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Original research article

Ginsenoside Rb2 improves heart failure by down-regulating miR-216a-5p to promote autophagy and inhibit apoptosis and oxidative stress

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Abstract
Background: Ginsenoside Rb2 is beneficial in cardiovascular disease treatment, yet its role in heart failure (HF) is obscure. This study aimed to investigate the effect and mechanism of ginsenoside Rb2 on HF.
Methods: The left anterior descending branch-ligated HF rat model and oxygen-glucose deprivation/reoxygenation (OGD/R) H9c2 cell model were constructed. Ginsenoside Rb2 were applied for intervention. Heart function indexes, miR-216a-5p expression, autophagy, oxidative stress, apoptosis, cell morphology, and proliferation were detected to explore the effect of ginsenoside Rb2 on HF. Overexpression of miR-216a-5p was employed to explore the specific mechanism of ginsenoside Rb2 on HF.
Results: Ginsenoside Rb2 improved the heart function of HF rats, including the reduction of heart rate, LVEDP, and heart weight/body weight ratio, and the increase of LVSP, $+dP/dt_{max}$, $-dP/dt_{max}$, LVEF, and LVFS. It also down-regulated miR-216a-5p expression and enhanced OGD/R-induced cardiomyocyte viability. Ginsenoside Rb2 up-regulated Bcl2, LC3B II/I, and Beclin1, and down-regulated Bax, Caspase-3, and p62 in the myocardium of HF rats and OGD/R-induced H9c2 cells. Moreover, ginsenoside Rb2 increased the levels of SOD and CAT, but decreased the levels of MDA and ROS in the myocardium of HF rats and OGD/R-induced H9c2 cells. However, overexpression of miR-216a-5p promoted the apoptosis and oxidative stress of cardiomyocytes and inhibited autophagy, thus reversing the therapeutic effect of ginsenoside Rb2 on HF *in vivo* and *in vitro*.
Conclusion: Ginsenoside Rb2 demonstrated potential as a therapeutic intervention for HF by enhancing autophagy and reducing apoptosis and oxidative stress through miR-216a-5p downregulation. Further research could explore its application in clinical trials and investigate the complex mechanism networks underlying its effects.

Keywords: Apoptosis; Autophagy; Ginsenoside Rb2; Heart failure; miR-216a-5p; Oxidative stress

Highlights:
- Ginsenoside Rb2 improved the heart function of HF rats.
- Ginsenoside Rb2 inhibited miR-216a-5p expression, reduced myocardial apoptosis and oxidative stress, and promoted autophagy in HF rats.
- Ginsenoside Rb2 improved the viability loss of OGD/R-induced cardiomyocytes.
- Ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in OGD/R-induced cardiomyocytes by down-regulating miR-216a-5p expression.

Abbreviations:
ANOVA, analysis of variance; Bax, BCL2-associated X protein; CAT, catalase; CCK-8, cell counting kit-8; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HF, heart failure; HRP, horseradish peroxidase; IF, immunofluorescence; LC3B, microtubule-associated protein 1 light chain 3 beta; LVEDP, left ventricular end-diastolic pressure; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVSP, left ventricular systolic pressure; $+dP/dt_{max}$, maximum rate of rise of left ventricular pressure; $-dP/dt_{max}$, maximum rate of drop of left ventricular pressure; MDA, malondialdehyde; MI/R, myocardial ischemia/reperfusion; MST, microscale thermophoresis; miRNA, microRNA; NC, nitrocellulose; Nrf2/HO-1, nuclear factor erythroid 2-related factor 2/heme oxygenase-1; OD, optical density; OGD/R, oxygen-glucose deprivation/reoxygenation; PBS, phosphate-buffered saline; p27/Cdk2/mTOR, p27/cyclin-dependent kinase 2/mammalian target of rapamycin; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SD, Sprague Dawley; SOD, superoxide dismutase

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Introduction

Heart failure (HF) is a clinical syndrome of cardiac insufficiency caused by myocardial remodeling and blood-pumping disorders (Liu and Eisen, 2014). Despite great advances in treatment, HF is a leading cause of high morbidity and mortality from cardiovascular diseases (Nabeebaccus et al., 2016). HF imposes a very serious health and economic burden on patients, their families, and society (Roger et al., 2012). The current therapeutic drugs for HF include β blockers, angiotensin-converting enzyme inhibitors, and aldosterone antagonists. Due to the existence of certain toxic side effects, their therapeutic effect is still unsatisfactory (Muiesan et al., 2017). Due to the advantages of multi-target, multi-pathway, small toxic and side effects, adjustment of immune function, and so on, natural products have become one of the hot spots of new drug development.

Ginsenoside Rb2 is an active proginsenodiol saponin, widely present in the stems, leaves, and berries of ginseng, and is one of the main bioactive components of ginseng (Miao et al., 2022). A pharmacological study has shown the beneficial effects of Rb2 in the treatment of diabetes, obesity, cancers, and cardiovascular diseases (Miao et al., 2022). In the study of myocardial ischemia/reperfusion (MI/R) injury and atherosclerosis, ginsenoside Rb2 displays a good therapeutic effect by inhibiting oxidative stress, reactive oxygen species (ROS) production, and inflammatory response (Fu et al., 2016, Liu et al., 2020). In addition, studies on coronary heart disease have found that ginsenoside Rb2 plays a cardioprotective role *in vitro* by inhibiting oxidative stress *via* the nuclear factor erythroid 2-related factor 2/heme oxygenase-1 (Nrf2/HO-1) signaling pathway (Li and Zhang, 2022). However, the therapeutic effect of ginsenoside Rb2 on HF remains unclear. Thus, it is essential to conduct studies to investigate the effect of ginsenoside Rb2 on HF and elucidate its potential mechanisms of action.

Studies have found that cardiomyocyte apoptosis is important in the occurrence and development of HF. Excessive cardiomyocyte apoptosis causes structural changes in the myocardium, impairs the perfusion capacity of the myocardium, and thus leads to HF (Chang et al., 2017). In addition, a normal level of autophagy is essential in regulating cardiomyocyte size and the structure and function of the heart, but abnormal autophagy can affect myocardial remodeling and HF progression (Huang et al., 2010; Lavandero et al., 2015). Besides, research advancements in the cardiovascular field have recognized oxidative stress as a crucial pathophysiological pathway in the advancement and development of HF (van der Pol et al., 2019). Oxidative stress induced by high levels of ROS can cause cellular dysfunction and damage, leading to myocardium remodeling and failure (Tsutsui et al., 2011). Therefore, targeted regulation of cardiomyocyte apoptosis, autophagy, and oxidative stress may be strategies for the treatment of HF.

Further study on HF has revealed that microRNA (miRNA) participates in the pathological process of myocardial remodeling and improves the occurrence and development of HF by regulating miRNA expression (Melman et al., 2014). Circulating miRNAs are expected to be a new biomarker of HF for diagnostic and prognostic purposes and to determine patient response to treatment. Some of them have been implicated in the important pathogenesis of HF, which is important to improve our understanding of the pathophysiology of this disease (Vegter et al., 2016). miR-216a is highly expressed in the plasma of HF patients, which may be a new biomarker for the diagnosis of HF and related diseases (Ding et al., 2020). However, the effect of miR-216a in HF needs to be further verified. According to the sequence and structural characteristics of ginsenoside Rb2, bioinformatics analysis verified the binding of ginsenoside Rb2 to miR-216a. It was further confirmed that ginsenoside Rb2 had a specific and high binding affinity for miR-216a by microscale thermophoresis (MST) (Chen et al., 2021). Therefore, ginsenoside Rb2 may be a therapeutic agent of HF by binding to miR-216a.

Based on the above results, we conducted a left anterior descending branch-ligated HF rat model and an oxygen-glucose deprivation/reoxygenation (OGD/R) cell model to explore the therapeutic effect of ginsenoside Rb2 on HF by targeting miR-216a *in vivo* and *in vitro*, providing new insights for the diagnosis and treatment of HF.

Materials and methods

Experimental animals and intervention

Male Sprague Dawley (SD) rats (SPF grade, 250–300 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. After one week of adaptive feeding, the experiments were carried out. All the rat groups in the experiment included Sham, Model, Ginsenoside Rb2-L, Ginsenoside Rb2-H, Ginsenoside Rb2+mimics NC, and Ginsenoside Rb2+miR-216a-5p mimics groups, 6 rats/group. After rats were connected to the small animal artificial ventilator (ZS-MV-HXB, ZS Dichuang, Beijing, China), the left anterior descending branch-ligated HF model was established according to the previously described method (Gao et al., 2020; Liu et al., 2017). In the Sham group, other operations were the same except that the sutures were not knotted. Four weeks after the operation, the echocardiography was observed using the biological signal collection and processing system (Madelab-4C/5H, ZS Dichuang, Beijing, China) to confirm the successful construction of the HF model. Rats in Ginsenoside Rb2-L and Ginsenoside Rb2-H groups were gavaged with ginsenoside Rb2 (10 mg/kg, 20 mg/kg) (HY-N0040, MCE, Monmouth Junction, NJ, USA) once a day for 3 days (Fu et al., 2016). Rats in Sham and Model groups were gavaged with the same dose of ddH2O. After the first ginsenoside Rb2 intervention, 100 μl of mimics NC or miR-216a-5p mimics (HonorGene, Changsha, China) was injected once through the tail vein into rats in Ginsenoside Rb2+mimics NC and Ginsenoside Rb2+miR-216a-5p mimics groups (Deng et al., 2022). Rats in Model and Ginsenoside Rb2 groups were injected with the same dose of ddH_2O.

The heart rate, left ventricular end-diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), maximum rate of rise of left ventricular pressure ($+dP/dt_{max}$), and maximum rate of drop of left ventricular pressure ($-dP/dt_{max}$), left ventricular ejection fraction (LVEF, %), and left ventricular fractional shortening (LVFS, %) were measured 24 h after the last intervention. After the rats were killed, the hearts were collected and left ventricular tissue of an area far from the infarction site were isolated for subsequent detection. Moreover, the heart weight/body weight ratio was measured. All animal experiments were approved by the Animal Ethics Committee of Hunan Provincial People's Hospital (2023-01).

Cell culture

Rat cardiomyocytes H9c2 (AW-CNR083, Abiowell, Changsha, China) were cultured in Dulbecco's modified Eagle medium (DMEM, D5796, Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) (10099141, Gibco, Waltham, MA, USA) and 1% Penicillin/Streptomycin (AWI0070a, Abiowell,

Changsha, China), and placed in a humidified incubator (DH-160I, SANTN, Shanghai, China) containing 5% CO_2 at 37 °C. In addition, cell slides were prepared for immunofluorescence (IF) staining.

Cell grouping and treatment

Grouping 1: Control, OGD/R, Ginsenoside Rb2-L, Ginsenoside Rb2-M and Ginsenoside Rb2-H. H9c2 cells in the OGD/R group were cultured in Earle's balanced salt solution (E485468, Aladdin, Shanghai, China) under hypoxia conditions for 8 h, and then reoxygenated for 12 h to construct the OGD/R cell model (Chen et al., 2019). H9c2 cells in Ginsenoside Rb2 groups were treated with ginsenoside Rb2 (10, 25, and 50 μM) for 12 h after OGD/R induction (Li and Zhang, 2022). Cell morphology was observed by the inverted biological microscope (DSZ2000X, Cnmicro, Beijing, China).

Grouping 2: Control, OGD/R, Ginsenoside Rb2, Ginsenoside Rb2+mimics NC and Ginsenoside Rb2+miR-216a-5p mimics. H9c2 cells in the Ginsenoside Rb2 group were treated with 50 μM ginsenoside Rb2 after OGD/R induction. The mimics NC or miR-216a-5p mimics were transfected into the H9c2 cells in Ginsenoside Rb2 + mimics NC and Ginsenoside Rb2+miR-216a-5p mimics groups by using Lipofectamine 2000 (11668019, Invitrogen, Carlsbad, CA, USA). After OGD/R induction, the H9c2 cells were treated with 50 μM ginsenoside Rb2.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA in the myocardium and H9c2 cells was extracted by Trizol reagent (15596026, Thermo, Waltham, MA, USA). mRNA was reverse transcribed into cDNA according to the guidance of the mRNA reverse transcription kit (CW2569, CWBIO, Beijing, China), followed by qRT-PCR with UltraSYBR Mixture (CW2601, CWBIO, Beijing, China). The primers used were as follows: miR-216a-5p: forward: GGCAACTGTGAGATGTCCCT; reverse: TCAAGGAAATTGCTCTGTTTAGCA; 5S: forward: GCCTACAGCCATACCACCCGGAA; reverse: CCTACAGCACCCGGTATCCCA. The expression level of miR-216a-5p was calculated by the $2^{-\Delta\Delta Ct}$ method with 5S as the internal reference.

Western blot

RIPA lysate (AWB0136, Abiowell, Changsha, China) was used for the extraction of total proteins from the myocardium and H9c2 cells. After separation, proteins were transferred to nitrocellulose (NC) membranes and blocked with 5% skimmed milk (AWB0004, Abiowell, Changsha, China) for 1.5 h. The NC membranes were incubated with primary antibodies at 4°C overnight separately, including B-cell lymphoma-2 (Bcl2, 1:5,000, 60178-1-Ig, Proteintech, Chicago, IL, USA), BCL2-associated X protein (Bax, 1:5,000, ab32503, Abcam, Cambridge, UK), Caspase-3 (1:1,000, 19677-1-AP, Proteintech, Chicago, IL, USA), p62 (1:10,000, 66184-1-Ig, Proteintech, Chicago, IL, USA), microtubule-associated protein 1 light chain 3 beta (LC3B, 2 μg/ml, ab48394, Abcam, Cambridge, UK), Beclin1 (1:2,000, 11306-1-AP, Proteintech, Chicago, IL, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5,000, 10494-1-AP, Proteintech, Chicago, IL, USA). The NC membranes were then incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1.5 h. Finally, the NC membranes were incubated with ECL reagent (AWB0005, Abiowell, Changsha, China) and followed by imaging. The protein expressions were analyzed by Quantity One 4.6.6 (Bio-Rad Inc., Hercules, CA, USA) with GAPDH as the reference protein.

Flow cytometry

The myocardium was rinsed with phosphate-buffered saline (PBS) and then cut into small pieces using sterile ophthalmic scissors. They were mixed with trypsin (AWC0232, Abiowell, Changsha, China) and Type II collagenase (AWH0565a, Abiowell, Changsha, China) and incubated at 37 °C (Louch et al., 2011). To accelerate the digestion process, the culture plate can be gently shaken or rotated to allow cells to better come into contact with enzymes. After 30 min, pure myocardial cells were obtained using a filter. We then harvested and washed the cells with PBS and resuspended them in the medium. For H9c2 cells, digestion was done with EDTA-free trypsin. After PBS washing, about 3.2×10^5 cells were collected. According to the instructions of the apoptosis kit (KGA1030, KeyGEN BioTECH, Nanjing, China), the cell suspension was mixed with 5 μl of Annexin V-FITC and 5 μl of Propidium Iodide successively, then reacted for 10 min without light before detection.

Immunofluorescence (IF) staining

Myocardium slices were dewaxed in xylene and dehydrated in gradient ethanol (75–100%). After antigen repair and enzyme inactivation, the slices were washed with PBS. The slices were sequentially placed in sodium borohydride solution, 75% ethanol solution, and Sudan black dye solution, and then rinsed with tap water. For H9c2 cell slides, they were fixed with 4% paraformaldehyde and mixed with 0.3% Triton X-100 at 37 °C. The myocardium slices and cell slides were sealed with 5% BSA for 1 h, and then cultured with the primary antibody of LC3 (1:50, 14600-1-AP, Proteintech, Chicago, IL, USA) overnight at 4 °C. Next, 100 μl of CoraLite488-conjugated Affinipure Goat Anti-Rabbit IgG (H + L) was added and incubated at 37 °C for 1.5 h. Nuclei were stained with DAPI reagent (AWI0331a, Abiowell, Changsha, China) at 37 °C for 20 min. The myocardium slices and cell slides were sealed with glycerin and observed under a fluorescence microscope.

Cell counting kit-8 (CCK-8) assay

After digestion, H9c2 cells were inoculated into 96-well plates at a density of 5×10^3 cells/well. After adhesion, H9c2 cells in each group were treated accordingly. Then, 100 μl of medium containing 10% CCK-8 reagent (NU679, DOJINDO, Kyushu, Japan) was added to replace the original medium. Finally, H9c2 cells were cultured for another 4 h at 37 °C, and the optical density (OD) at 450 nm was determined by a multifunctional microplate reader (MB-530, HEALES, Shenzhen, China).

Biochemical detection

The levels of malondialdehyde (MDA), ROS, superoxide dismutase (SOD), and catalase (CAT) in rat myocardium and H9c2 cells were measured according to the instructions of MDA assay kits (A003-1), ROS assay kits (E004-1-1), SOD assay kits (A001-1), and CAT assay kits (A007-1-1). These kits were all bought from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) was applied for statistical analysis. Experimental data were all expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and two-way ANOVA were conducted in comparison between multiple groups, and $P < 0.05$ was considered statistically significant.

Results

Ginsenoside Rb2 improved the heart function of HF rats

To investigate the effect of ginsenoside Rb2 on HF, rats were subjected to left anterior descending branch ligation and then gavaged with low and high doses of ginsenoside Rb2 for intervention, and their indexes of heart function were detected. Rats in the Model group displayed heart dysfunction characterized by increased heart rate, LVEDP, and heart weight/body weight ratio, as well as decreased LVSP, +dP/dt$_{max}$, –dP/dt$_{max}$, LVEF, and LVFS, suggesting the successful construction of the HF rat model. However, the intervention of ginsenoside Rb2 resulted in a reduction in heart rate, LVEDP, and heart weight/body weight ratio, as well as an increase in LVSP, +dP/dt$_{max}$, –dP/dt$_{max}$, LVEF, and LVFS in HF rats. This effect was more pronounced at the higher dose of ginsenoside Rb2 (Fig. 1A–E). These results confirmed that ginsenoside Rb2 improved the heart function of HF rats.

Ginsenoside Rb2 inhibited miR-216a-5p expression, reduced myocardial apoptosis and oxidative stress, and promoted autophagy in HF rats

To further explore the effect of ginsenoside Rb2 on the myocardial function of HF rats, miR-216a-5p expression, cell apoptosis, and markers of oxidative stress and autophagy were detected. Remarkably, miR-216a-5p was highly expressed in the myocardium of HF rats. However, the intervention of ginsenoside Rb2 significantly reduced miR-216a-5p expression

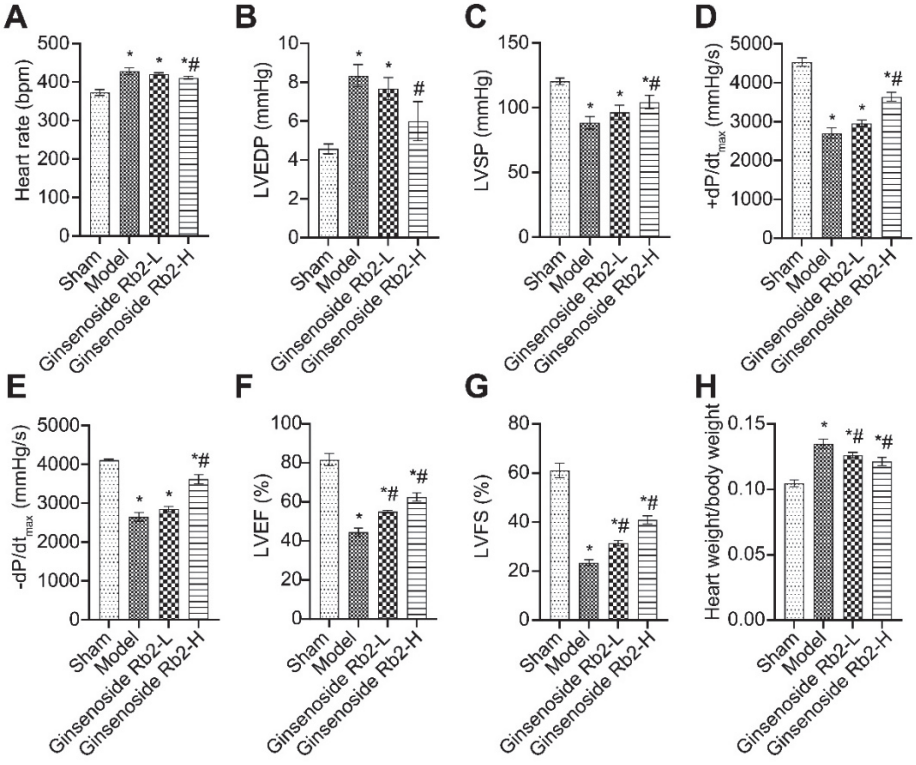

Fig. 1. Ginsenoside Rb2 improved the heart function of HF rats. (**A**) Heart rate; (**B**) LVEDP; (**C**) LVSP; (**D**) +dP/dt$_{max}$; (E) –dP/dt$_{max}$; (**F**) LVEF (%); (**G**) LVFS (%); (**H**) Heart weight/body weight ratio. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Model.

(Fig. 2A). Additionally, Western blot analysis results displayed that the expressions of Bax and Caspase-3 were significantly up-regulated in the Model group, while that of Bcl2 was the opposite. However, this situation was reversed by the intervention of ginsenoside Rb2, resulting in reduced low expression of Bax and Caspase-3, and high expression of Bcl2 (Fig. 2B). Flow cytometry results proved that apoptosis of rat cardiomyocytes was significantly induced by HF, but inhibited after the intervention of ginsenoside Rb2 (Fig. 2C). The intervention of ginsenoside Rb2 effectively inhibited HF-induced oxidative stress, displaying decreased levels of MDA and ROS and increased levels of SOD and CAT (Fig. 2D). The intervention of ginsenoside Rb2 significantly raised the levels of LC3B II/I and Beclin1, and reduced that of p62 (Fig. 2E). IF staining results further verified the remission effect of the intervention of ginsenoside Rb2 on the inhibition of HF-induced LC3B level in the myocardium (Fig. 3). These results proved that ginsenoside Rb2 inhibited miR-216a-5p expression, reduced myocardial apoptosis and oxidative stress, and promoted autophagy in HF rats.

Ginsenoside Rb2 improved the viability loss of OGD/R-induced cardiomyocytes

To investigate the effect of ginsenoside Rb2 on the viability loss of OGD/R-induced cardiomyocytes, low, medium, and high doses of ginsenoside Rb2 were applied for intervention, and the morphological integrity and proliferation ability of cardiomyocytes were measured. The cardiomyocytes in the

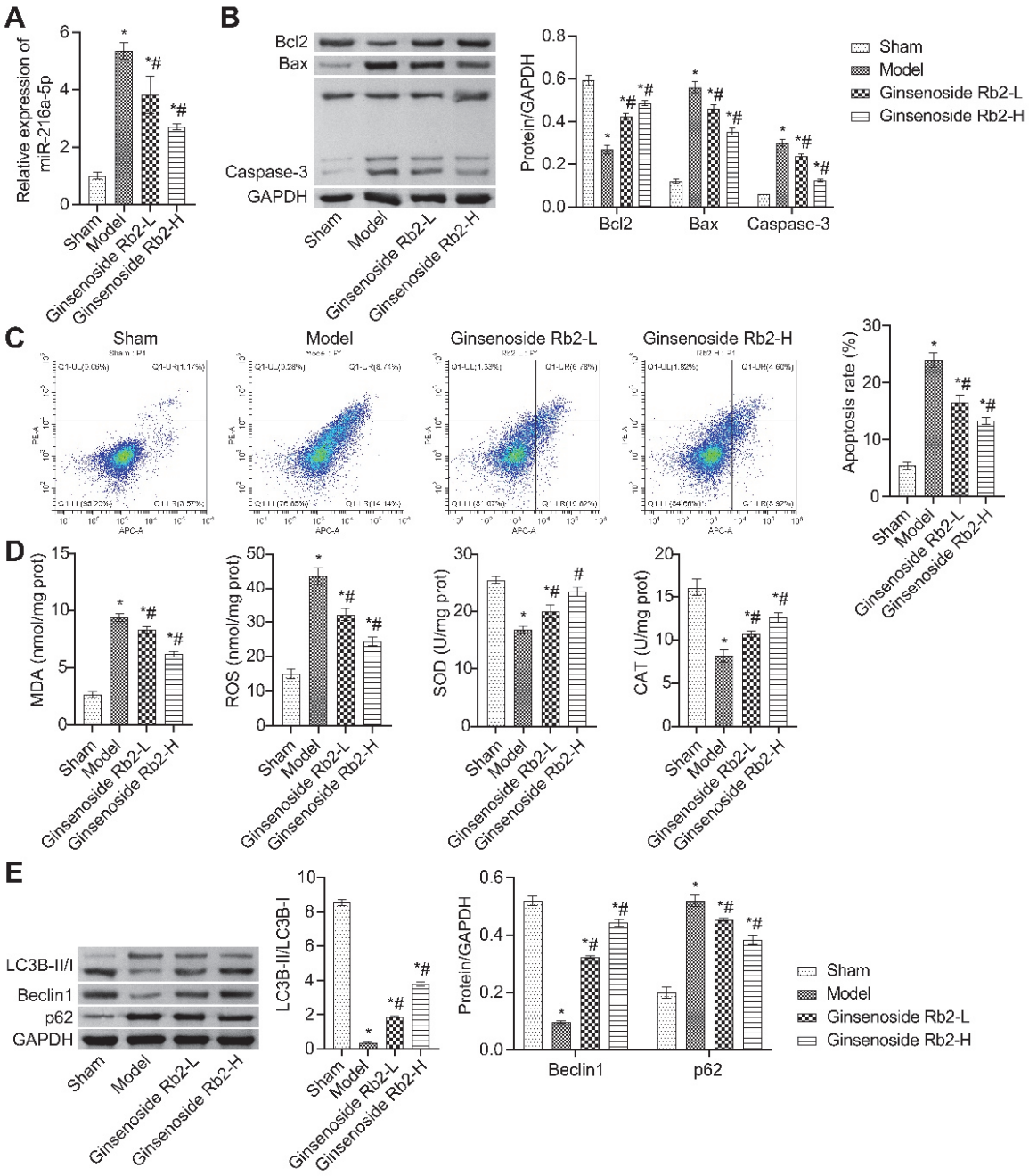

Fig. 2. Ginsenoside Rb2 inhibited miR-216a-5p expression, reduced myocardial apoptosis and oxidative stress, and promoted autophagy in HF rats. (**A**) qRT-PCR results of miR-216a-5p expression in rat myocardium; (**B**) Western blot analysis of expressions of Bcl2, Bax, and Caspase-3 in rat myocardium; (**C**) The apoptosis of rat myocardium was assessed by flow cytometry; (**D**) Levels of MDA, ROS, SOD, and CAT in rat myocardium were measured by biochemical kits; (**E**) Western blot analysis of levels of LC3B II/I, Beclin1, and p62 in rat myocardium.
* $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Model.

Control group showed a long spindle shape, complete morphology, and clear structure. However, OGD/R induction led to cell contraction, shape change, and cell space widening. Remarkably, the intervention of high and medium doses of ginsenoside Rb2 partially restored the morphology and structure of cardiomyocytes, but the low dose of ginsenoside Rb2 had little effect (Fig. 4A). In addition, the proliferation ability of cardiomyocytes was significantly reduced after OGD/R induction. However, with the intervention of different doses of ginsenoside Rb2, their proliferation ability was successfully restored, with the degree of recovery being concentration-dependent (Fig. 4B). These results proved that ginsenoside Rb2 improved the viability loss of OGD/R-induced cardiomyocytes.

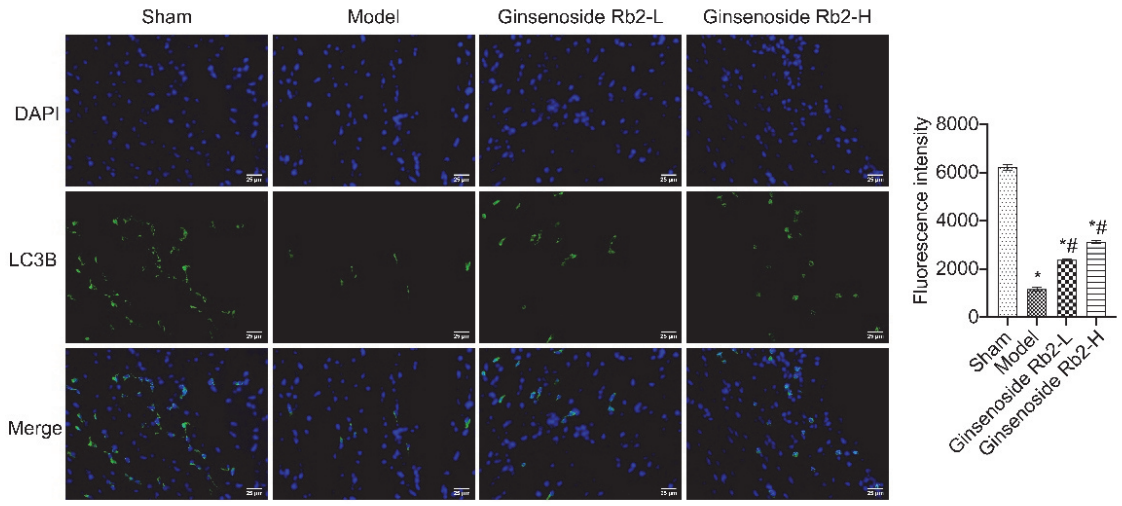

Fig. 3. LC3B content in cardiomyocytes was measured by IF staining. * $P < 0.05$ vs. Sham, # $P < 0.05$ vs. Model.

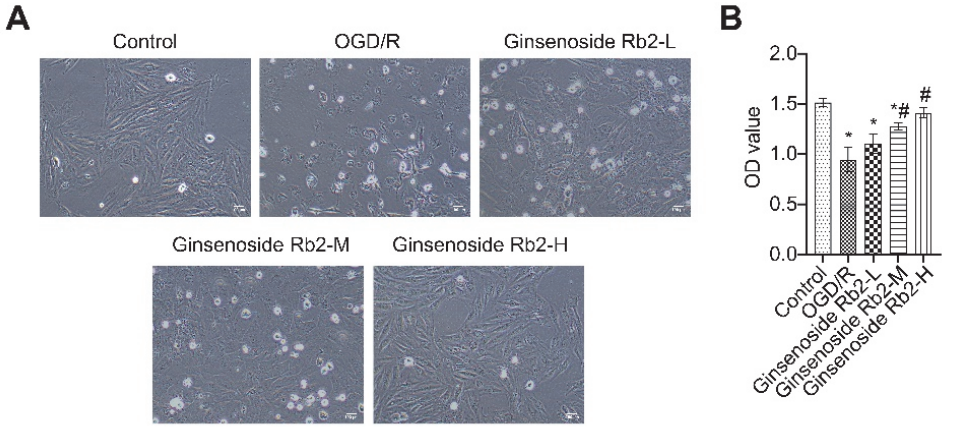

Fig. 4. Ginsenoside Rb2 improved the viability loss of OGD/R-induced cardiomyocytes. (**A**) Cell images; (**B**) CCK-8 assay of the proliferative ability of cardiomyocytes proliferation. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. OGD/R.

Ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in OGD/R-induced cardiomyocytes by down-regulating miR-216a-5p expression

To explore the specific mechanism of ginsenoside Rb2 in the treatment of HF, cardiomyocytes were sequentially treated with miR-216a-5p mimics, OGD/R induction, and ginsenoside Rb2. The increased expression of miR-216a-5p in cardiomyocytes in the Ginsenoside Rb2+miR-216a-5p mimics group verified the successful and efficient transfection (Fig. 5A). Western blot analysis results further displayed that ginsenoside Rb2 treatment significantly reduced OGD/R-induced high apoptosis level in cardiomyocytes. However, compared with the Ginsenoside Rb2+mimics NC group, the expressions of Bax and Caspase-3 were up-regulated while the expression of Bcl2 was down-regulated in the Ginsenoside Rb2+miR-216a-5p mimics group (Fig. 5B). Flow cytometry further confirmed that miR-216a-5p mimics transfection reversed the inhibition of ginsenoside Rb2 on cardiomyocyte apoptosis induced by OGD/R (Fig. 5C). Furthermore, miR-216a-5p mimics transfection partially counteracted the inhibition of ginsenoside Rb2 on HF-induced oxidative stress, as indicated by increased levels of MDA and ROS and decreased levels of SOD and CAT (Fig. 5D). Additionally, ginsenoside Rb2 treatment significantly raised the level of autophagy in OGD/R-induced cardiomyocytes. However, compared with the Ginsenoside Rb2+mimics NC group, the levels of LC3B II/I and Beclin1 were decreased and that of p62 was increased in the Ginsenoside Rb2+miR-216a-5p mimics group (Fig. 5E). IF staining results further verified that miR-216a-5p mimics transfection reversed the promotion of ginsenoside Rb2 on autophagy in OGD/R-induced cardiomyocytes (Fig. 6). These results displayed that ginsenoside Rb2 inhibited apoptosis and oxidative stress, and promoted autophagy in OGD/R-induced cardiomyocytes by down-regulating miR-216a-5p expression.

Upregulation of miR-216a-5p reversed the improvement of ginsenoside Rb2 on heart function in HF rats

weight/

Next, the impact of miR-216a-5p on the improvement of heart function by ginsenoside Rb2 was investigated *in vivo*. As already mentioned, intervention with ginsenoside Rb2 led to a decrease in heart rate, LVEDP, and heart weight/body weight ratio, as well as an increase in LVSP, $+dP/dt_{max}$, $-dP/dt_{max}$, LVEF, and LVFS in HF rats. However, transfection

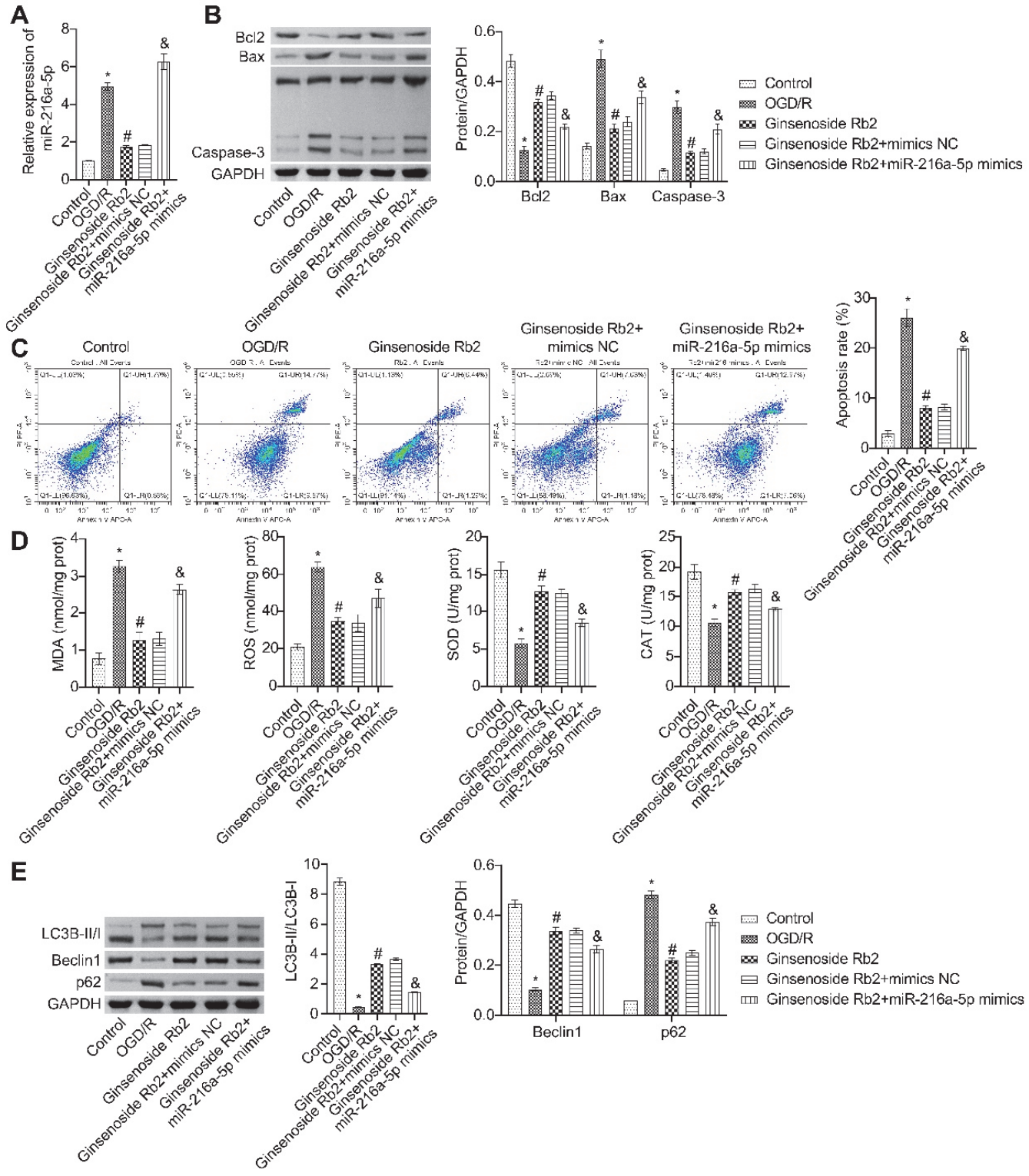

Fig. 5. Ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in OGD/R-induced cardiomyocytes by down-regulating miR-216a-5p expression. (**A**) qRT-PCR results of miR-216a-5p expression in cardiomyocytes; (**B**) Western blot analysis of protein expressions of Bcl2, Bax, and Caspase-3 in cardiomyocytes; (**C**) The myocardial apoptosis was assayed by flow cytometry; (**D**) Levels of MDA, ROS, SOD, and CAT in cardiomyocytes were measured by biochemical kits; (**E**) Western blot analysis of levels of LC3B II/I, Beclin1, and p62 in cardiomyocytes. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. OGD/R, and $P < 0.05$ vs. Ginsenoside Rb2+mimics NC.

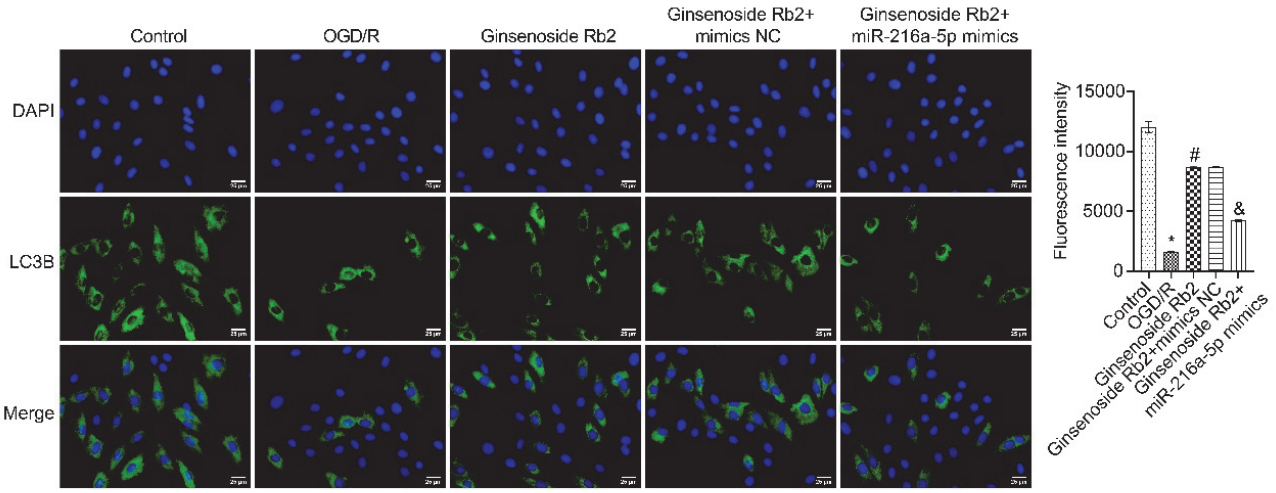

Fig. 6. LC3B content in cardiomyocytes was measured by IF staining. * $P < 0.05$ vs. Control, # $P < 0.05$ vs. OGD/R, and $P < 0.05$ vs. Ginsenoside Rb2+mimics NC.

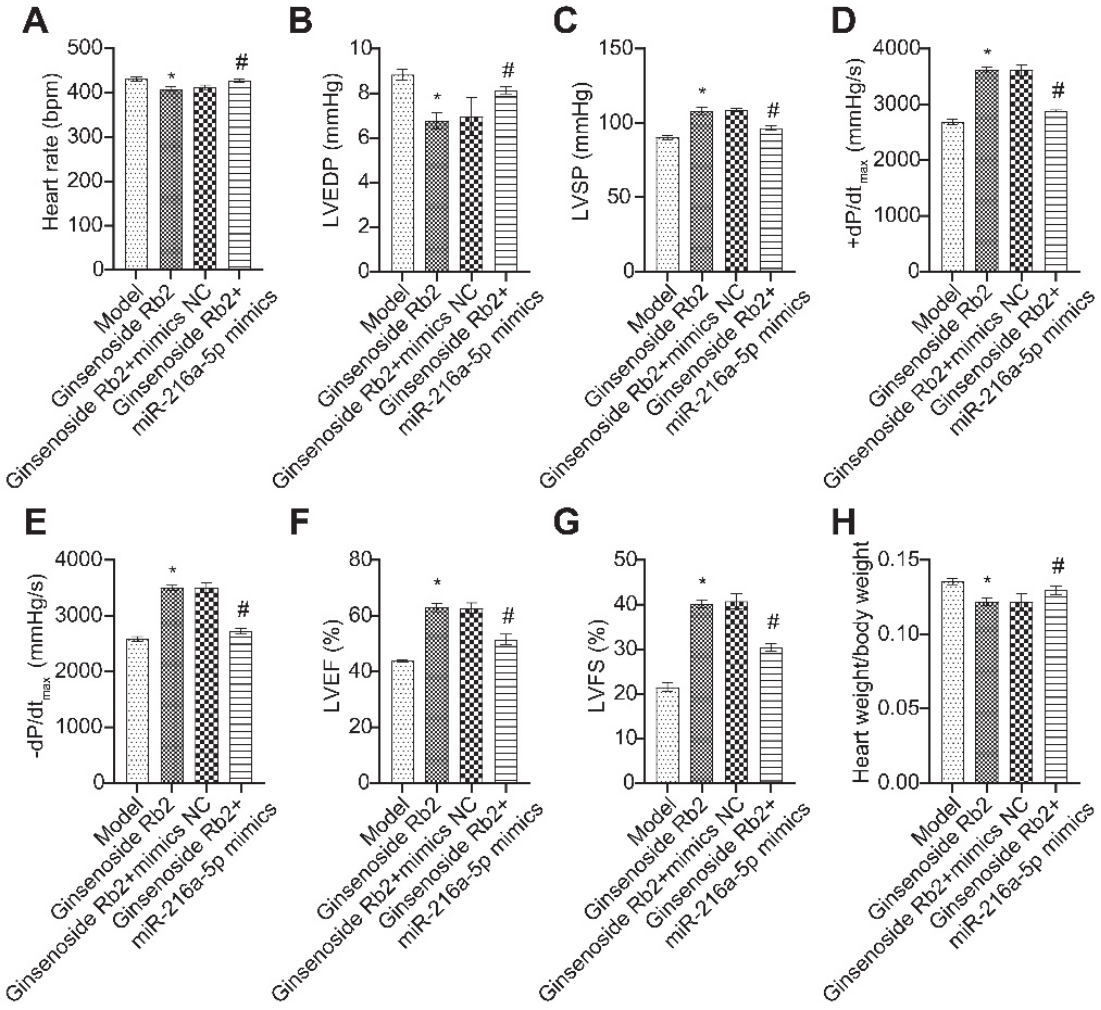

Fig. 7. Upregulation of miR-216a-5p reversed the improvement of ginsenoside Rb2 on heart function in HF rats. (**A**) Heart rate; (**B**) LVEDP; (**C**) LVSP; (**D**) +dP/dtmax; (**E**) –dP/dtmax; (**F**) LVEF (%); (**G**) LVFS (%); (**H**) Heart weight/body weight ratio. * $P < 0.05$ vs. Model, # $P < 0.05$ vs. Ginsenoside Rb2+mimics NC.

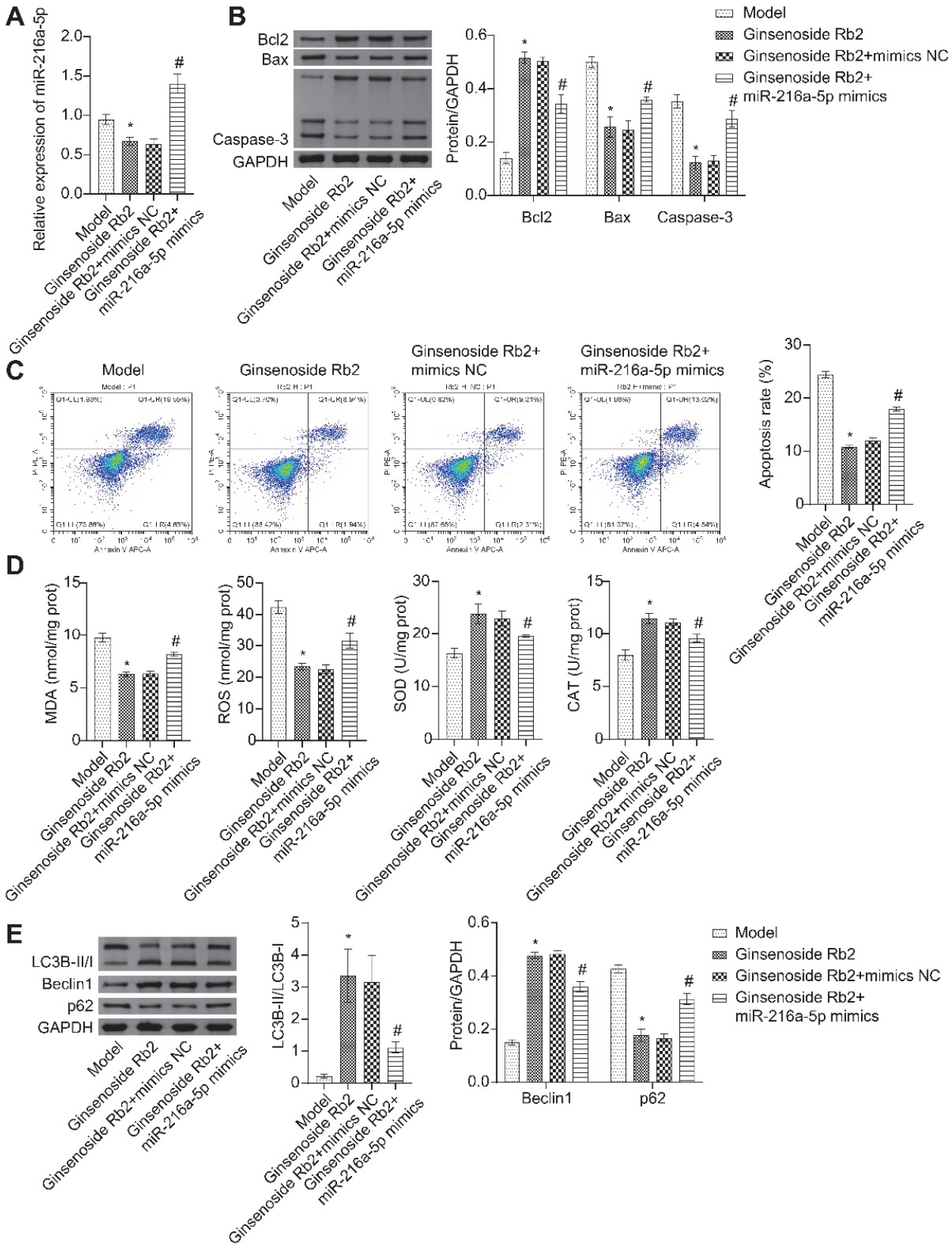

Fig. 8. Ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in HF rats by down-regulating miR-216a-5p expression. (**A**) qRT-PCR results of miR-216a-5p expression in rat myocardium; (**B**) Western blot analysis of expressions of Bcl2, Bax, and Caspase-3 in rat myocardium; (**C**) The apoptosis of rat myocardium was assessed by flow cytometry; (**D**) Levels of MDA, ROS, SOD, and CAT in rat myocardium were measured by biochemical kits; (**E**) Western blot analysis of levels of LC3B II/I, Beclin1, and p62 in rat myocardium. * $P < 0.05$ vs. Ginsenoside Rb2, # $P < 0.05$ vs. Ginsenoside Rb2+mimics NC.

of miR-216a-5p mimics significantly increased heart rate, LVEDP, and heart weight/body weight ratio, and decreased LVSP, $+dP/dt_{max}$, $-dP/dt_{max}$, LVEF, and LVFS in HF rats (Fig. 7A–E). Taken together, the above results indicated that upregulation of miR-216a-5p expression reversed the improvement of ginsenoside Rb2 on heart function in HF rats.

Ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in HF rats by down-regulating miR-216a-5p expression

To verify the mechanism of ginsenoside Rb2 in the treatment of HF *in vivo*, HF rats were sequentially treated with miR-216a-5p mimics and ginsenoside Rb2. The results showed that miR-216a-5p mimics effectively reversed the downregulation of HF-induced miR-216a-5p expression by ginsenoside Rb2 (Fig. 8A). Western blot analysis further displayed that compared with the Ginsenoside Rb2+mimics NC group, the expressions of Bax and Caspase-3 were up-regulated while the expression of Bcl2 was down-regulated in the Ginsenoside Rb2+miR-216a-5p mimics group (Fig. 8B). Flow cytometry also confirmed that miR-216a-5p mimics reversed the inhibition of ginsenoside Rb2 on cardiomyocyte apoptosis in HF rats (Fig. 8C). Furthermore, miR-216a-5p mimics partially counteracted the inhibition of ginsenoside Rb2 on HF-induced oxidative stress, as indicated by increased levels of MDA and ROS, and decreased levels of SOD and CAT (Fig. 8D). Compared with the Ginsenoside Rb2+mimics NC group, the levels of LC3B II/I and Beclin1 were decreased and that of p62 was increased in the Ginsenoside Rb2+miR-216a-5p mimics group (Fig. 8E). IF staining results further verified that miR-216a-5p mimics reversed the promotion of ginsenoside Rb2 on autophagy in HF rats (Fig. 9). These findings displayed that ginsenoside Rb2 inhibited apoptosis and oxidative stress and promoted autophagy in HF rats by down-regulating miR-216a-5p expression.

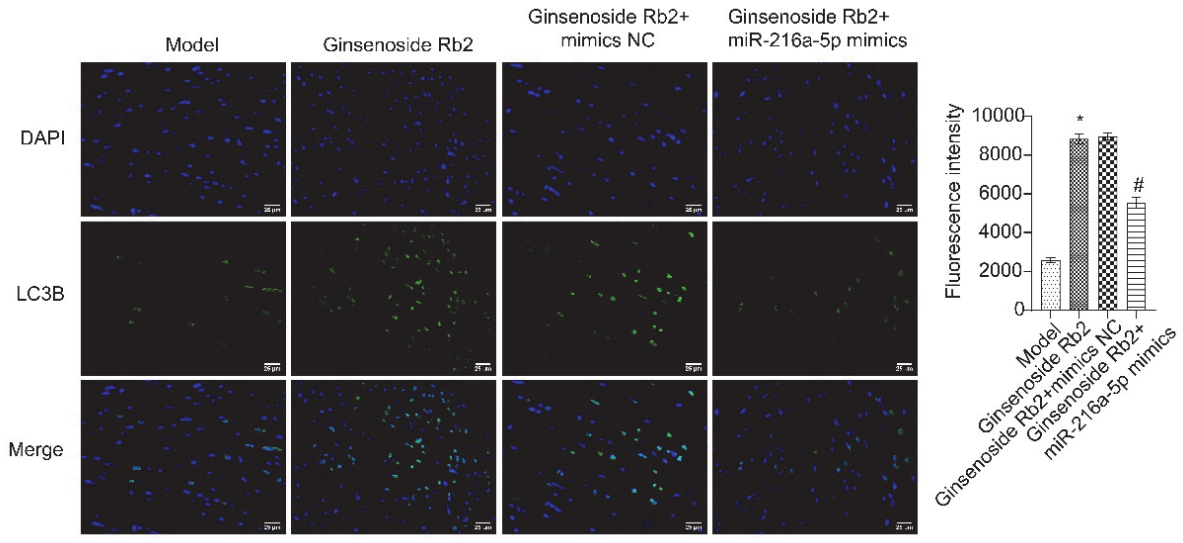

Fig. 9. LC3B content in cardiomyocytes was measured by IF staining. * $P < 0.05$ vs. Ginsenoside Rb2, # $P < 0.05$ vs. Ginsenoside Rb2+mimics NC.

Discussion

HF is a common cardiovascular disease, which has high morbidity and mortality (Orso et al., 2017). Traditional HF drugs have certain limitations, such as more adverse reactions (Yancy et al., 2013). Thus, it is urgent to find other HF drugs and explore their therapeutic mechanisms. Ginsenoside Rb2, one of the main active ingredients in ginseng, has been proven to have a protective effect on the heart (Xue et al., 2020). However, the treatment of HF by ginsenoside Rb2 and its possible mechanism remains unclear. Here, ligation surgery of the left anterior descending branch was used to cause myocardial infarction, and the HF rat model was obtained 4 weeks later. After the intervention of ginsenoside Rb2, the heart rate, LVEDP, and heart weight/body weight ratio were reduced, LVSP, $+dP/dt_{max}$, $-dP/dt_{max}$, LVEF, and LVFS were increased in HF rats, and the myocardial relaxation effect was effectively improved to restore the heart function. It has been discovered that ginsenosides can play a strong role in improving hemodynamics and weakening myocardial remodeling to a certain extent (Lim et al., 2013). Myocardial remodeling is a compensatory mechanism in HF, but it also changes the shape and function of the myocardium and accelerates the progression of HF (Wang et al., 2002). This study further verified that ginsenoside Rb2 reduced the myocardial remodeling induced by HF, kept the morphological integrity of cardiomyocytes, and restored cell viability.

Autophagy has been explained to be related to the death of cardiomyocytes. By regulating the autophagy level of cardiomyocytes and the expression of related proteins, it exerts a protective effect on cardiomyocytes and thus delays the progression of HF (Gurusamy et al., 2009, 2010). Here, the autophagy levels of HF rats and OGD/R-induced cardiomyocytes were significantly decreased. The autophagy of cardiomyocytes was induced by the intervention of ginsenoside Rb2, which showed up-regulated LC3B II/I and Beclin1 levels and down-regulated p62 expression. In related studies, apoptosis was inhibited by inducing autophagy of H9c2 cells from the myocardium (Jia et al., 2015). LC3 is often used as a sensitive marker to detect autophagy (Corsetti et al., 2019). The regulation of Beclin 1 significantly affects autophagy and ap-

optosis, and thus profoundly affects the survival and death of cardiomyocytes (Maejima et al., 2016). The transcriptional and post-translational regulation of p62 constitutes an important mechanism for regulating autophagy (Jeong et al., 2019). In the development of HF, cardiomyocyte apoptosis is the key, which seriously affects the recovery of heart function. Apoptosis is regulated by a variety of genes, and the classic genes are Bcl-2, Bax, and Caspase-3 (Yuan et al., 2021). Bax and Bcl-2 are positive and negative regulators of cardiomyocyte apoptosis in ischemic cardiovascular diseases. The Bcl-2/Bax ratio is an important indicator of myocardial viability, and apoptosis occurs when the ratio decreases (Kilbride and Prehn, 2013). Here, the apoptosis level of HF rats and OGD/R-induced cardiomyocytes was significantly increased. The intervention of ginsenoside Rb2 significantly reduced cardiomyocyte apoptosis, which was characterized by down-regulation of Bax and Caspase-3 expressions, and up-regulation of Bcl2 expression.

The study has found that HF may be associated with antioxidant deficiencies as well as increased myocardial oxidative stress (Hill and Singal, 1996). SOD and CAT were the main antioxidant enzymes responsible for ROS inactivation in cardiomyocytes (van der Pol et al., 2019). This study revealed an increase in oxidative stress in OGD/R-induced H9c2 cells and HF rat myocardia, marked by reduced SOD and CAT levels and heightened MDA and ROS levels, aligning with previous literature trends (Khaper et al., 2003; Waldman et al., 2018). However, these changes were reversed after ginsenoside Rb2 intervention. The above findings indicated that the mechanism of ginsenoside Rb2 in treating HF was related to the reduction of apoptosis and oxidative stress and the induction of autophagy.

In studies, miRNAs can regulate many aspects of heart disease, such as myocardial hypertrophy, myocardial remodeling, heart failure, and arrhythmia (Bauersachs and Thum, 2007; Divakaran and Mann, 2008; Thum et al., 2007; van Rooij and Olson, 2007). Recently, inhibition or reversal of myocardial remodeling has been a hot topic in the treatment of HF. In addition, miRNAs have been reported to play an important role in the pathogenesis, diagnosis, treatment, and prognosis evaluation of HF (Divakaran and Mann, 2008; Foinquinos et al., 2020; Matsumoto et al., 2013; Tijsen et al., 2010). miR-125b has been found to inhibit cardiomyocyte apoptosis in HF by targeting Bcl-2 Antagonist/Killer 1 (BAK1) (Zhang et al., 2021). Furthermore, miR-221 inhibits autophagy and promotes HF by regulating the p27/cyclin-dependent kinase 2 (Cdk2)/mammalian target of rapamycin (mTOR) axis (Su et al., 2015). Several microRNAs are involved in oxidative stress-related processes that contribute to the development of HF (Klimczak-Tomaniak et al., 2022). Here, miR-216a-5p was up-regulated in HF rats and OGD/R-induced cardiomyocytes, which was consistent with the results in the literature (Tao et al., 2019). The intervention of ginsenoside Rb2 significantly down-regulated miR-216a-5p expression. However, overexpression of miR-216a-5p reversed the therapeutic effect of ginsenoside Rb2 on HF by induction of myocardial apoptosis and oxidative stress and inhibition of autophagy. Relevant studies have found that autophagy is down-regulated in HF and negatively correlated with miR-216a expression (Menghini et al., 2014). Therefore, ginsenoside Rb2 could regulate the expression of miR-216a-5p, thus becoming a potentially effective drug for HF therapy.

There are also some limitations to this study. First, the experimental results were based on the left anterior descending branch-ligated HF rats and OGD/R-induced H9c2 cells. However, these models may differ from HF in human patients, so caution should be exercised when interpreting the results for translation into clinical applications in humans. Moreover, this study primarily focused on evaluating the effects on cardiac function and cellular level, lacking in-depth exploration of underlying mechanisms such as drug pharmacology, pharmacokinetics, safety, molecular signaling pathways, *etc.* Furthermore, this study only focused on the therapeutic effects of ginsenoside Rb2 and did not consider other factors that may influence the development and treatment outcomes of HF. HF is a complex disease that can be influenced by multiple factors, such as hypertension, diabetes, hyperlipidemia, *etc.* Therefore, further research is needed to validate the results of this study and to conduct more comprehensive

Conclusion

In summary, our findings demonstrated that ginsenoside Rb2 had therapeutic effects on HF both *in vivo* and *in vitro*. These beneficial effects may be due to the down-regulation of miR-216a-5p, resulting in reduced apoptosis and oxidative stress while simultaneously promoting the autophagy of cardiomyocytes. This study will provide a basis for the research of pathogenesis and new ginsenoside Rb2-based drugs for HF.

Authors' contributions

Study conception and design: You Peng. Data collection: You Peng, Bin Liao, Yan Zhou, Wei Zeng. Analysis and interpretation of results: You Peng, Bin Liao. Draft manuscript preparation: You Peng. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare regarding the present study.

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Ethics approval

All animal experiments were approved by the Animal Ethics Committee of Hunan Provincial People's Hospital (2023-01).

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