling, et al., Polydatin post-treatment alleviates myocardial ischaemia/reperfusion injury by promoting autophagic flux, Clin. Sci. 130 (18) (2016) 1641–1653.
[28] J. Kocot, et al., Antioxidant potential of propolis, bee pollen, and royal jelly: possible medical application, Oxid. Med. Cell. Longev. (2018. 2018) 1–29.
[29] M. You, et al., Trans-10-hydroxy-2-decenoic acid protects against LPS-induced neuroinflammation through FOXO1-mediated activation of autophagy, Eur. J. Nutr. 59 (7) (2020) 2875–2892.
[30] A. Aslan, et al., Royal jelly abrogates flouride-induced oxidative damage in rat heart tissue by activating of the nrf-2/NF-kappaB and bcl-2/bax pathway, Toxicol. Mech. Methods 31 (9) (2021) 644–654.
[31] J. Makino, et al., Royal jelly constituents increase the expression of extracellular superoxide dismutase through histone acetylation in monocytic THP-1 cells, J. Nat. Prod. 79 (4) (2016) 1137–1143.
[32] Y. Chen, et al., In vitro anti-inflammatory effects of three fatty acids from royal jelly, Mediat. Inflamm. 2016 (2016), 3583684-11.
[33] D. Gatica, et al., The role of autophagy in cardiovascular pathology, Cardiovasc. Res. 118 (4) (2022) 934–950.
[34] Y. Zhang, et al., NAD+ administration decreases microvascular damage following cardiac ischemia/reperfusion by restoring autophagic flux, Basic Res. Cardiol. 115 (5) (2020).
[35] R. Zhao, et al., Alliin alleviates myocardial ischemia-reperfusion injury by promoting autophagy, Biochem. Biophys. Res. Commun. 512 (2) (2019) 236–243.
[36] S. Qiao, et al., Sevoflurane postconditioning protects against myocardial ischemia/reperfusion injury by restoring autophagic flux via an NO-dependent mechanism, Acta Pharmacol. Sin. 40 (1) (2019) 35–45.
[37] Q. Su, et al., Exosomal LINC00174 derived from vascular endothelial cells attenuates myocardial I/R injury via p53-mediated autophagy and apoptosis, Mol. Ther. Nucleic Acids 23 (2021) 1304–1322.
[38] C. Gao, et al., TXNIP/Redd1 signalling and excessive autophagy: a novel mechanism of myocardial ischaemia/reperfusion injury in mice, Cardiovasc. Res. 116 (3) (2020) 645–657.
[39] L.Y. Zhou, et al., The circular RNA ACR attenuates myocardial ischemia/reperfusion injury by suppressing autophagy via modulation of the Pink1/FAM65B pathway, Cell Death Differ. 26 (7) (2019) 1299–1315.
[40] Z. Li, et al., Inhibition of lncRNA XIST improves myocardial I/R injury by targeting miR-133a through inhibition of autophagy and regulation of SOCS2, Mol. Ther. Nucleic Acids 18 (2019) 764–773.
[41] X. Ma, et al., Impaired autophagosome clearance contributes to cardiomyocyte death in ischemia/reperfusion injury, Circulation 125 (25) (2012) 3170–3181.
[42] A.V. Kuznetsov, et al., The role of mitochondria in the mechanisms of cardiac ischemia-reperfusion injury, Antioxidants 8 (10) (2019) 454.
[43] H.K. Kim, et al., Novel GSK-3β inhibitor neopetroside A protects against murine myocardial ischemia/reperfusion injury, JACC (J. Am. Coll. Cardiol.): Basic to Translational Science 7 (11) (2022) 1102–1116.
[44] N.V. Naryzhnaya, L.N. Maslov, P.R. Oeltgen, Pharmacology of mitochondrial permeability transition pore inhibitors, Drug Dev. Res. 80 (8) (2019) 1013–1030.
[45] J. Wang, S. Toan, H. Zhou, New insights into the role of mitochondria in cardiac microvascular ischemia/reperfusion injury, Angiogenesis 23 (3) (2020) 299–314.