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Original Article

Berberine protects cardiac cells against ferroptosis

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Abstract

Objectives: Cardiovascular diseases are one of the primary causes of death. Cardiomyocyte loss is a significant feature of cardiac injury. Ferroptosis is iron-dependent cell death, which occurs due to excess iron and reactive oxygen species (ROS) accumulation causing lipid peroxidation, and subsequent cell death. Ferroptosis has been confirmed to mediate ischemia/reperfusion-induced cardiomyopathy and chemotherapy-induced cardiotoxicity. Berberine (BBR) has been proven to protect the heart from cardiomyopathies, including cardiac hypertrophy, heart failure, myocardial infarction, and arrhythmias. It protects cardiomyocytes from apoptosis and autophagy. However, the relation between BBR and ferroptosis is still unknown. This study aimed to confirm if BBR reduces cardiac cell loss via inhibiting ferroptosis. **Materials and Methods:** We used erastin and Ras-selective lethal small molecule 3 (RSL3) to establish a ferroptosis model in an H9c2 cardiomyoblast cell line and rat neonatal cardiomyocytes to prove that BBR has a protective effect on cardiac cells via inhibiting ferroptosis. **Results:** In H9c2 cardiomyoblasts, the results showed that BBR reduced erastin and RSL3-induced cell viability loss. Moreover, BBR decreased ROS accumulation and lipid peroxidation in cells induced with ferroptosis. Furthermore, quantitative polymerase chain reaction results showed that *Ptgs2* mRNA was reduced in BBR-treated cells. In rat neonatal cardiomyocytes, BBR reduced RSL3-induced loss of cell viability. **Conclusion:** These results indicated that BBR inhibited ferroptosis via reducing ROS generation and reducing lipid peroxidation in erastin and RSL3-treated cardiac cells.

Keywords: *Berberine, Cardiomyocyte, Ferroptosis, Ischemia-reperfusion injury, Lipid peroxidation*

Introduction

Heart diseases remain the leading cause of morbidity and mortality worldwide. Abnormal cardiac cell loss leads to pathological ventricular remodeling, and subsequently induced cardiomyopathy. The different kinds of cell death involved in cardiac cell loss include apoptosis, necrosis, autophagy, and ferroptosis [1-4]. Ferroptosis is a new type of programmed nonapoptotic cell dependent on iron and characterized by the accumulation of lipid peroxides [5]. Morphologically, ferroptosis is characterized by typical features, such as shrinkage of mitochondria, with increased membrane density and diminished mitochondria cristae. The main characteristic of ferroptosis is lipid reactive oxygen species (ROS) generation, which results in membrane lipid peroxidation and DNA damage [6]. In cells, ferroptosis is induced when ferrous ions accumulate and cause subsequent ROS generation. Excess-free ferrous ions catalyze the conversion of hydrogen peroxide (H_2O_2) into hydroxyl radicals (OH^-) via the Fenton reaction. Free ferrous ions or OH^- can react with polyunsaturated fatty acids (PUFAs) to induce the formation of lipid peroxyl radicals, resulting in lipid peroxidation, which can be prevented by using iron chelators (e.g., deferoxamine, dexrazoxane) or lipid peroxidation scavengers, including ferrostatin-1 (Fer-1), liproxstatin-1 (Lip-1).

Recently, ferroptosis has been found to participate in various pathological conditions and diseases, including neurodegeneration, stroke, acute kidney injury, cancers, and heart ischemia/reperfusion (I/R) injury [7,8]. Some studies have shown that inhibition of glutaminolysis, the essential component of ferroptosis, can reduce heart injury triggered by I/R [9]. In addition, ferroptosis plays a pivotal role in chemotherapy-induced cardiotoxicity. A detailed analysis of chemotherapy-induced cardiotoxicity showed that ferroptosis induces cardiotoxicity in a doxorubicin-treated animal model. It indicates that doxorubicin causes heme degradation to

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release free iron, which accumulates in the mitochondria, and triggers lipid peroxidation to induce ferroptosis in mice [10]. Furthermore, another study describes how doxorubicin reduces glutathione peroxidase 4 (GPX4) expression and destroys mitochondria to induce ferroptosis in doxorubicin-induced cardiomyopathic mice [11].

Previously, a study showed that puerarin acts against ferroptosis-induced heart failure by reducing cardiac cell loss, lipid peroxidation, and labile iron pool [12]. Further, Astragaloside IV, through activating the nuclear factor erythroid factor 2 related factor 2 (NRF2) signaling pathway and suppressing oxidative stress, inhibits adriamycin-induced cardiac ferroptosis [13]. Nevertheless, so far, there has been no effective therapy for ferroptosis-induced heart disease. In this study, we showed that berberine (BBR) can protect cardiac cells from ferroptosis. BBR, isolated from *Coptis chinensis*, is an isoquinoline alkaloid [14]. In recent studies, BBR was shown to exhibit anti-bacterial, anti-viral, anti-inflammatory, anti-oxidative, and also anti-cancer activity [15-17]. Several studies proved that BBR protected the heart from I/R injury, heart failure, arrhythmias, and hypertension; therefore, it may be a drug for cardiovascular treatment [18-20].

However, the relationship between BBR and cardiac ferroptosis is still unknown. This study explored the ability of BBR to inhibit ferroptosis-induced cell death or a loss in cell viability by reducing ROS levels and suppressing lipid peroxidation in erastin and Ras-selective lethal small molecule 3 (RSL3)-treated cardiac cells.

Materials and methods

H9c2 cardiomyoblast cell line culture

The H9c2 cardiomyoblast cell line was grown in Dulbecco's modified Eagle medium-high glucose (DMEM-HG) (GIBCO; Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin/streptomycin (GIBCO) at 37°C in 5% CO_2 atmosphere. At 70% confluency, the cells were trypsinized with 0.25% trypsin-EDTA (GIBCO) for 5 min and neutralized with DMEM containing 10% FBS. They were then centrifuged at 1200 rpm for 5 min. Next, cell pellets were collected and suspended with DMEM-HG in a 15 cm petri dish.

Neonatal cardiomyocytes culture

The neonatal cardiomyocytes were isolated and cultured in Sprague-Dawley rats (1–2 days; Experimental Animal Center, Tzu-Chi University). The hearts were excised from neonatal rats, washed twice with Hank's solution, and then minced into small pieces. The tissues were digested with Hank's solution (0.051% pancreatin, 0.01% collagenase) in 37°C water, neutralized by adding F-12 medium (80% F-12 nutrient mixture, 10% horse serum, 10% FBS, and 1% penicillin), and then seeded on a 6 cm petri dish. The medium was replaced daily during the experiment. All animal modules were performed following the recommended procedures approved by the Institutional Animal Care and Use Committee of Tzu Chi University (approval number: 110029).

Cell viability assay

The H9c2 cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) colorimetric assay. The cell density of 35000 cells/cm^2 was cultured in 24-well plates, which were coated with 0.1% gelatin. Different reagents treated H9c2 cell 16 h. After 16 h, medium was removed and then MTT was added and incubated for 2 h. Then, dimethyl sulfoxide (DMSO) was added (Sigma-Aldrich) to dissolve formazan crystals. Using the ELISA plate reader (Multiskan Ex; Thermo Fisher, Waltham, MA.), we measured cell viability at a wavelength of 570 nm.

Reactive oxygen species analysis

The H9c2 cell ROS expression level was determined by CM-H_2DCFDA reagent (Molecular Probes; Eugene, OR). Cells were treated with different drugs for 10 h. After 10 h, the drugs were removed, and the cells were incubated with 2.5 μM CM-H_2DCFDA/HEPES for 30 min at 37°C. We detected the intracellular levels of ROS by Gallios™ Flow Cytometer (BECKMAN COULTER; Bera, CA); the excitation/emission was detected at 488/525 nm wavelength.

Thiobarbituric acid reactive substances assay

The H9c2 cell density of 35000 cells/cm^2 were cultured in a 0.1% gelatin-coated 10 cm^2 petri dish. Cells were treated with DMSO, erastin, RSL3, or BBR for 16 h. Cells were collected and sonicated. The H9c2 cell lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company) following the manufacturer's instructions.

Apoptosis and necrosis assay

Apoptotic cells were detected by Annexin V/propidium iodide (PI) staining. The H9c2 cells were treated with BBR, H_2O_2, or DMSO and incubated for 1 h. Cells were harvested by 0.25% trypsin-EDTA and centrifuged at 1200 rpm for 5 min. Binding buffer was used to wash the cell pellets, and they were centrifuged at 1200 rpm for 5 min, twice. The cell pellets were resuspended in binding buffer with Annexin V and PI, which incubated for 15 min at 4°C in the dark. Apoptotic cells were analyzed by Gallios™ Flow Cytometer (BECKMAN COULTER; Bera, CA).

Quantitative real time-polymerase chain reaction

Total RNA was extracted from H9c2 cells using Trizol reagent (Ambion; Carlsbad, CA) according to the manufacturer's instructions. Each sample containing a total of 3 μg RNA was converted to cDNA using the Veros™ cDNA kit (Thermo). We performed the quantitative real-time-polymerase chain reaction (PCR) using Fast SYBR™ Green Master Mix (Thermo) in the LightCycler 480. The following PCR primers were used: Ptgs-2, forward: 5′-ATG TTC GCA TTC TTT GCC CAG-3′, reverse: 5′-TAC ACC TCT CCA CCG ATG AC-3′; and GAPDH, forward: 5′-ATG TTC CAG TAT GAC TCC ACT CAC G-3′, reverse: 5′-GAA GAC ACC AGT AGA CTC CAC GAC A-3′. All gene expression was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$), where $\Delta\Delta Ct = \Delta Ct$ (sample) − ΔCt (reference) relative to GAPDH levels.

Statistical analyses

In this study, all results were presented as means ± standard error of the mean and compared to those of the unpaired *t*-test. One-way ANOVA was measured by the data obtained from three or more groups, which was followed by Fisher's least significant difference procedure. Results were considered statistically significant at $P < 0.05$.

Results

Ferroptosis inducer induced ferroptosis of H9c2 cardiomyoblast cells

This study established a ferroptotic cell death model in cardiac cells to investigate whether a Chinese herb (BBR) can protect cardiac cells from ferroptotic cell death. We used H9c2 cardiomyoblast cells as our model system because a study showed that H9c2 cardiomyoblast cells are similar to primary cardiomyocytes [21]. We used two ferroptosis inducers: Erastin and RSL3. Erastin induces ferroptosis by inhibiting cystine-glutamate antiporter uptake of cysteine to reduce the synthesis of glutathione (GSH). RSL3 inhibits GPX4 activity to induce ferroptosis [22]. We further checked the viability of H9c2 cells, which were treated with three doses of erastin (0.5, 1, 2 μM). Moreover, compared to various inhibitors (ferroptosis inhibitor: Fer-1 and liproxststain-1, apoptosis inhibitor: Z-VAD-FMK) cells were co-treated with erastin (1 μM) for 16 h. The MTT data showed that compared to the control, erastin dose-dependently induced cell viability loss [Figure 1a]. Furthermore, the ferroptosis inhibitor compared to erastin (1 μM) showed that the ferroptosis inhibitor inhibited erastin induced-cell viability loss. Nevertheless, apoptosis inhibitor Z-VAD-FMK did not inhibit erastin induced-cell viability loss [Figure 1a]. Similar to the results with erastin, RSL3 (0.05, 0.1, 0.2 μM) also reduced cell viability, and both ferroptosis inhibitors recovered the cell

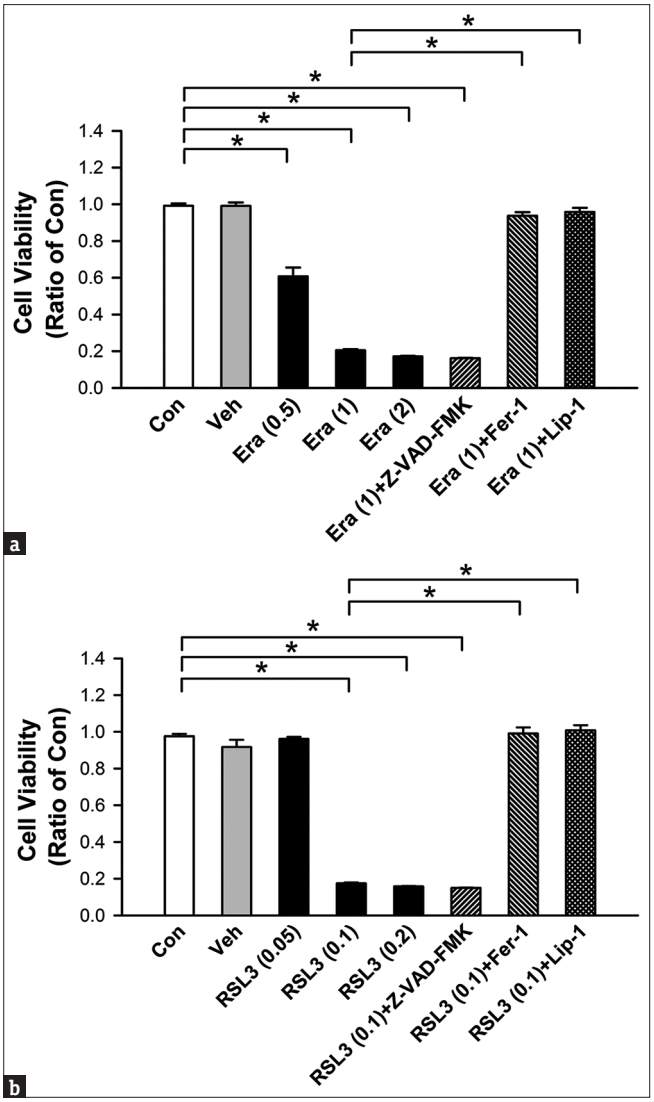

Figure 1: Erastin and ras-selective lethal small molecule 3 both induced ferroptosis in H9c2 cardiomyoblast cells. (a) Cells were treated with different concentrations of erastin (0.5, 1, 2 μM) or indicated inhibitors (Z-VAD-FMK, 10 μM; Fer-1, 1 μM; Lip-1, 2 μM) for 16 h. (b) Cells were treated with different concentrations of ras-selective lethal small molecule 3 (0.05, 0.1, 0.2 μM) or indicated inhibitors (Z-VAD-FMK, 10 μM; Fer-1, 1 μM; Lip, 2 μM) for 16 h. The cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. ($n = 8$) *$P < 0.05$. All data represent mean ± standard error of the mean. Con: Control, Veh: Vehicle, Era: Erastin, RSL-3: Ras-selective lethal small molecule 3

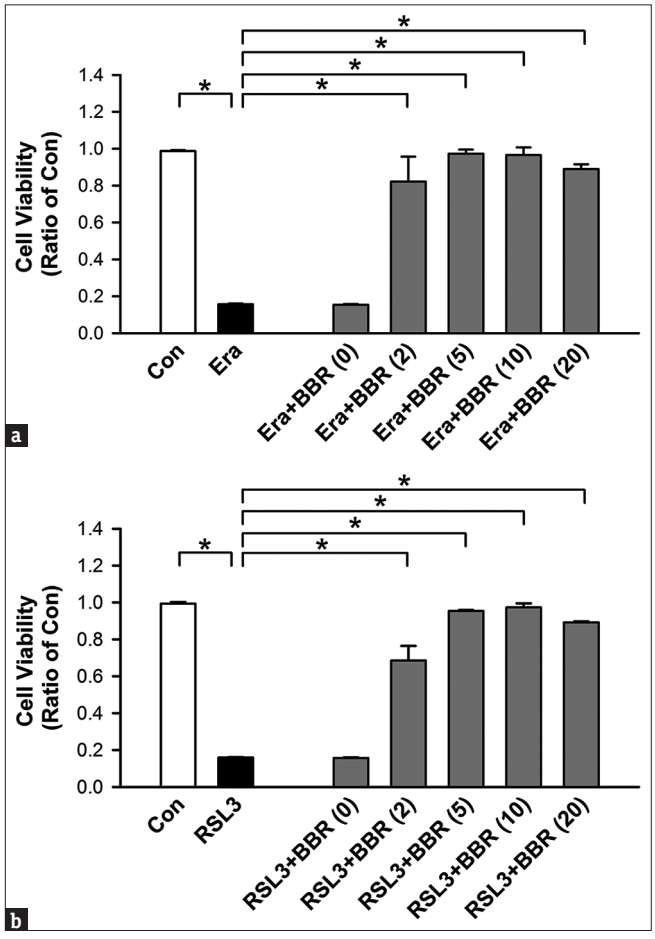

Figure 2: Berberine inhibited the erastin- and ras-selective lethal small molecule 3-induced cardiac cell loss via inhibition of ferroptosis. (a) H9c2 cells were treated with either erastin (1 μM) only or co-treated with erastin (1 μM) and different concentrations of berberine (2, 5, 10, 20 μM) for 16 h. (b) Cells were treated with ras-selective lethal small molecule 3 (0.1 μM) only or ras-selective lethal small molecule 3 (0.1 μM) co-treatment different concentrations berberine (2, 5, 10, 20 μM) for 16 h. The cell viability was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. ($n = 8$) *$P < 0.05$. All data represent mean ± standard error of the mean. Con: Control, Era: Erastin, BBR: Berberine, RSL-3: Ras-selective lethal small molecule 3

viability of RSL3-treated cells [Figure 1b]. These data suggest that erastin and RSL3 both induce cell death only through ferroptosis and not because of apoptosis or necrosis.

Berberine restored cell viability in erastin or RSL3-treated H9c2 cardiomyoblast cells

Recently, studies have shown that BBR exerts beneficial effects on the heart and protects cardiomyocytes from apoptosis and autophagy [23]. However, the relationship between BBR and ferroptosis remains unclear. Therefore, we investigated whether BBR protects H9c2 cardiomyoblast cells by inhibiting ferroptosis. The MTT assay was used to detect the cell viability of erastin (1 μM) co-treated with different concentrations of BBR (2, 5, 10, 20 μM) for 16 h in H9c2 cells. The result showed that BBR inhibited erastin-induced cell viability loss [Figure 2a]. A similar result was observed in RSL3 (0.1 μM)-treated cells, and the BBR increased the cell viability in a dose-dependent manner [Figure 2b]. These data indicated that BBR reduced ferroptosis in erastin or RSL3-treated H9c2 cardiomyoblast cells.

Berberine did not induce cytotoxicity in H9c2 cardiomyoblast cells

To determine if BBR induces cytotoxicity in H9c2 cardiomyoblast cells, we used the AnnexinV/PI assay. The result showed no apoptosis or necrosis in BBR-treated H9c2 cells [Figure 3]. These data indicated that BBR did not induce cytotoxicity in the H9c2 cardiomyoblast cells.

Berberine inhibited ferroptosis by reducing intracellular ROS production

The significant process of ferroptosis is the reaction of excessive ROS with PUFAs to induce lipid peroxidation. Therefore, ROS levels have a significant effect on ferroptosis. To investigate whether BBR inhibits ferroptosis in H9c2 cardiomyoblast cells, we measured intracellular ROS levels and lipid peroxidation by CM-H_2DCFDA (general ROS indicator) intensity and TBARS assay in H9c2 cells for 10 h. Figure 4a shows that BBR reduced erastin-or RSL3-induced intracellular ROS production. The TBARS assay showed that BBR reduced erastin-or RSL3-induced lipid peroxidation in H9c2 cells [Figure 4b]. We detected the mRNA expression level of *Ptgs2*, a marker of ferroptosis, by quantitative PCR in H9c2 cells. We observed that BBR reduced erastin-or RSL3-induced *Ptgs2* mRNA expression levels [Figure 4c]. These data indicated that BBR inhibited ferroptosis by reducing ROS production and lipid peroxidation in H9c2 cardiomyoblast cells.

Berberine inhibited ferroptosis in rat neonatal cardiomyocytes

To determine if BBR can inhibit RSL3-induced ferroptotic cell death in neonatal cardiomyocytes, we used Annexin V/PI assay to exclude apoptotic and necrotic cells in RSL3-induced cell death. We treated neonatal cardiomyocytes with RSL3 and BBR for 24 h. RSL3 did not induce apoptotic and necrotic cell death in rat neonatal cardiomyocytes [Figure 5a]. Furthermore, using the MTT assay, we confirmed whether BBR reduced RSL3-induced ferroptotic cell death in rat neonatal cardiomyocytes. Both BBR and ferroptosis inhibitors (Fer-1, Lip-1) reduced RSL3-induced cell death [Figure 5b]. These data indicated that BBR protected neonatal cardiomyocytes from ferroptotic cell death.

Discussion

Several compounds induce ferroptosis (e.g., erastin, RSL3) by inhibiting the activity of enzymes that have

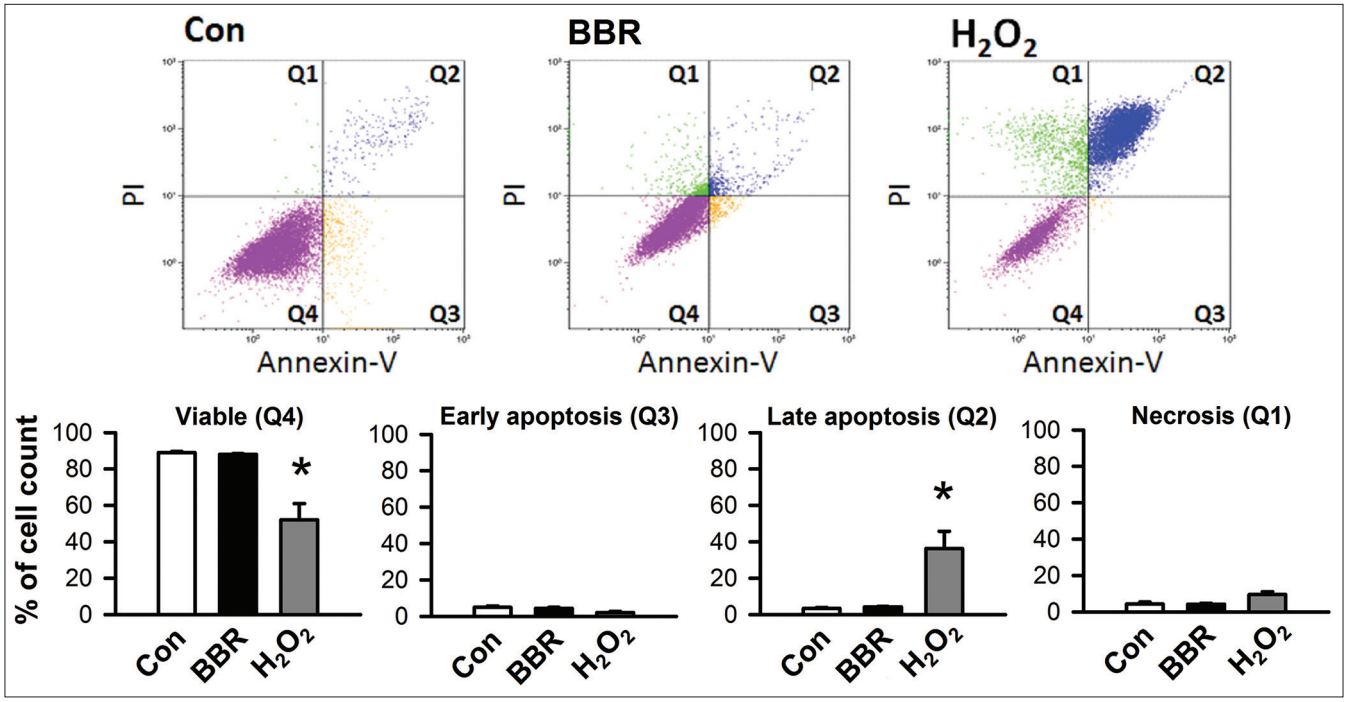

Figure 3: Analysis of apoptosis and necrosis by flow cytometry in berberine-treated H9c2 cardiomyoblast cells. Cells were treated with dimethyl sulfoxide, berberine (10 μM), or H_2O_2 (20 μM) for 1 h. Detection of apoptotic cells and necrotic cells by Annexin V and PI staining. (n = 6) *P < 0.05. All data represent mean ± standard error of the mean. Con: Control, BBR: Berberine

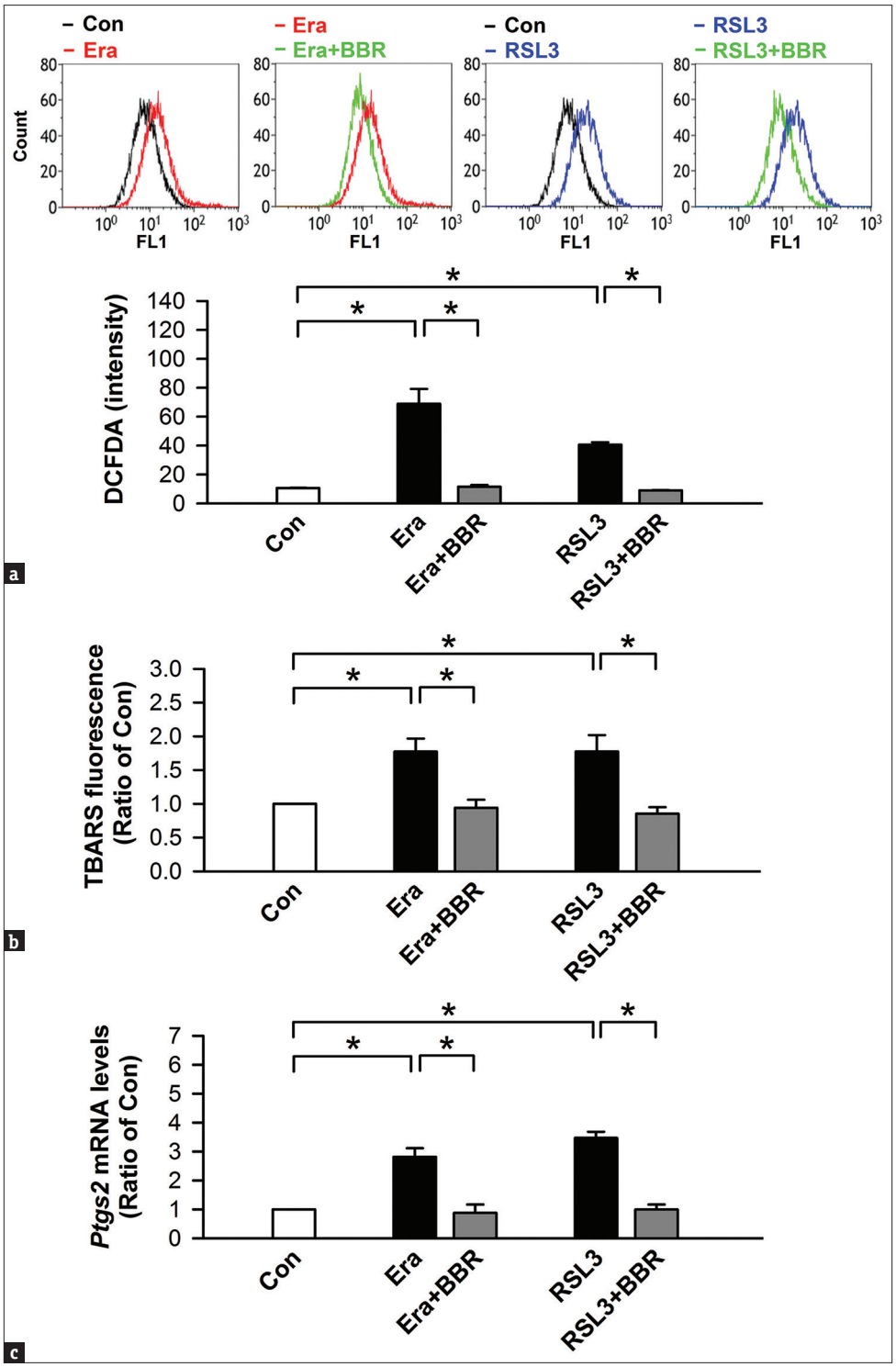

Figure 4: Berberine inhibited erastin- or ras-selective lethal small molecule 3-induced ferroptosis in H9c2 cardiomyoblast cells. (a) Cells treated 0.1% dimethyl sulfoxide, erastin (1 μM), ras-selective lethal small molecule 3 (0.1 μM), or berberine (10 μM) for 10 h. To measure intracellular reactive oxygen species by DCFDA assay ($n = 4$) (b) To measure lipid peroxidation measured by thiobarbituric acid reactive substances assay ($n = 3$). (c) To detect *Ptgs2* mRNA expression by quantitative polymerase chain reaction. ($n = 4$) *$P < 0.05$. All data represent mean ± standard error of the mean. Con: Control, Era: Erastin, RSL-3: Ras-selective lethal small molecule 3, BBR: Berberine

been identified as regulators of ferroptosis, such as GPX4, activation of NRF2, p53, heme oxygenase-1 (HO-1), and acyl-CoA synthetase long-chain family member 4 (ACSL4). Many studies have shown that different pathways participate in ferroptosis, such as activation of mitogen-activated protein kinase (MAPK) pathway, inhibition of cystine-glutamate antiporter to decreased cysteine uptake resulting in GSH depletion, increased NADPH oxidase (NOX)-mediated ROS, the release of arachidonic acid mediators, and mitochondrial dissipation [24]. Several previous studies also confirmed the relationship between NOX4 and ferroptosis. Erastin, via mediated activation of the JNK/p38/MAPK pathways and

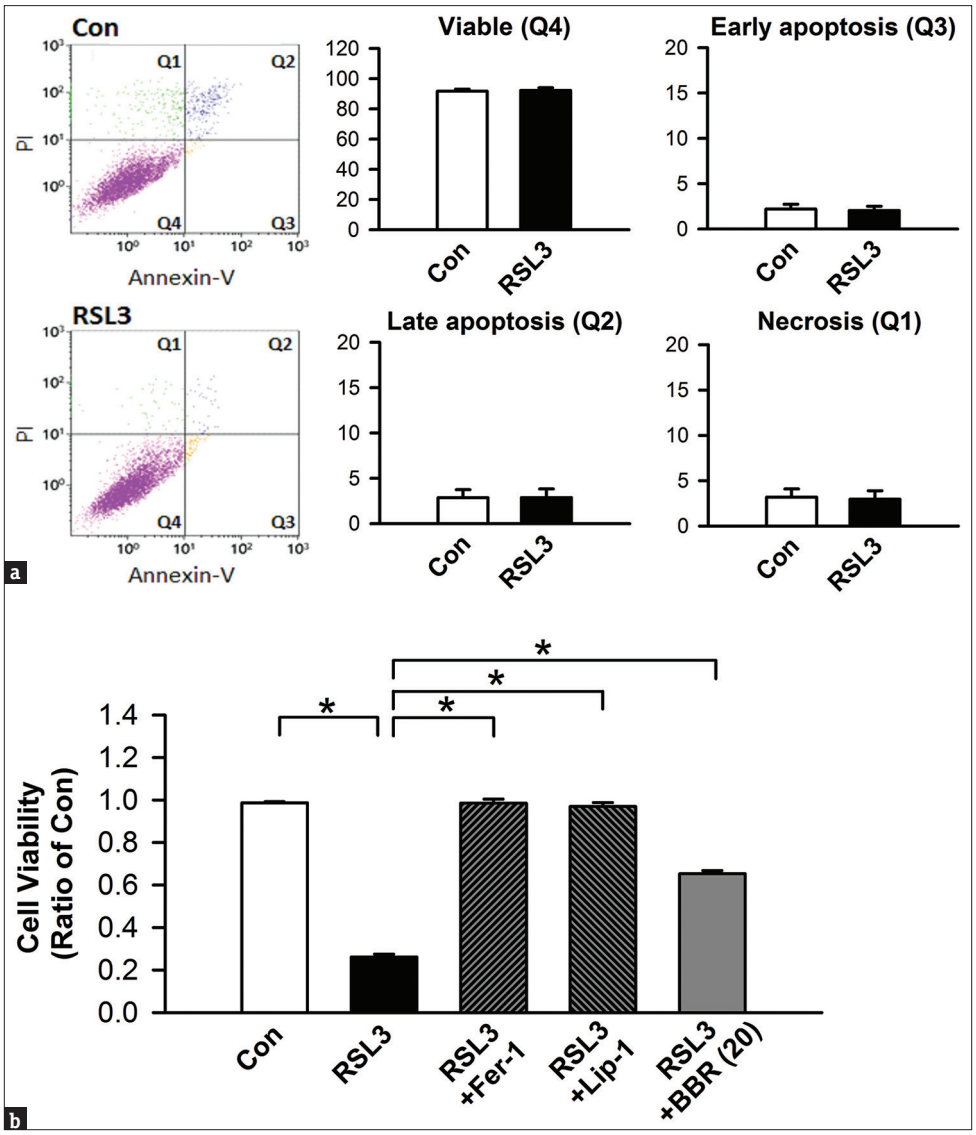

Figure 5: Berberine reduced ras-selective lethal small molecule 3-induced cell viability loss of rat neonatal cardiomyocytes. (a) Cells were treated with 0.1% dimethyl sulfoxide or ras-selective lethal small molecule 3 (0.5 μM) for 24 h, then measured ras-selective lethal small molecule 3-induced apoptotic cells and necrotic cells were by Annexin V and PI ($n = 4$). (b) Cells were treated with ras-selective lethal small molecule 3 (0.5 μM), or indicated inhibitors (Fer-1, 1 μM; Lip-1, 2 μM; berberine, 10 μM) for 24 h. To measure the cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. ($n = 8$) *$P < 0.05$. All data represent mean ± standard error of the mean. Con: Control

upregulation of NOX4 expression, induces ferroptosis in human pancreatic islet-like cell clusters [25]. Using lentiviral delivery siRNA to knockdown NOX4 in the myocardium inhibited ferroptosis in aortic banding heart failure rats [26].

In ferroptosis, free ferrous ions react with PUFAs to induce excessive lipid-ROS generation, resulting in lipid peroxidation. Malondialdehyde (MDA) is one of the most commonly used biomarkers for lipid peroxidation. Consistent with our results, Cheng *et al*. and Shin *et al*. report that erastin and RSL3 significantly increase MDA and ROS levels compared with the control group [27,28]. In the present study, we observed that BBR effectively reduced lipid peroxidation in erastin-and RSL3-induced ferroptosis in H9c2 cells. Other studies indicated that BBR inhibits doxorubicin-induced apoptosis by reducing ROS generation and mitochondrial damage [29]. In addition, previous studies indicated that BBR modulates NOX4, which catalyzes oxygen to produce ROS, in cardiomyopathy [23]. In human umbilical vein endothelial cells, BBR ameliorates palmitate-induced endothelial dysfunction by downregulating NOX4 expression to reduce ROS levels [30]. A comprehensive compilation of previously conducted research and our results leads us to speculate that BBR reduces NOX4 to inhibit erastin-and RSL3-induced ferroprotic cell death in H9c2 cells.

In this study, although we found that BBR significantly inhibited ferroptosis of H9c2 and cardiomyocytes, it also improved ROS and lipid peroxidation caused by erastin and RSL3. However, the mechanism by which BBR produces a protective effect is still unclear. A study showed that BBR improves transverse aorta constriction (TAC)-induced cardiac hypertrophy in rats by inhibiting the mTOR pathway [31]. Particularly, some evidence indicates that hyperactive

mutation of PI3K-AKT-mTOR signaling protects cancer cells from ferroptosis and oxidative stress through SREBP1/SCD1-mediated lipogenesis [32]. Furthermore, BBR upregulated PINK1/Parkin-mediated mitophagy to ameliorate TAC-induced chronic heart failure in mice [33]. The PINK1/Parkin pathway has been identified to participate in mitophagy. Knockdown of PINK1 reduced BAY 87-2243-induced mitophagy stimulation, ferroptosis, and ROS increase in cells [34]. Of note, Phospho-Erk1/2 is regarded as a molecular feature of ferroptosis [35]. Chronic infusion of BBR reduces the NOX2/NOX4/Erk1/2 (extracellular-signal-regulated kinase ½)/iNOS signaling pathway to attenuate hypertensive and sympathoexcitation in two-kidney, one-clip renovascular hypertensive rats [36]. The hepatoprotective effect of BBR appears to be via the activation of AMP-activated protein kinase (AMPK), and the subsequent decrease in NOX4 and Akt expression [37].

Additionally, a study demonstrated that energy stress-mediated AMPK activation inhibits ferroptosis [38].

Recently, it was shown that ferroptosis played a vital role in doxorubicin-induced cardiotoxicity [10,11]. Moreover, some studies showed that doxorubicin reduces GPX4 levels, and excess free iron accumulated in mitochondria resulted in lipid peroxidation. In addition, BBR enhances doxorubicin-induced cell death in many cancers [39-41]. Furthermore, our data indicated that BBR could inhibit ferroptosis in neonatal cardiomyocytes. Taken together, these results suggest that BBR can be used in combination with doxorubicin to treat different cancers, enhance doxorubicin-induced cancer cell death, and reduce cardiotoxicity.

Despite the positive results, this study had some limitations. The mechanism of the cell damage caused by ischemia-reperfusion or other cardiomyopathy is very complex, including the increase of free radicals, inflammation, apoptosis, necrosis, and ferroptosis. In the animal ischemia-reperfusion mode, it is very difficult to clarify whether BBR protects against ischemia-reperfusion injury by inhibiting ferroptosis or improving heart function. Therefore, this study used cultured H9c2 cardiomyoblast cells and neonatal cardiomyocytes, and used RSL3 and erastin to induce cardiac cell ferroptosis mode to verify whether BBR can inhibit ferroptosis. The results of cell experiments confirmed that BBR had a good protective effect. Therefore, to increase the clinical applicability of BBR, other pathological events, such as I/R and cardiac hypertrophy-induced ferroptosis, must be considered in the future to clarify whether BBR has the same effect of inhibiting ferroptosis in an animal mode and can improve cardiac function.

Conclusion

Our study demonstrated that erastin-and RSL3-induced ferroptosis was inhibited by BBR by reducing intracellular ROS accumulation and suppressing lipid peroxidation in cardiac cells, thereby helping to recover cell viability. The results from this study in combination with the aforementioned research lead us to believe that BBR has the potential to treat ferroptosis-induced cardiomyopathy.

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Conflicts of interest

There are no conflicts of interest.

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