

# Hydrogen therapy after resuscitation improves myocardial injury involving inhibition of autophagy in an asphyxial rat model of cardiac arrest

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**Abstract.** Hydrogen (H<sub>2</sub>) therapy is a therapeutic strategy using molecular H<sub>2</sub>. Due to its ability to regulate cell homeostasis, H<sub>2</sub> therapy has exhibited marked therapeutic effects on a number of oxidative stress-associated diseases. The present study investigated the effectiveness of H<sub>2</sub> therapy in protecting against myocardial injury in a rat model of asphyxial cardiac arrest and cardiopulmonary resuscitation.

Rats underwent 10-min asphyxia-induced cardiac arrest (CA) and cardiopulmonary resuscitation (CPR), and were randomly divided into control and H<sub>2</sub> therapy groups. After resuscitation, the H<sub>2</sub> therapy group was administered room air mixed with 2% H<sub>2</sub> gas for respiration. During CA/CPR, the arterial pressure and heart rate were measured every minute. Survival rate, cardiac function, myocardial injury biomarkers creatine kinase-MB and cardiac troponin-T, and histopathological changes were evaluated to determine the protective effects of H<sub>2</sub> therapy in CA/CPR. Immunohistochemistry and western blot analysis were used to determine the expression levels of autophagy-associated proteins. *In vitro*, H9C2 cells were subjected to hypoxia/reoxygenation and H<sub>2</sub>-rich medium was used in H<sub>2</sub> treatment groups. Western blotting and immunofluorescence were used to observe the expression levels of autophagy-associated proteins. Moreover, an adenovirus-monomeric red fluorescent protein-green fluorescent protein-LC3 construct was used to explore the dynamics of autophagy in the H9C2 cells. The results showed that H<sub>2</sub> therapy significantly improved post-resuscitation survival and cardiac function. H<sub>2</sub> therapy also improved mitochondrial mass and decreased autophagosome numbers in cardiomyocytes after resuscitation. The treatment inhibited autophagy activation, with lower expression levels of autophagy-associated proteins and decreased autophagosome formation *in vivo* and *in vitro*. In conclusion, H<sub>2</sub> gas inhalation after return of spontaneous circulation improved cardiac function via the inhibition of autophagy.

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## Introduction

Cardiac arrest (CA) is a global public health concern with a low resuscitation rate and a high mortality rate (1,2). Despite improvements in cardiopulmonary resuscitation (CPR) and post-resuscitation care in recent years, the post-discharge survival rate of patients with return of spontaneous circulation (ROSC) is less than one-third of all cases (3-6). Myocardial dysfunction along with the systemic ischemia/reperfusion (I/R) injury that occurs during CPR is a primary cause for the poor prognosis of patients after ROSC (7). Thus, protection of myocardial function after CA is essential and of significant value.

Although the mechanism of CPR-induced myocardial injury is not fully understood, systemic I/R-induced reactive oxygen species (ROS) has been widely demonstrated as a critical factor (8-10). CA/ROSC is a global I/R event accompanied by ROS generation. Moreover, the accumulation of ROS is further exacerbated after ROSC owing to oxygenated blood returning to the tissues, which generates oxidation of cell macromolecular substances (9,11). Generally, oxidative stress events, featuring excessive production of ROS, have been recognized as serving a key role in cell damage, mitochondrial dysfunction, and ultimately cell apoptosis and death (12).

Restoring spontaneous circulation and preventing hypoxic ischemic tissue injury are the goals of CPR (13). As sufficient oxygen delivery is required to restore and maintain the energy state of the heart, the use of maximal inspired oxygen ( $O_2$ ) concentrations during CPR and of earlier post-resuscitation are recommended in the European Resuscitation Council Guidelines 2021 (14). However, a considerable amount of data has emerged challenging the appropriateness of the use of 100%  $O_2$  during and after resuscitation from CA (15,16). Hyperoxia has been shown to increase the generation of ROS, resulting in aggravated reperfusion myocardial injury and worsened CPR outcomes (17).

Hydrogen ( $H_2$ ) gas is a type of endogenous gas transmitter produced by the intestinal flora during the fermentation of nondigestible carbohydrates (18). Previous studies have demonstrated that molecular  $H_2$  is a new type of safe and effective therapeutic agent (19,20). In addition, studies to date have found that  $H_2$  therapy significantly protects against oxidative stress and inflammation-related diseases, such as cancer, atherosclerosis, diabetes, I/R injury, neurodegenerative diseases, arthritis, hepatitis and pancreatitis (21-24). Based on its safety and wide effectiveness,  $H_2$  therapy is attracting increasing attention and is undergoing an important evolution from bench to bedside (19,25). In previous studies,  $H_2$  inhalation without hyperoxia after resuscitation improved the neurological outcome in rat models of CA (26,27). However, the effects of  $H_2$  inhalation on CA/CPR-induced myocardial injury remain poorly understood. Therefore, the present study used  $H_2$  inhalation in post-resuscitation care to investigate its effect on myocardial injury induced by CA in rats.

## Materials and methods

**Animals.** A total of 60 adult male Wistar rats (age, 10-12 weeks; weight, 400-450 g) were purchased from the Experimental Animal Center of Shandong University (Jinan, China). All animals were housed together in a room maintained at 40-60%

relative humidity and  $23\pm 2^\circ\text{C}$ , with 12-h light/dark cycles and *ad libitum* access to food and water. All animal experiments were approved by the Animal Care and Use Committee of the Qilu Hospital of Shandong University (Jinan, China; approval no. KYLL-2020-ZM-122), and adhered to the Care and Use of Laboratory Animals guidelines and to the Animal Research: Reporting of *In Vivo* Experiments guidelines. All animal experiments took place at Shandong Provincial Engineering Laboratory for Emergency and Critical Care Medicine, Qilu Hospital of Shandong University. All animals were anesthetized with phenobarbital sodium and then sacrificed with carbon dioxide release devices.

**Rat model of asphyxial CA.** The 10-min asphyxial CA/CPR model was used in this study. Rats were randomly assigned to the following four groups ( $n=15$  per group): i) Sham + normoxia (anesthetized with 4% isoflurane mixed with room air and normoxia inhalation by ventilator for 2 h); ii) Sham +  $H_2$  (anesthetized with 4% isoflurane mixed with room air and  $H_2$  inhalation by ventilator for 2 h); iii) CPR + normoxia (anesthetized with 4% isoflurane mixed with room air, CA/CPR treatment and normoxia inhalation by ventilator for 2 h after ROSC); and iv) CPR +  $H_2$  (anesthetized with 4% isoflurane mixed with room air, CA/CPR treatment and  $H_2$  inhalation using a ventilator for 2 h after ROSC). Rats were anesthetized with 4% isoflurane mixed with room air and were under tracheal intubation with connection to a ventilator. Intravascular catheters were placed into the right femoral artery and right vein for blood pressure monitoring and drug administration, respectively. After stabilization for 20 min, the ventilator was disconnected to induce CA. Circulatory arrest was determined by cessation of the arterial pulse and a mean arterial pressure (MAP) of  $<20$  mmHg. After 10 min of asphyxia, CPR was performed, with inhalation of air with a ventilator and intravenous administration of epinephrine (0.01 mg/kg). In addition, the rate of artificial chest compressions was  $\sim 200$  per min. Epinephrine (0.02 mg/kg) was administered at 2-min intervals until ROSC was achieved. ROSC was defined by an MAP of  $>60$  mmHg that lasted for at least 10 min. A total of 3 rats with ROSC failure within 5 min or those that could not be disengaged from the ventilator after observation for 1 h were excluded from the study. The core temperature of each rat was maintained at  $37.0\pm 0.5^\circ\text{C}$ . After ROSC, gas inhalation was continued for 2 h (Fig. 1A): Premixed gas (1.3%  $H_2$  and 26%  $O_2$ ) was used in the  $H_2$  therapy groups, and normoxia (26%  $O_2$ ) was used as the control (27). Following this, the rats were euthanized by exposure to a gradually increasing concentration of  $CO_2$  (the flow rate was 50% of the chamber volume/min).

**Cell culture.** H9C2 cells were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin + 100 mg/ml streptomycin in a cell incubator (95% air and 5%  $CO_2$ ) at  $37^\circ\text{C}$ . In the cell hypoxia/reoxygenation (H/R) model, H9C2 cells were exposed to hypoxia (5%  $CO_2$  and 1%  $O_2$  at  $37^\circ\text{C}$ ) for 24 h and reoxygenation (95% air and 5%  $CO_2$ ) for 4 or 12 h.

**$H_2$  treatment *in vitro*.** In the  $H_2$  treatment group,  $H_2$ -rich medium was used to culture the H9C2 cells instead of normal

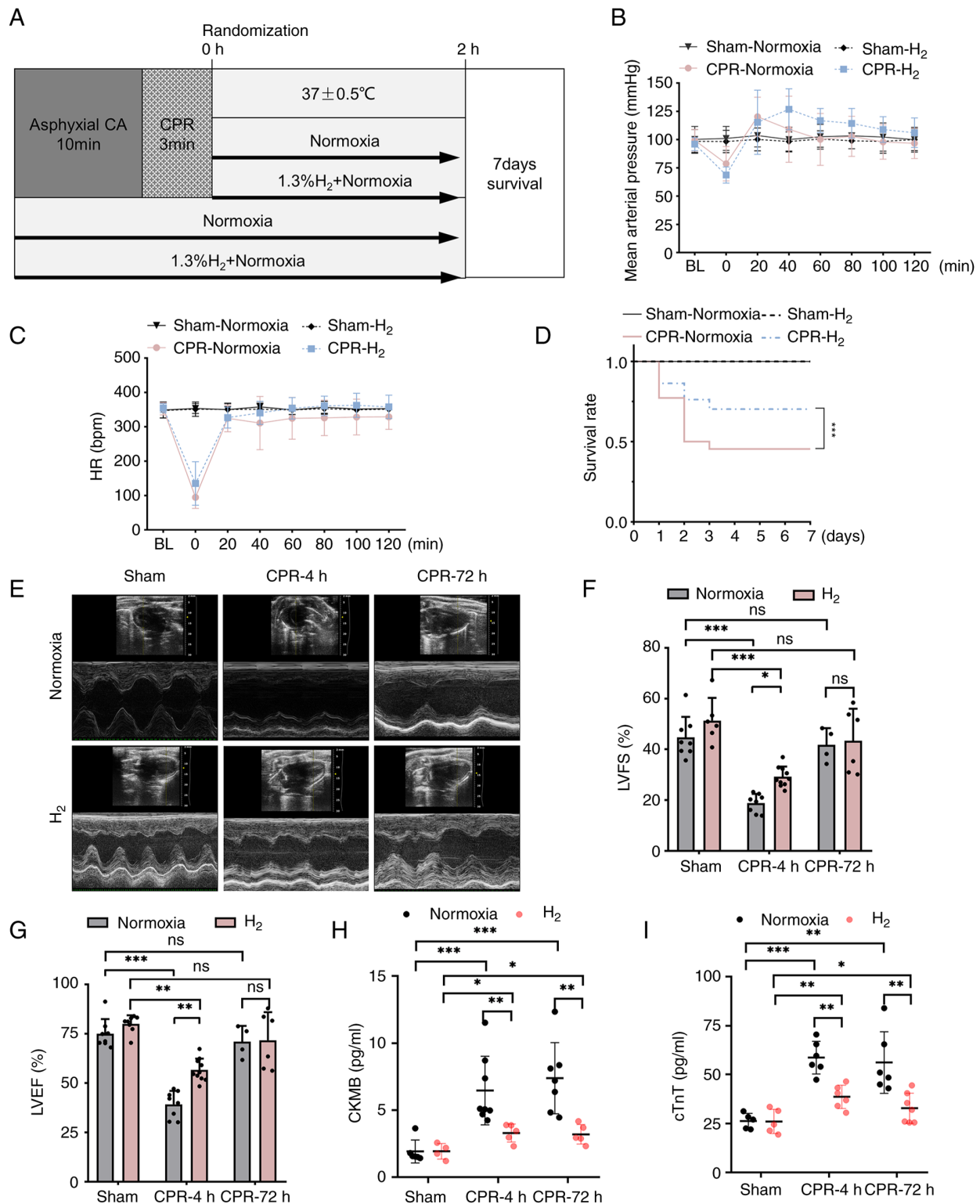


Figure 1. Inhalation of H<sub>2</sub> gas after ROSC improves post-resuscitation survival and cardiac function. (A) The experimental process for CPR and post-resuscitation care in the rat model of asphyxial CA/CPR. (B and C) The MAP and heart rate of rats during asphyxial CA/CPR. (D) The survival rate of rats in each group were recorded for 7 days after asphyxial CA/CPR (n=10). (E-G) Representative images and quantitative assessment of LVFS and LVEF evaluated by echocardiography (n=8-10). (H and I) The serum levels of CKMB and cTnT (n=5). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. ROSC, return of spontaneous circulation; MAP, mean arterial pressure; H<sub>2</sub>, hydrogen molecule; HR, heart rate; CA, cardiac arrest; CPR, cardiopulmonary resuscitation; LVFS, left ventricular fraction shortening; LVEF, left ventricular ejection fraction; CKMB, creatine kinase-MB; cTnT, cardiac troponin T.

DMEM. H<sub>2</sub> was diluted into cell culture medium to produce an H<sub>2</sub>-rich culture medium (0.6 mmol/l) (28). The H<sub>2</sub>-rich medium was freshly prepared for each experiment. DMEM was used as the vehicle.

*Transmission electron microscopy (TEM).* Part of the left ventricle (LV) was obtained from the rats and fixed quickly in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature for 2 h. After

3 washes in phosphate-buffered saline, tissues were fixed and stained using 1% osmium tetroxide on 4°C for 2 h and washed 3 times in phosphate-buffered saline. After ethanol dehydration, samples were embed in LX112 resin (Ladd Research Industries) at room temperature for 2 h. Ultrathin sections (~70 nm) were obtained with a MT-X ultramicrotome (Leica EM UC7; Leica Microsystems, Inc.) and observed using an electron microscope (H-7650; Hitachi, Ltd.). Images of sections were assessed using ImageJ (V1.8.0.112; National Institutes of Health).

**Mitochondrial mass quantification.** The TEM images were processed using ImageJ V1.8.0.112. The outline of each mitochondrion was precisely drawn using a Surface Pro 6 tablet equipped with a touch pen (Microsoft Corporation), and filled with a solid bright color. The image with color-filled mitochondria was converted into a binary black and white image using the 'color threshold' command, and areas of mitochondria were then generated using the 'analyze particles' command. Briefly, this command recognized the black objects (mitochondria) in the binary images and outlined them such that the area of each outlined object was automatically computed. Fractional area was calculated as total mitochondrial area divided by image area for the cardiomyocytes. Mitochondrial number and average mitochondrial area (an indication of mitochondrial size) were also measured (29).

**Western blotting.** Samples (left ventricle tissues from rats and H9C2 cells) were lysed with RIPA buffer (Beyotime Institute of Biotechnology) for 30 min on ice. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was transferred to a new tube. PMSF (1:100) was added, and a BCA kit (Beyotime Institute of Biotechnology) was used to determine protein concentration. Proteins (20 µg) were separated using 10% SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% milk in PBS at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. The membranes were washed three times with TBS-Tween-20 (TBS-T) and incubated with secondary antibody (dissolved in 1% BSA; 1:5,000) at room temperature for 1 h. After being washed with TBS-T three times, the membranes were exposed to ECL substrate (Beyotime Institute of Biotechnology; catalog no. P0018FS) and detected by the chemiluminescence method in an AI600 gel imaging system (GE Healthcare). The following primary antibodies were used: Anti-Beclin-1 (catalog no. ab223348; 1:1,000), anti-LC3B (catalog no. ab51520; 1:1,000), anti-p62 (catalog no. ab109012; 1:1,000) and anti-β-actin (catalog no. ab8226; 1:1,000) (all Abcam). The secondary antibodies were goat anti-rabbit IgG H&L (HRP) (catalog no. ab205718; 1:5,000) and goat anti-mouse IgG H&L (HRP) (catalog no. ab6789; 1:5,000) (both Abcam).

**Immunohistochemical analysis.** Rats tissues were fixed with 4% polyformaldehyde at room temperature for 24 h, then dehydrated with alcohol and washed with xylene. The tissues were embedded in a wax block and sliced to a 5-µm thickness. Dewaxing agent was used to dewax the sections at 55°C for 1 h, and different concentrations of alcohol (100, 95, 90, 80, 70, 60 and 50%, for 30 min each) were

used to wash the sections. According to the instructions of the SABC-POD kit (Boster Biological Technology; catalog no. SA1028), sections were incubated with 3% H<sub>2</sub>O<sub>2</sub> at room temperature for 5 min, and infiltrated into 0.01 M Citrate Antigen Retrieval solution (Wuhan Servicebio Technology Co., Ltd.; catalog no. G1201-1L) at 100°C for 20 min. To cool them down to room temperature, sections were washed three times with PBS (phosphate-buffered saline). After blocking with 5% BSA (Wuhan Servicebio Technology Co., Ltd.; catalog no. SW3015) at 37°C for 30 min, sections were incubated with primary antibodies at 4°C overnight. Next, the sections were washed three times with PBS, and incubated with anti-rabbit IgG H&L (HRP; 1:1,000; cat. no. ab205718; Abcam) and goat anti-mouse IgG H&L (HRP; 1:1,000; cat. no. ab6789; Abcam) at 37°C for 30 min. After washing three times with PBS, the sections were incubated with SABC at room temperature for 20 min. Finally, sections were incubated with DAB substrate (Boster Biological Technology; catalog no. AR1022) and washed with ddH<sub>2</sub>O. Sections were redyed with hematoxylin for 30 sec, dehydrated with inversed different concentrations of alcohol and sealed with Permount™ Mounting Medium (Sangon Biotech, Co., Ltd.; catalog no. E675007). Immunohistochemical staining was performed with anti-LC3B (1:1,000). Images of sections were captured (IX53; Olympus Corporation) and assessed (ImageJ V1.8.0.112) according to the percentage of stained cells (total original magnification, x40; area, 250x250 µm).

**Immunofluorescence analysis and confocal microscopy.** H9C2 cells were incubated with the aforementioned primary antibodies to evaluate the levels of Beclin-1 (1:100) and LC3B (1:100). Samples were incubated with DAPI after washing three times with PBS at room temperature, and then sealed with coverslips immediately. Images of cells were taken using confocal laser scanning fluorescence microscopy (SP8; Leica Microsystems GmbH).

**Evaluation of fluorescent LC3 punctae.** The changing fluorescent punctae of LC3 in H9C2 cells were observed with a tandem red fluorescent protein (RFP)-green fluorescent protein (GFP)-LC3 construct (Ad-RFP-GFP-LC3). Ad-RFP-GFP-LC3 adenovirus was purchased from ViGene Biosciences (Charles River Laboratories, Inc.). H9C2 cells (American Type Culture Collection; cat. no. CRL-1446) were transfected with Ad-RFP-GFP-LC3 at 50 MOI. In brief, H9C2 cells were inoculated into a 24-well plate at a density of 1x10<sup>5</sup> cells/well. A total of 250 µl of DMEM containing 1% FBS (both Thermo Fisher Scientific, Inc.), 100 µl adenovirus (10<sup>6</sup> PFU/ml; ViGene Biosciences; Charles River Laboratories, Inc.) and 100 µl Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added to each well. After mixing, the cells were cultured for 6 h in an incubator containing 5% CO<sub>2</sub> at 37°C. After 6 h, the adenovirus was moved off and the transfected cells were cultured for 48 h continuously. The green and red fluorescence intensities were assessed under laser scanning fluorescence microscopy (SP8; Leica Microsystems GmbH). Images of sections were assessed (ImageJ software V1.8.0.112; National Institutes of Health) according to the numbers of red and yellow dots in each cell (total original magnification, x63).



**Ultrasonic cardiogram.** Under isoflurane anesthesia, the spontaneous breathing of the rats was maintained. The two-dimensional images of the LV [left ventricular fraction shortening (LVFS) and left ventricular ejection fraction (LVEF)] were collected using ultrasonic cardiogram equipment (Vevo2100; VisualSonics, Inc.) on the short-axis and long-axis section of the parasternal papillary muscles. The two-dimension-guided M-mode or B-mode ultrasonic cardiogram of 10 cardiac cycles was obtained. Images were assessed using Vevo2100 software.

**Creatine kinase-MB (CKMB) and cardiac troponin-T (cTnT) measurement.** Blood was collected at sacrifice and then the serum was isolated using centrifugation (1,000 g; 4°C for 15 min). The serum concentrations of CKMB (Cloud-Clone Corp.; catalog no. SEA479Ra) and cTnT (Cloud-Clone Corp.; catalog no. SED232Ra) were measured by ELISA kit following the manufacturer's instructions.

**Statistical analysis.** The statistical significance was performed using GraphPad Prism 8 (GraphPad Software, Inc.) Differences between groups were estimated using one-way ANOVA followed by Tukey's post hoc test. Comparisons across two variables were used a two-way ANOVA followed by Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference. All data are expressed as the mean  $\pm$  standard error.

## Results

**Inhalation of  $H_2$  gas after ROSC improves post-resuscitation survival and cardiac function.** As shown in Fig. 1B and C, there were no statistical differences in MAP and heart rate during post-resuscitation care whether using inhalation of  $H_2$  gas or not. The survival rate of the rats was recorded continuously for 7 days after CA/CPR (Fig. 1D). In the Sham groups, the survival rate was 100% whether using inhalation of  $H_2$  gas or not. However, compared with that in the normoxia groups, the survival rate at 7 days after ROSC was significantly higher following inhalation of  $H_2$  gas during post-resuscitation care.

Ultrasonic cardiogram detection was used to further evaluate the effect of  $H_2$  on the cardiac function of rats after ROSC. As shown in Fig. 1E, cardiac function was evaluated by echocardiography at 4 and 72 h post-ROSC. Echocardiograms were analyzed by the LV trace of M mode images using VevoStrain software. Compared with the Sham group, the LVFS and LVEF were significantly decreased within 4 h following ROSC. However, the LVFS and LVEF significantly increased after ROSC in the CPR +  $H_2$  group (Fig. 1F and G). However, there was no significant difference in levels of LVFS and LVEF between CPR and CPR +  $H_2$  groups at 72 h. Moreover, compared with inhalation normoxia,  $H_2$  therapy also markedly decreased the levels of myocardial injury biomarker CKMB and cTnT in serum after CPR at 4 and 72 h (Fig. 1H and I). Thus, inhalation of  $H_2$  after ROSC significantly improved the cardiac function of rats.

**Inhalation of  $H_2$  gas after ROSC improves mitochondrial mass and decreases the number of autophagosomes in rat cardiomyocytes.** To investigate the potentially cardioprotective mechanism of the inhalation of  $H_2$  after ROSC, the

cross-section and TEM images of LV cardiomyocytes were observed. As shown in Fig. 2A and B, TEM analysis revealed the presence of extensive mitochondrial abnormalities, such as swelling, disorganization and loss of cristae, and the relative mitochondrial mass were significantly decreased in post-resuscitation rat LV cardiomyocytes. However, inhalation of  $H_2$  gas after ROSC significantly decreased the number of abnormal mitochondria and increased the relative mitochondrial mass in rat LVs, and cardiac tissue seemed to have returned to its original morphology at 72 h.

Moreover, autophagic lysosomal structures were common in post-resuscitation rat LV cardiomyocytes, suggesting the activation of autophagic cell death mechanisms. In the CPR groups, a significant decrease in autophagic vesicles was observed at 4 and 72 h in post-resuscitation rats after inhalation of  $H_2$  gas compared with normoxia (Fig. 2A and C).

**Inhalation of  $H_2$  gas after ROSC decreases the expression levels of Beclin-1 and suppresses autophagy activation in rat cardiomyocytes.** The study next investigated how  $H_2$  affects autophagy in the heart. As show in Fig. 2D-G, western blot analysis revealed increased expression levels of the autophagy promotor protein Beclin1 in normoxic post-resuscitation rat hearts. The expression levels of LC3B were also higher in the CPR + Normoxia groups at 4 and 72 h compared with that in the Sham-Normoxia group, and the ratio of LC3BII/I was also significantly higher, indicating an enrichment of LC3BII. However, the expression levels of p62 were significantly lower in the same groups. These results suggested excessive autophagy activation. However, compared with the CPR + Normoxia groups,  $H_2$  treatment suppressed autophagy activation with significantly lower Beclin-1 and LC3B levels, and a higher p62 protein level. Furthermore, immunohistochemical staining revealed that LC3B levels were significantly higher in cardiomyocytes from rats after ROSC, while  $H_2$  therapy significantly decreased the LC3B protein levels (Fig. 2H and I).

**$H_2$  treatment suppresses H/R-induced autophagy activation in H9C2 cells.** All H/R-induced injury experiments were performed on the rat heart embryonic H9C2 cell line. The present study data (Figs. 1 and 2) had shown that  $H_2$ -treatment protected cardiomyocytes from CA/resuscitation by inhibiting autophagy activation. To clarify the role of  $H_2$  therapy in CPR-induced myocardial I/R injury, H9C2 cells were subjected to 24 h of hypoxia followed by 4 or 12 h of reoxygenation. According to a previous study protocol,  $H_2$ -rich culture medium was used as  $H_2$  treatment for cells *in vitro* (28).

The expression levels of autophagic markers Beclin-1, LC3B and p62 were measured by western blot analysis in H/R-treated H9C2 cells. After H/R, H9C2 cells showed an increase in expression levels of autophagic proteins Beclin-1, LC3B and p62, compared with the control (Fig. 3A and B).  $H_2$  significantly decreased Beclin-1 and LC3B expression in the H/R-treated cardiomyocytes, suggesting the inhibition of autophagy. However, the p62 expression was increased significantly in these H/R +  $H_2$  groups.

Consistent with the western blot analysis, the immunofluorescence staining also indicated that the expression of Beclin-1 and LC3B was increased in H/R-treated cells, while  $H_2$  administration significantly decreased the expression of

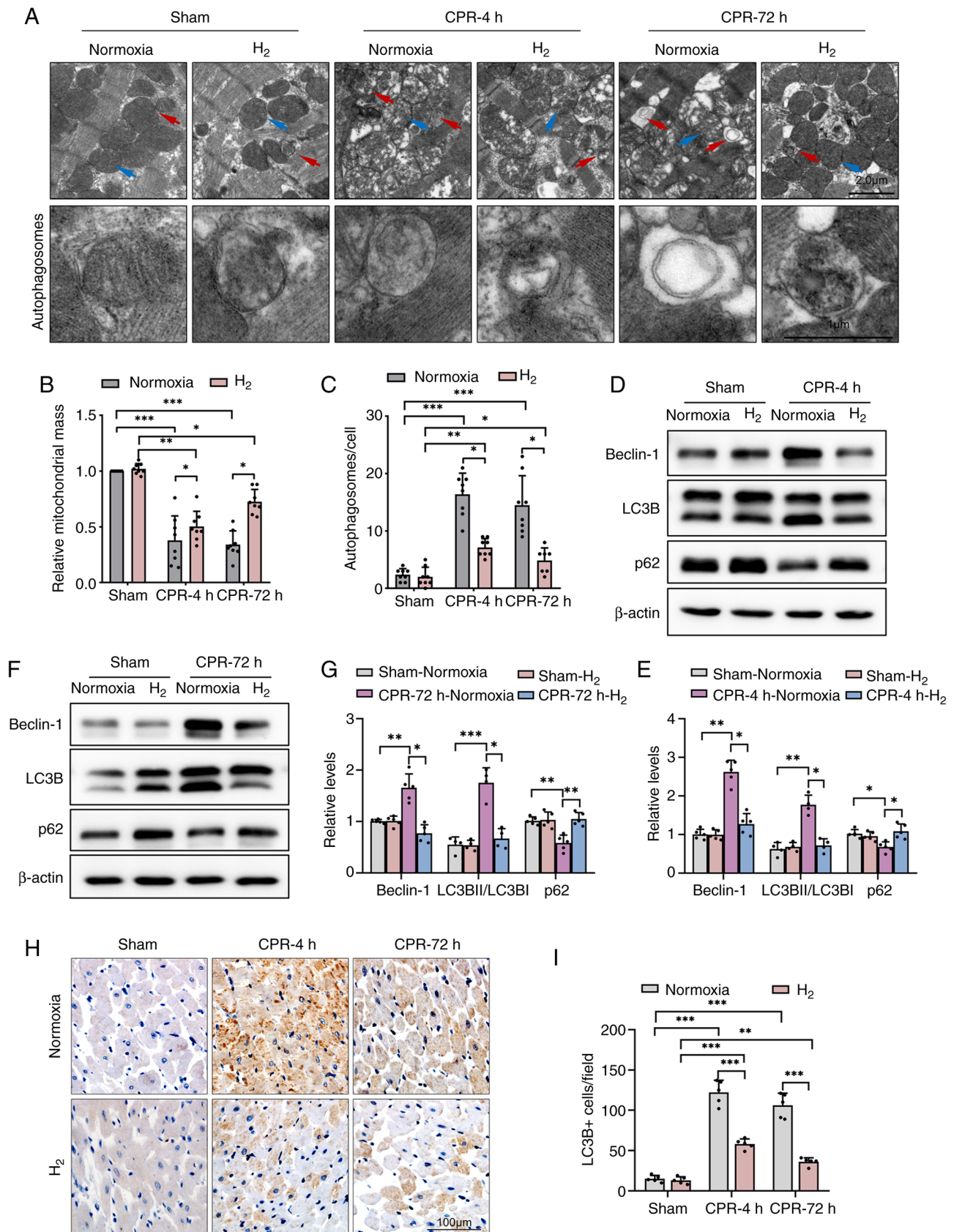


Figure 2. Inhalation of H<sub>2</sub> gas after ROSC improves mitochondrial mass and suppresses autophagy activation in rat cardiomyocytes. (A) The representative images of TEM exhibited the morphology of mitochondria (blue arrows) and autophagosomes (red arrows) in rat cardiomyocytes. The numbers of autophagosomes from at least 10 images in each group were analyzed for the quantification results. Scale bar: 2 μm (top) and 1 μm (bottom). (B) Quantification of mitochondrial mass in TEM images from rat cardiomyocytes (n=8). (C) Quantification of autophagosomes in TEM images from rat cardiomyocytes (n=8). (D-G) The representative images and quantification of immunoblotting analysis of Beclin-1, LC3B and p62 in rat left ventricles after ROSC at 4 and 72 h. (H and I) The representative images and quantification analysis of immunohistochemical staining of LC3B in rat cardiomyocytes (n=5). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. ROSC, return of spontaneous circulation; H<sub>2</sub>, hydrogen molecule; CPR, cardiopulmonary resuscitation; TEM, transmission electron microscopy; LC3B, microtubule-associated protein 1 light chain 3-B.



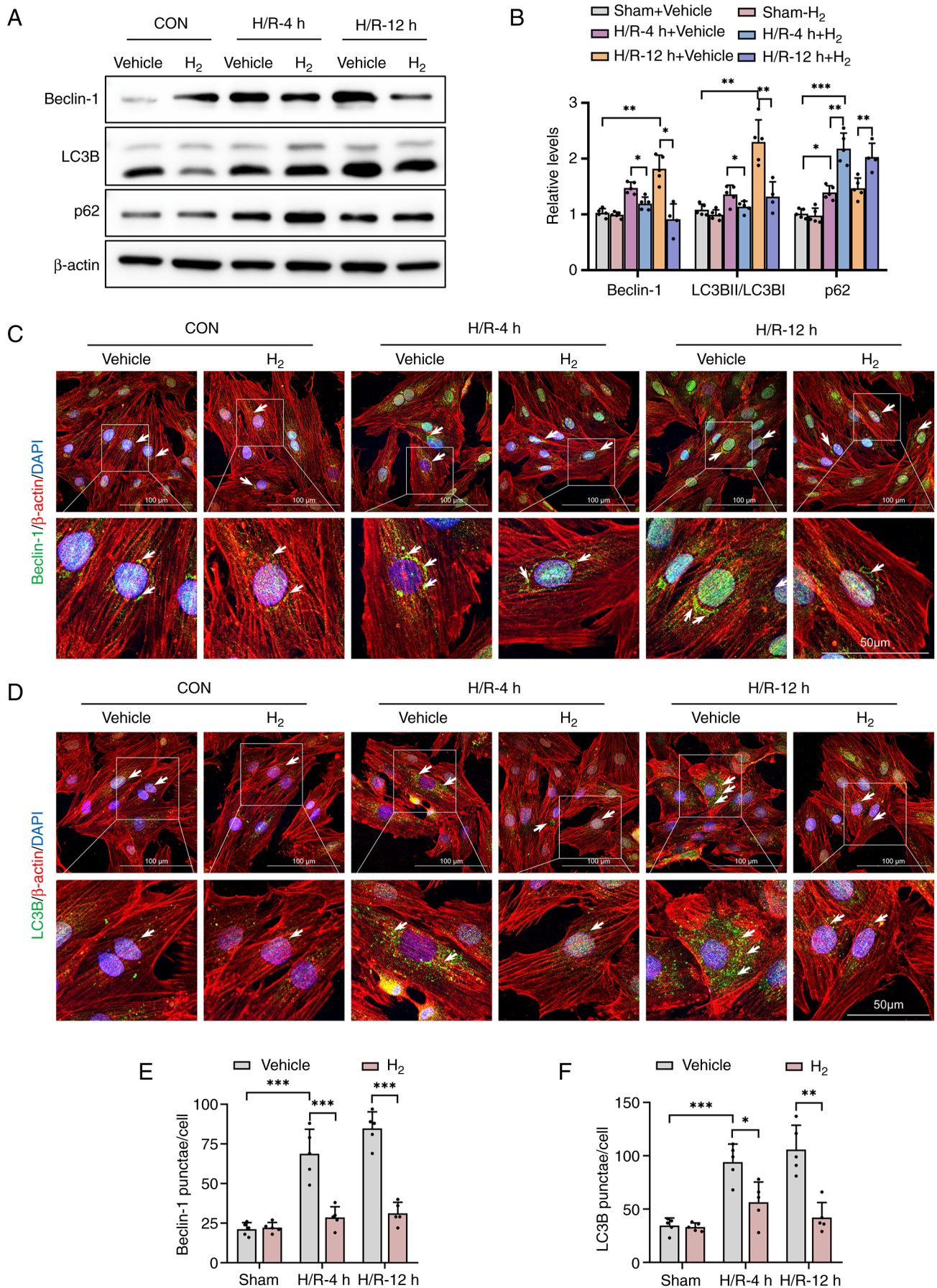


Figure 3. H<sub>2</sub> treatment suppresses H/R-induced autophagy activation in H9C2 cells. (A and B) The representative images and quantification of immunoblotting analysis of Beclin-1, LC3B and p62 in H9C2 cells after H/R. (C-F) The representative images and quantification analysis of immunofluorescence staining of Beclin-1 and LC3B in H9C2 cells (n=5). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. CON, control; H<sub>2</sub>, hydrogen molecule; H/R, hypoxia/reoxygenation; LC3B, microtubule-associated protein 1 light chain 3-B.

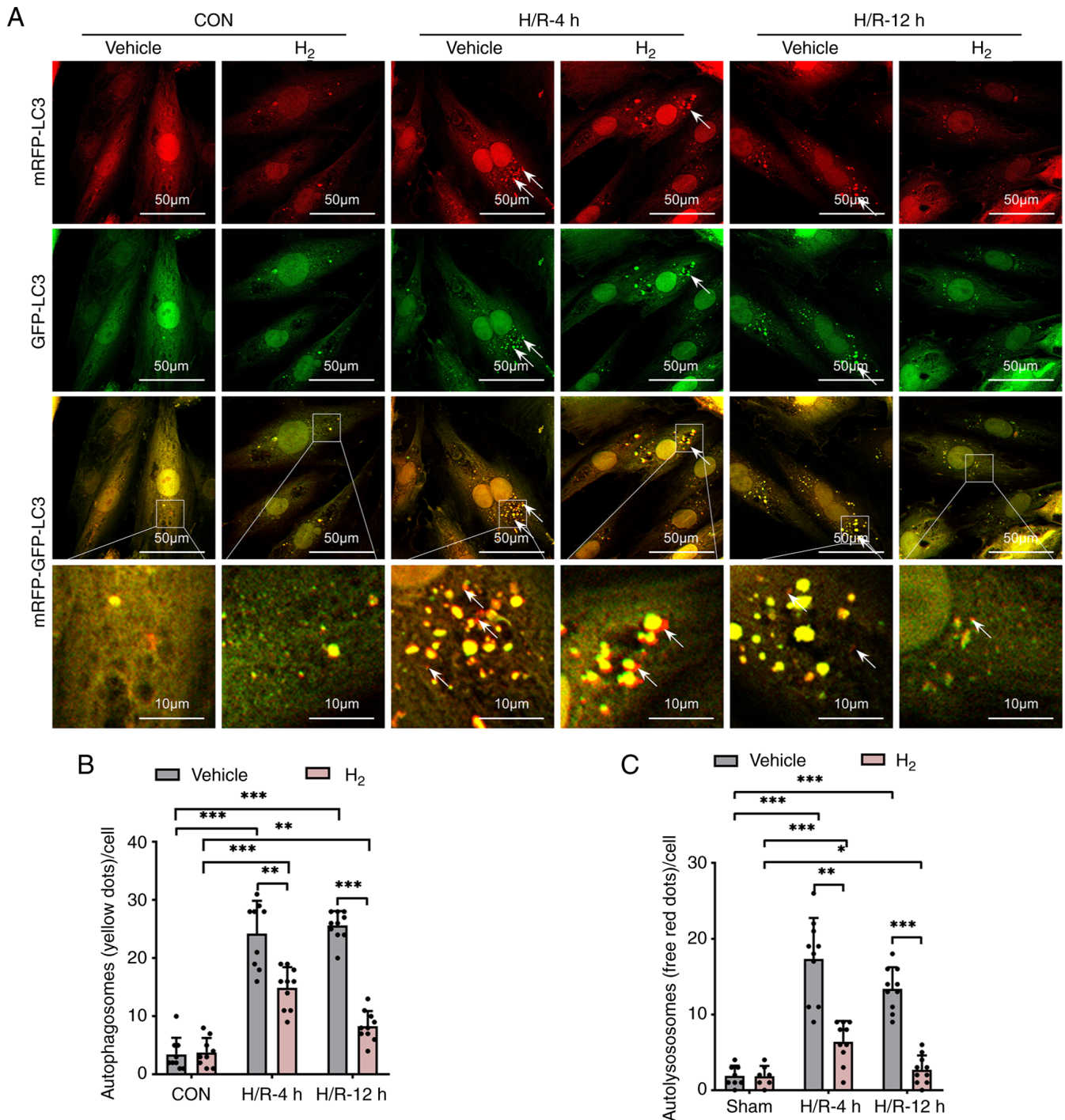


Figure 4. H<sub>2</sub> treatment suppresses H/R-induced accumulation of autophagosomes and autolysosomes in H9C2 cells. (A) The representative images and quantification analysis of the formation of (B) autophagosomes and (C) autolysosomes in H9C2 cells transfected with adenovirus-RFP-GFP-LC3 (n=7-10). Scale bars, 50  $\mu$ m (top three rows) and 10  $\mu$ m (bottom row). \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001. CON, control; H<sub>2</sub>, hydrogen molecule; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; LC3, microtubule-associated protein 1 light chain 3; H/R, hypoxia/reoxygenation.

these proteins (Fig. 3C-F). These data support the proposal that H<sub>2</sub> protects against H/R-induced injury by decreasing H/R-mediated autophagy.

*H<sub>2</sub> treatment suppresses H/R-induced accumulation of autophagosomes and autolysosomes in H9C2 cells.* In order to study the formation and process of autophagy, H9C2 cells were transfected with Ad-monomeric RFP (mRFP)-GFP-LC3 and observed at different time points after H/R. As shown

in Fig. 4, the generation of both GFP- and mRFP-positive autophagosomes was significantly increased in the H9C2 cells after H/R, while H<sub>2</sub> treatment resulted in a significant decrease in both autophagosome types.

## Discussion

The present study investigated the cardioprotective effect of H<sub>2</sub> inhalation after ROSC in a rat CPR model. The results



revealed that H<sub>2</sub> treatment ameliorated animal survival and myocardial abnormalities. The echocardiography at 4 and 72 h after ROSC revealed improved cardiac function in H<sub>2</sub>-treated animals compared with the CPR group. Consistent with previous studies (30–33), using electron microscopy analysis, extensive mitochondrial abnormalities and autophagosomes were observed in myocardial cells at 4 and 72 h after ROSC in rats subjected to asphyxial CA/CPR. However, H<sub>2</sub> treatment after ROSC improved mitochondrial morphology and decreased the numbers of autophagosomes. These data demonstrated that the excessive activation of autophagy might exist for a long period in rats subjected to asphyxial CA/CPR. H<sub>2</sub> treatment significantly suppressed autophagy activation.

The present data have defined the role of autophagy in myocardial survival/death after ROSC in an asphyxial CA/CPR model. Furthermore, the role of H<sub>2</sub> in the regulation of autophagy against myocardial injury has not been investigated in asphyxial CA/CPR. In previous studies, autophagy activation was considered as a double-edged sword with both pro-survival and death-causing potential in myocardial I/R injury (34,35). Excessive activation of autophagy leads to degeneration of organelles and drives cell death after reperfusion. Additionally, excess autophagosome clearance has been determined as a major cause of cardiomyocyte death as a result of the observation of autophagosome generation in necrotic cardiomyocytes (36).

In the present study, the data showed that autophagosomes significantly accumulated in cardiomyocytes after ROSC, indicating excessive activation of autophagy. Moreover, the expression levels of Beclin-1 and LC3B were also increased in the LV from 4 to 72 h after asphyxial CA/CPR. Beclin-1, a component of phosphatidylinositol type III kinase complex, has been confirmed to serve a crucial role in regulating autophagosome formation (34). Previous studies have reported the association between Beclin-1 and autophagy-associated cell death in cerebral ischemia (37–40). The present data also revealed that H<sub>2</sub> treatment significantly inhibited autophagy, with decreased Beclin-1 and LC3B expression in cardiomyocytes. Moreover, compared with those in the CPR or H/R groups, the expression levels of p62 significantly decreased in the H<sub>2</sub> treatment groups *in vitro* and *in vivo*, indicating the inhibition of autophagy. However, in contrast to the results in the animal experiments, the expression levels of p62 were significantly increased in the H/R groups in cells. These data may be related to the different amounts of time suffering hypoxia *in vitro* and *in vivo*. In H/R-induced injury experiments, a longer period of hypoxia increased the expression levels of p62 in the H9C2 cells. In summary, these results evaluated the potential cardioprotection of H<sub>2</sub> treatment in CA/CPR.

*In vitro*, Ad-mRFP-GFP-LC3 were used to observe the formation and process of autophagy. After transfection with Ad-mRFP-GFP-LC3, H9C2 cells ubiquitously express the autophagosome-building microtubule-associated protein LC3 linked with both mRFP and GFP. With autophagy activation, fluorescent signals of mRFP and GFP significantly increase with the formation of phagosomes. Moreover, GFP signals are quenched where autophagosomes eventually fuse with lysosomes (41). In the *in vitro* H/R experiments of the present study, a decrease in autophagy-associated proteins (Beclin-1 and LC3B) and autophagosomes was observed after H<sub>2</sub> treatment,

which suggested that the cell homeostasis mechanisms of H<sub>2</sub> therapy in cardioprotection are associated with the inhibition of autophagy. In previous studies, the anti-apoptotic properties of H<sub>2</sub> have also been demonstrated, with the alleviation of hyperoxia inducing lung epithelial cell apoptosis via the induction of Bcl-2 and the suppression of Bax expression (42–44). These results revealed a potential mechanism of H<sub>2</sub>-mediated cell fate under stress.

In conclusion, the present study demonstrated that H<sub>2</sub> inhalation after resuscitation suppressed autophagy activation and improved cardiac function and survival in a rat model of CA. These findings suggest a potentially novel and easily applicable treatment for cardiac dysfunction in post-cardiac arrest syndrome; however, further investigation is required to confirm the cell homeostasis mechanisms of H<sub>2</sub> therapy for cardioprotection.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

XG, TX, XH and YC conceived and designed the study. XG, XF, XY, TX, JL, JG and XZ performed the study. XG, TX, SW, QY, JW and XF contributed to the data analysis. XG, TX, XH and YC wrote the manuscript. XG and TX confirm the authenticity of all the raw data. All authors have read and approved the manuscript.

### Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of the Qilu Hospital of Shandong University (Jinan, China; approval no. KYLL-2020-ZM-122), and adhered to the guidelines from the Care and Use of Laboratory Animals.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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