

# Curcumin attenuates hypoxia/reoxygenation-induced myocardial injury

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**Abstract.** Curcumin (Cur) has been reported to function as an antioxidant and anti-inflammatory agent and to play a role in anti-atherosclerosis. The present study aimed to explore the protective effect of Cur on hypoxia/reoxygenation (H/R) injury. The morphological changes in H9c2 cardiomyocytes were observed under an inverted microscope. Cell viability was determined by Cell Counting Kit-8 (CCK-8). Lactate dehydrogenase (LDH) level, malondialdehyde (MDA) level and the antioxidant superoxide dismutase (SOD) activity were determined by corresponding kits. Apoptosis and reactive oxygen species (ROS) levels were determined by flow cytometry. Endoplasmic reticulum (ER) stress-related factors, which were examined by quantitative real-time polymerase chain reaction (qPCR) and western blot analysis, included 78-kDa glucose-regulated protein (GRP78) and C/EBP homologous protein (CHOP). Extracellular signal regulating kinase 1/2 (ERK1/2), p38, c-Jun NH2-terminal kinase (JNK) and the phosphorylation levels of key proteins in the mitogen-activated protein kinase (MAPK) signaling pathway were all determined by western blot analysis. Compared to the control group, the cell morphology of the H9c2 cells was obviously altered upon H/R. Cell viability was significantly decreased, while apoptosis was significantly increased by H/R. We also observed that the levels of LDH and MDA were elevated and the activity of SOD was decreased in the H/R group. Notably, LDH, MDA and SOD levels were reversed following treatment with Cur; while apoptosis and ROS levels in the H/R injury group were decreased by Cur. H/R injury-triggered ER stress and the MAPK signaling pathway were suppressed by Cur. These results demonstrated that Cur has a protective effect on cardiomyocytes via suppression of ER stress and the MAPK pathway.

## Introduction

Myocardial cell death caused by ischemia-reperfusion (I/R) is one of the main causes of high morbidity and mortality worldwide (1). It is generally believed that the use of thrombolysis agents or direct percutaneous coronary intervention treatment of myocardial reperfusion could increase the survival rate of patients. However, myocardial reperfusion may induce oxidative stress and lead to inflammation and apoptosis; therefore, the morbidity and mortality of ischemic cardiomyopathy patients remain at a high level (2,3). To the best of our knowledge, no effective therapeutic agents for I/R injury have been discovered in clinical practice (4,5).

Researchers have indicated that severe endoplasmic reticulum (ER) stress leads to apoptosis of cardiomyocytes *in vitro* and *in vivo* (6,7). Inhibitors of ER stress protect the heart by inhibiting pathological changes and apoptosis (8). C/EBP homologous protein (CHOP) plays a key role in ER stress-induced apoptosis; the ablation of CHOP attenuates ER-mediated apoptosis (9). The development of ER stress is caused by dissociating abundant molecular chaperone BiP/78-kDa glucose-regulated protein (GRP78) signaling molecules in the ER cavity (10). ER stress signals can eventually trigger apoptotic CHOP expression (11,12).

Cells respond to exogenous stimuli by regulating intracellular signaling pathways. The mitogen-activated protein kinase (MAPK) signaling pathway, which is widely distributed in the cell, contains extracellular signal regulating kinase 1/2 (ERK1/2), p38 and c-Jun NH2-terminal kinase (JNK). These two signaling pathways are known to play vital roles in cell differentiation, proliferation and apoptosis as well as in cell apoptosis induced by ER stress (11,13,14). Specifically, *in vivo* animal studies have shown that inhibition of sustained phosphorylation of MAPK (ERK1/2, JNK, p-38) not only reduces myocardial damage (15,16), but also enhances cardiac function (17). The MAPK pathway has attracted much attention due to its critical involvement in the functions of the heart (15,18).

Curcumin (Cur) is a polyphenol from *Curcuma longa* (turmeric plant). Curcumin is an alcohol-based molecule that exists in an organic solvent (19). Studies have shown that curcumin is an effective molecule which exerts a variety of positive pharmacological effects including anti-inflammatory (20,21), antioxidant (22) and anti-apoptotic effects (23). However, the functional roles of Cur in H/R injury still remain

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largely unexplored. Therefore, the present study aimed to determine whether Cur relieves H/R injury and whether Cur can be used as an effective therapeutic agent for clinical cardiac I/R injury.

## Materials and methods

**Reagents and cell line.** Curcumin was obtained from Sigma-Aldrich; Merck KGaA (cat. no. 08511; HPLC >98%; powder). The primary antibodies for GRP78, CHOP, p-p38, p-JNK and p-ERK1/2 were purchased from Cell Signaling Technology (CST), and the primary antibody for GAPDH was purchased from Santa Cruz Biotechnology. The Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), and lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) assay kits were all purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The H9c2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

**Establishment of a hypoxia/reoxygenation cell model.** It has been reported that H9c2 cells are used as a cell model of cardiac ischemia-reperfusion injury *in vitro* (24). H9c2 cardiomyocytes were incubated in an incubator at 37°C with 95% N<sub>2</sub> and 5% CO<sub>2</sub> and used for experiments when the cell confluency reached ~90%. In brief, the cells were cultured with phosphate-buffered saline (PBS) which was then replaced with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc.), and then placed in the hypoxic chamber (Stem Cell Technologies) with 95% N<sub>2</sub> in an incubator for 10 min at 37°C. Four hours later, 10% FBS in DMEM medium was added to the cells and the cells were incubated under a normoxic condition (20% O<sub>2</sub>, 5% CO<sub>2</sub>) without the chamber for another 4, 8 and 12 h at 37°C. The cultured cardiomyocytes in the control group were then cultured in an incubator without any treatment. Three complexes were set in each group.

**Evaluation of cell morphology and determination of cell viability.** H9c2 cells were seeded in a 96-well plate at a density of 5x10<sup>3</sup> cells/well. After being pre-treated with Cur, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation. Then the H9c2 cells were placed under an inverted light microscope (magnification, x200) to observe changes in cell morphology. After adding 10 µl CCK-8 solution, the cells were incubated for 2 h at 37°C. Optical density (OD) at 450 nm was detected by a microplate reader (Thermo Fisher Scientific, Inc.). Cell viability was detected using the CCK-8 assay kit (Dojindo, Kumamoto, Japan), according to the manufacturer's protocol.

**LDH concentration.** H9c2 cells were seeded in a 96-well plate at a density of 5x10<sup>3</sup> cells/well. After being pre-treated with Cur, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation, and an LDH assay kit was prepared to detect the LDH activity in the culture medium, according to the manufacturer's protocol. Briefly, the culture medium was centrifuged at 400 x g for 10 min at room temperature to collect 20 µl of the supernatant, which was then mixed with 2,4-dinitrophenylhydrazine and incubated for 15 min at 37°C.

NaOH (0.4 M) was added into the mixture and incubated for another 15 min at 37°C. The mixture was maintained at room temperature for 5 min, and subsequently the OD (450 nm) was measured on a microplate reader (Thermo Fisher Scientific, Inc.).

**Measurement of MDA activity.** H9c2 cells were seeded in a 96-well plate at a density of 5x10<sup>3</sup> cells/well. After being pre-treated with Cur, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation. Next, the H9c2 cells were collected in a centrifuge tube and the supernatant was discarded after being centrifuged at 12,000 x g at 4°C for 10 min. RIPA lysis buffer was added to fully isolate the cell protein lysate. The cells were centrifuged at 12,000 x g for 30 min at 4°C and then the supernatant was extracted and placed on ice for testing. A mixture of MDA test working solution and crude enzyme solution was used at a ratio of 3:1, and the mixture was placed in a water bath at 100°C for 30 min and then cooled on ice. According to the manufacturer's protocol, the OD at 532 nm was read using a microplate reader (Thermo Fisher Scientific, Inc.).

**Measurement of SOD activity.** H9c2 cells were seeded in a 96-well plate at a density of 5x10<sup>3</sup> cells/well. After being pre-treated with Cur, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation. The H9c2 cells were then collected. Next, an enhanced bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) was used to measure the protein concentration after the protein was isolated using RIPA lysis buffer. To detect SOD levels, the protein was incubated with an SOD kit for 20 min at 37°C, and the SOD value was examined at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

**Cell apoptosis.** H9c2 cells were seeded in a 6-well plate at a density of 1.3x10<sup>5</sup>/well. After being pre-treated with Cur, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation. Next, the supernatant was collected into a 15-ml centrifuge tube, and the culture flask was gently washed once by adding 2 ml of PBS. The cells were digested with trypsin (1 ml) without ethylenediaminetetraacetic acid (EDTA) and shaken gently. The supernatant was aspirated after the well became wet. The mixture was maintained at room temperature for 1 min, and DMEM containing 10% FBS was added to terminate the digestion. The cells were centrifuged at 1,000 x g for 3 min and the supernatant was removed. Next, the cells were washed twice with pre-cooled PBS and resuspended in 1X Annexin V binding buffer. According to the instructions included in the Annexin V-FITC cell apoptosis detection kit (cat. no. K201-100; BioVision, Milpitas, CA, USA), the H9c2 cells were collected and stained with Annexin V-FITC and propidium iodide (PI) for 15 min and counted by flow cytometry (version 10.0, FlowJo, FACSCalibur™; BD Biosciences) at room temperature. Based on the flow cytometry scatter diagrams, the lower left quadrant represented living cells (Annexin V-/PI-), the lower right quadrant indicated early apoptotic cells (Annexin V+/PI-), and the upper right quadrant indicated late apoptotic cells (Annexin V+/PI+) and the upper left quadrant represented necrotic cells (Annexin V-/PI+). The

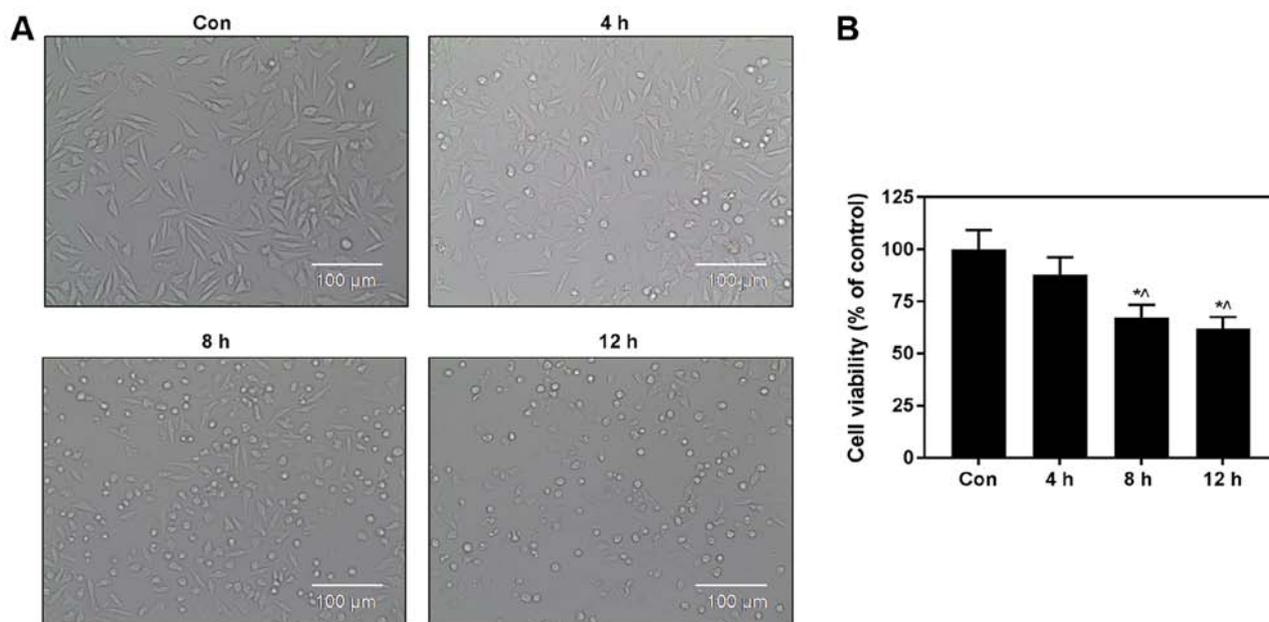


Figure 1. Effects of reoxygenation time on H9c2 cardiomyocyte cell morphology and cell viability. H9c2 cardiomyocytes were exposed to 4 h of hypoxia and then subjected to various time (4, 8 and 12 h) of reoxygenation. (A) Changes in cell morphology were observed under an inverted microscope following H/R injury. (B) CCK-8 kit was used to detect an obvious reduction in cell viability in the H/R-injured cells.  $n=3$  wells/group. All data are expressed as the means  $\pm$  SEM. \* $P<0.05$  vs. Con; ^ $P<0.05$  vs. 4 h. H/R, hypoxia/reoxygenation; 4 h indicates 4 h hypoxia/4 h reoxygenation; 8 h indicates 4 h hypoxia/8 h reoxygenation; 12 h indicates 4 h hypoxia/12 h reoxygenation.

total apoptosis was calculated as the sum of the right upper quadrant and right lower quadrant (25).

**ROS measurement by flow cytometry.** H9c2 cells were seeded in a 6-well plate at a density of  $1.3 \times 10^5$ /well and were exposed to hypoxia/reoxygenation (4/8 h) with or without pretreated with Cur for 2 h. Next, the cells were incubated with  $10 \mu\text{mol/l}$  carboxylated 2',7'-dichlorodihydrofluorescein diacetate ester (c-H<sub>2</sub>DCFDA, Beyotime Biotechnology, Shanghai, China,  $5 \mu\text{M}$ ) for 30 min to determine the oxidation of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) mediated into fluorescent compound DCF at 37°C. Fluorescent compound DCF model was used for measuring the fluorescence. The excitation wavelength of the flow cytometer (version 10.0; FlowJo) was 480 nm and the emission wavelength was 525 nm (26), and the x-axis indicated the fluorescence intensity.

**Western blot analysis.** H9c2 cells were seeded in a 6-well plate at a density of  $1.3 \times 10^5$ /well and exposed to hypoxia/reoxygenation (4/8 h) with or without pretreatment with  $10 \mu\text{M}$  Cur for 2 h. After drug treatment, the H9c2 cells were flushed by cold PBS for 3 times and put on ice with protein lysis buffer (Radio Immunoprecipitation Assay, RIPA, cat. #9806; Cell Signaling Technology, Inc.) for 2 h. The cells were centrifuged at  $13,500 \times g$  for 30 min at 4°C and then the supernatant was extracted. The concentration of protein was determined using a BCA protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). SDS-PAGE with 10% running gels was used to separate the proteins (at least  $40 \mu\text{g}$ ), which were then transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). To block the non-specific signals, the membranes were incubated with 5% non-fat milk for at least 2 h at room temperature. Protein strips were then incubated with

a primary antibody overnight at 4°C and then washed with 5% bovine serum albumin (BSA, Gibco; Thermo Fisher Scientific, Inc.) in PBS/0.1% Tween-20 (PBS/T) and incubated with the secondary antibody for 1 h at room temperature. The protein strip was developed with a developer (EZ-ECL kit; Biological Industries; BI) and the protein quantity was analyzed using ImageJ software (version 5.0; National Institutes of Health, Bethesda, MD, USA). The antibodies used in the study were as follows: Anti-GAPDH (mouse; dilution 1:1,000; cat. no. sc-47724; Santa Cruz Biotechnology), anti-GRP78 (rabbit; dilution 1:500; cat. no. 3477; CST), anti-CHOP (mouse; dilution 1:1,000; cat. no. 2895; CST), anti-p-ERK1/2 (mouse; dilution 1:1,000; cat. no. 4307; CST), anti-ERK1/2 (mouse; dilution 1:1,000; cat. no. 4695; CST), anti-p-p38 (rabbit; dilution 1:1,000; cat. no. 4511; CST), anti-p38 (rabbit; dilution 1:1,000; cat. no. 9212; CST) anti-p-JNK (mouse; dilution 1:1,000; cat. no. 9255; CST) and anti-JNK (mouse; dilution 1:1,000; cat. no. 9252; CST) as well as the secondary antibody [horseradish peroxidase-labeled goat anti-rabbit or -mouse IgG (1:5,000, cat. nos. sc-516102/sc-2357; Santa Cruz Biotechnology, Inc.)].

**RNA isolation and quantitative real-time PCR.** H9c2 cells were seeded in a 6-well plate at a density of  $1.3 \times 10^5$ /well and exposed to hypoxia/reoxygenation (4/8 h) with or without pretreatment with  $10 \mu\text{M}$  Cur for 2 h. According to the instructions, total RNA of H9c2 cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Chloroform (Sigma Aldrich; Merck KGaA) was added to the tube and incubation was carried out at room temperature for 5 min and then the cells were centrifuged at  $14,000 \times g$  for 20 min at 4°C. Next, the supernatant was transferred into a new tube and isopropanol was added. The aqueous phase was

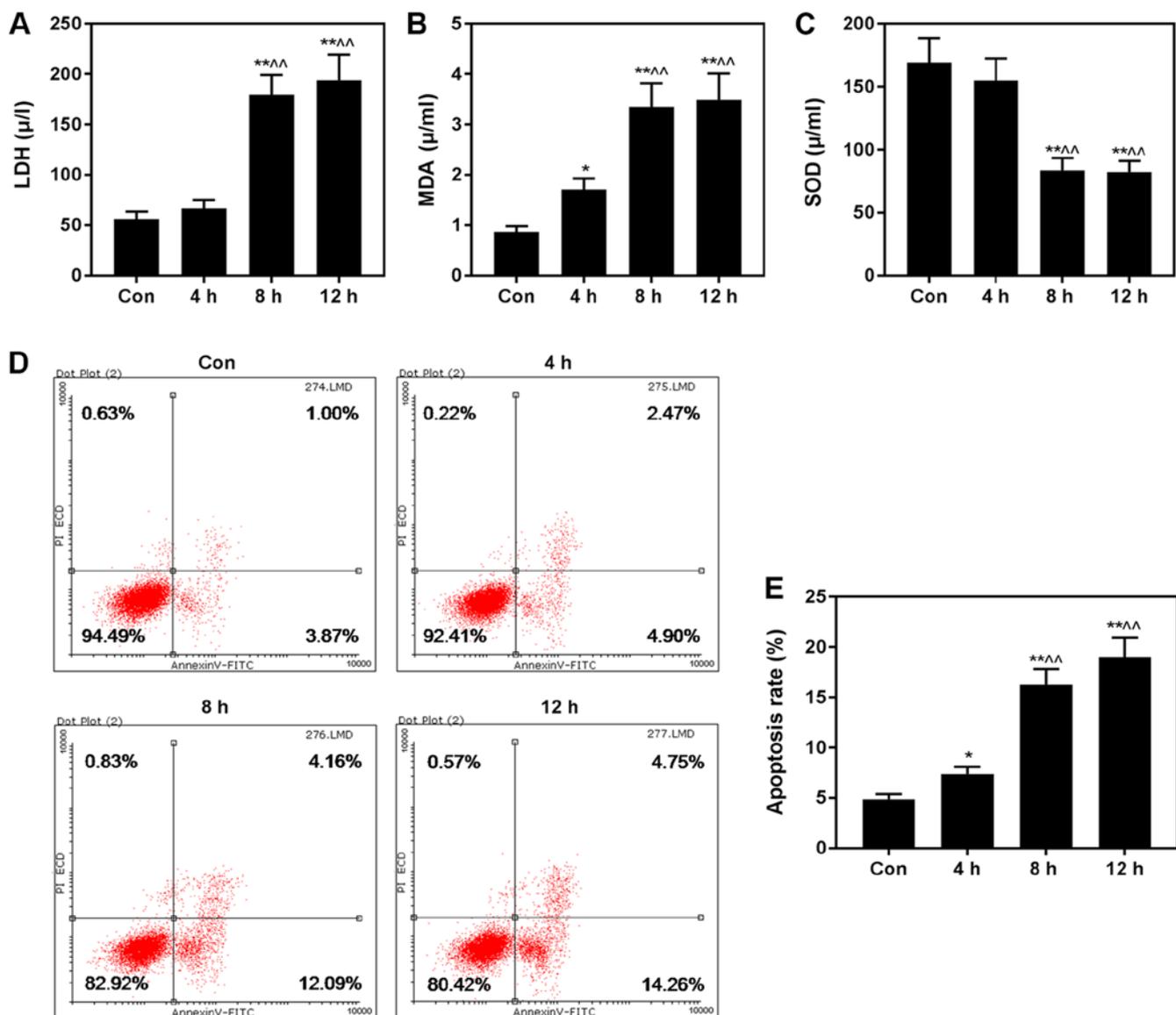


Figure 2. Changes in biochemical indicators in relation to reoxygenation time in H9c2 cardiomyocytes. (A) LDH content in the cell culture fluid was increased as assessed by LDH kits. (B) MDA content was increased as determined by MDA kits. (C) SOD was evidently lower. (D and E) The percentage of apoptotic H9c2 cardiomyocytes was analyzed using flow cytometry, and Annexin V<sup>-</sup>/PI<sup>-</sup> (lower right quadrant) and Annexin V<sup>+</sup>/PI<sup>+</sup> (upper right quadrant) populations are presented for H9c2 cardiomyocyte apoptosis. n=3 wells/group. All data are expressed as means ± SEM. \*P<0.05, \*\*P<0.01 vs. Con; ^P<0.01 vs. 4 h. H/R, hypoxia/reoxygenation; 4 h indicates 4 h hypoxia/4 h reoxygenation; 8 h indicates 4 h hypoxia/8 h reoxygenation; 12 h indicates 4 h hypoxia/12 h reoxygenation. LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase.

centrifuged at 14,000 x g for 20 min at 4°C. The precipitate was washed with 70% ethanol and suspended again in water treated with diethyl carbamate (DEPC). The purity and concentration of RNA was tested by the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the absorbance was read at 260 and 280 nm. According to the program provided by the manufacturer (Thermo Fisher Scientific, Inc.), reverse transcription cDNA kit was used to reversely transcribe 1 μg total RNA for synthesis of cDNA (at 42°C for 60 min, at 70°C for 5 min, at 4°C preservation). SYBR-Green PCR Master Mix (Roche, Basle, Switzerland) was used to perform quantitative real-time polymerase chain reaction (qPCR) experiment using Opticon Real-Time PCR Detection System (ABI 7500; Life Technology, USA). The PCR cycle was as follows: pretreatment at 95°C for 10 min; followed by 40 cycles of 94°C for 15 sec, 60°C for 1 min,

finally at 60°C for 1 min and at 4°C for preservation. The relative mRNA quantity was determined using the comparative quantification cycle (ΔΔCq) method (27). GAPDH expression was used for normalization. The primer sequences were used for RT-qPCR analysis as follows: GRP78s: 5'-GAACCAACT CACGTCCAACC-3' (F) and 5'-AACCACCTTGAATGG CAAGA-3' (R), CHOP: 5'-GAAATCGAGCGCCTGACC AG-3' (F) and 5'-GGAGGTGATGCCAACAGTTCA-3' (R), GAPDH: 5'-CTCAACTACATGGTCTACATGTTCCA-3' (F) and 5'-CCATTCTCGGCCTTGACTGT-3' (R).

**Statistical analysis.** Data are shown as the mean ± SEM. Differences between the experimental groups were assessed by ANOVA, followed by Dunnett-t test, and analyzed by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

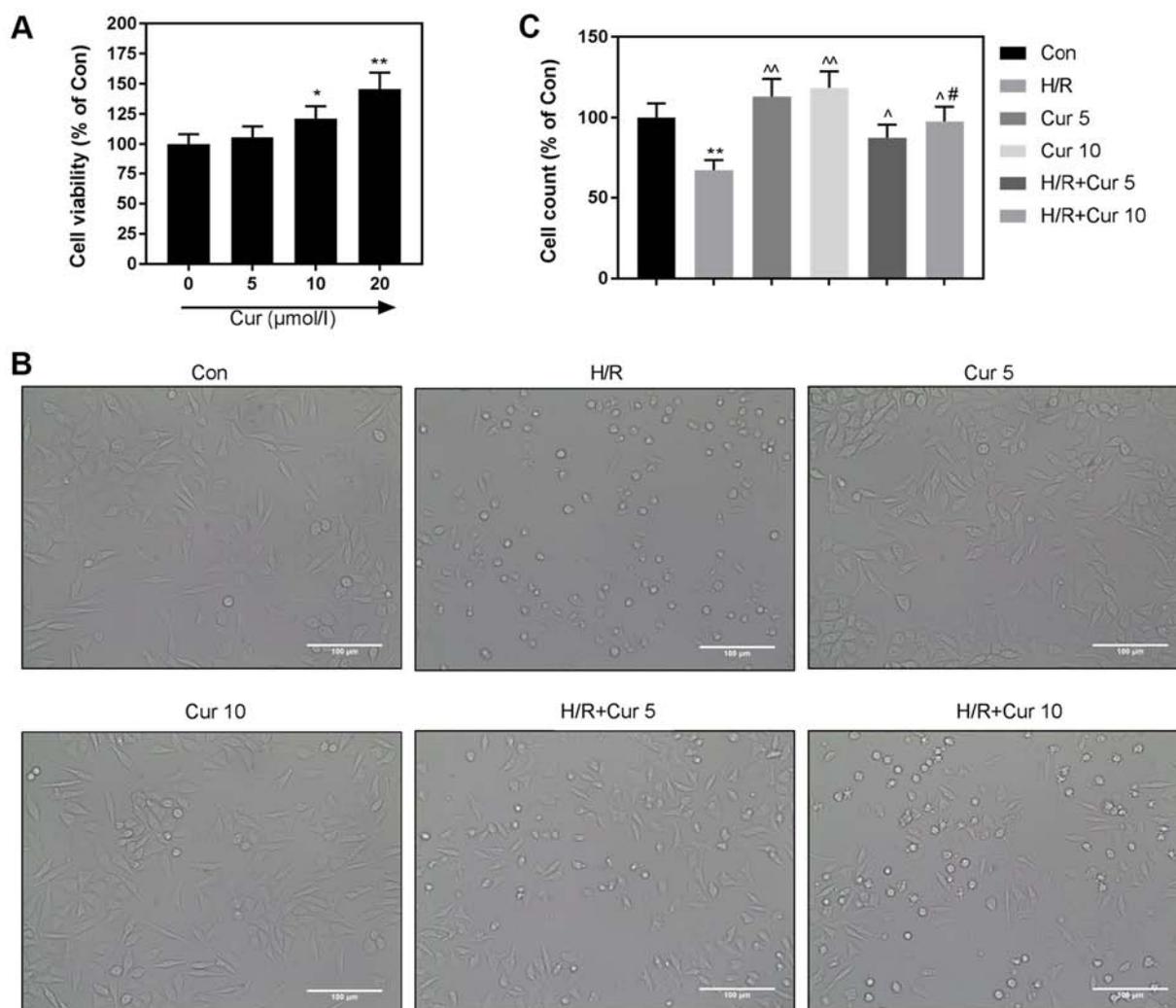


Figure 3. Cur (10  $\mu$ M) has a better protective effect under H/R. (A) There was no negative effect on cell cytotoxicity in H9c2 cardiomyocytes pretreated with Cur for 2 h at different doses (5, 10 and 20  $\mu$ M). \* $P$ <0.05, \*\* $P$ <0.01 vs. 0  $\mu$ mol/l Cur. (B and C) Cur (10  $\mu$ M) had a slightly better protective effect under H/R as determined by CCK-8 assay.  $n$ =3 wells/group. All data are expressed as means  $\pm$  SEM. \* $P$ <0.05, \*\* $P$ <0.01 vs. Con; ^ $P$ <0.05, ^^ $P$ <0.01 vs. H/R; # $P$ <0.05 vs. H/R+Cur5. H/R, hypoxia/reoxygenation; Cur, Curcumin.

## Results

*Hypoxia/reoxygenation injury model was established in H9c2 cells.* A model of hypoxia/reoxygenation (H/R) injury in H9c2 cardiomyocytes was established in this study. Then, the cells were exposed to 4 h of hypoxia and then 12 h of reoxygenation, and the differences in cell morphology between the control group and H/R groups were observed. Cell morphology was typical and regular in the Con group, while a large number of cells exhibited a dot state in the 4 h hypoxia/8 h reoxygenation and 4 h hypoxia/12 h reoxygenation injury group, indicating that these cells may have died in the H/R injury group (Fig. 1A). H9c2 cell viability was reduced in the 4 h hypoxia/8 h reoxygenation and 4 h hypoxia/12 h reoxygenation injury groups, compared with control group (Fig. 1B). These experimental results demonstrated that an H/R injury cell model was successfully established.

*H/R injury induces apoptosis and biomarkers.* Furthermore, we assessed the levels of LDH, MDA and SOD, and found that the levels of LDH (Fig. 2A) and MDA (Fig. 2B) were

increased, while SOD (Fig. 2C) was decreased in the H/R groups, compared with control group. The rate of apoptosis was significantly higher in the H/R injury groups than that in the control group (Fig. 2D and E). Thus, 4 h of hypoxia, followed by 8 h of reoxygenation was selected to construct the H/R cell model for subsequent experiments.

*Cur exhibits no cytotoxicity on H9c2 cardiomyocytes.* For the purpose of determining the cytotoxicity of Cur on H9c2 cardiomyocytes, CCK-8 assay was used to determine the viability of H9c2 cardiomyocytes following treatment of Cur at different doses. The results revealed that no negative effect was observed (Fig. 3A). Furthermore, we found that 10  $\mu$ M Cur had a better protective effect under H/R (Fig. 3B and C). Thus, 10  $\mu$ M Cur was selected for subsequent experiments.

*Cur inhibits ROS accumulation and apoptosis caused by H/R injury.* It has been reported that Cur protects heart cells (28,29); however, its potential protective mechanism still remains unclear. Thus, we determined whether Cur protects H9c2 cardiomyocytes against H/R injury. We found that the

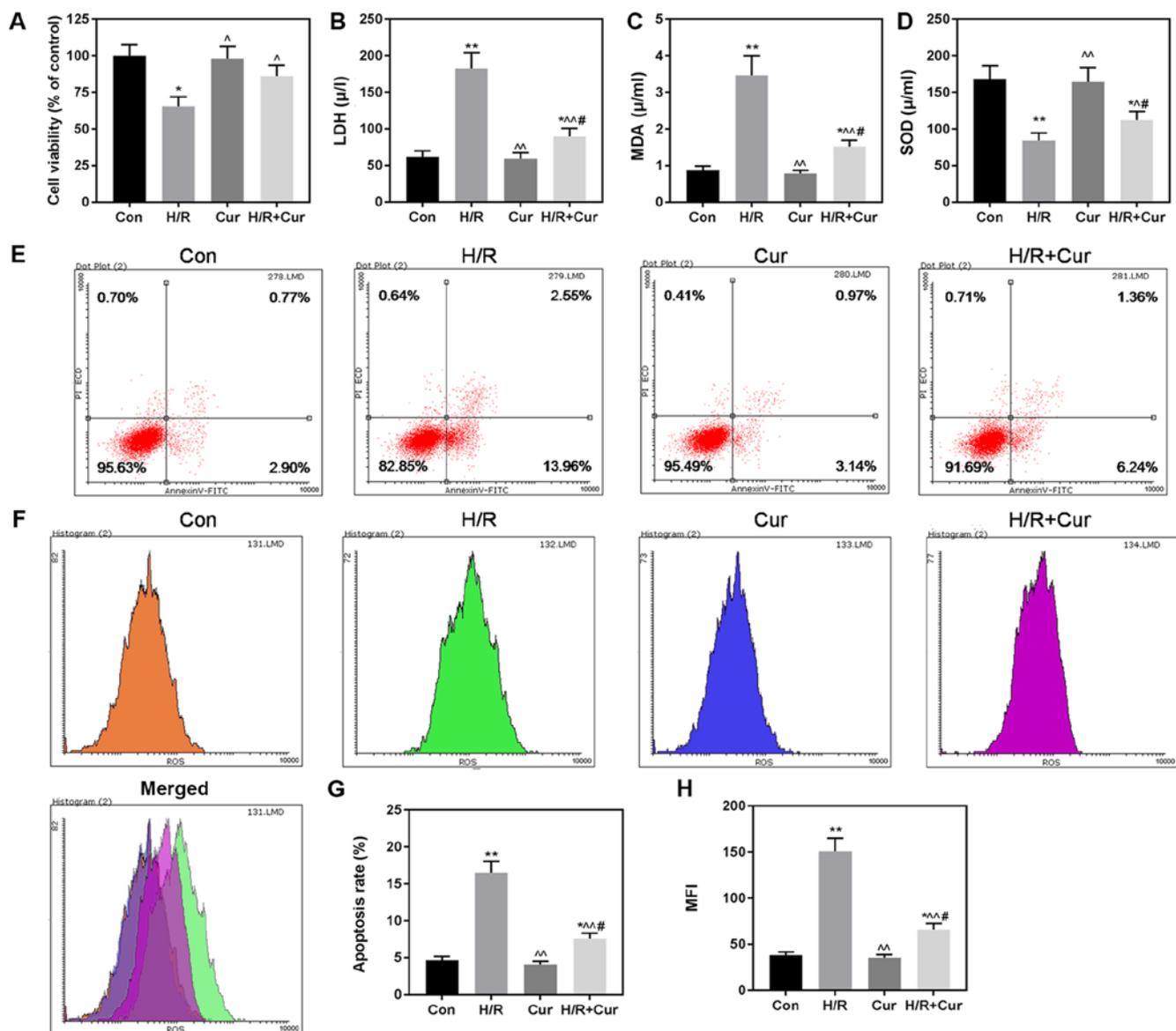


Figure 4. Cur protects H9c2 cardiomyocytes against H/R-induced cell death. (A) Cell viability was determined by CCK-8 assay. The release level of LDH (B), and levels of MDA (C) and SOD (D) in H9c2 cells were evaluated by relevant kits in line with the manufacturer's instructions. (E and G) Apoptosis of H9c2 cardiomyocytes was analyzed and quantified by flow cytometry. (F and H) ROS level in H9c2 cardiomyocytes was determined and quantified by flow cytometry.  $n=3$  wells/group. All data are expressed by means  $\pm$  SEM. \* $P<0.05$ , \*\* $P<0.01$  vs. Con; <sup>^</sup> $P<0.05$ , <sup>^^</sup> $P<0.01$  vs. H/R; <sup>#</sup> $P<0.05$  vs. Cur. H/R, hypoxia/reoxygenation. LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase; ROS, reactive oxygen species; MFI, mean fluorescence intensity; Con, control; Cur, Curcumin.

cell viability was higher in the H/R+Cur group than that in the H/R group (Fig. 4A), and that the levels of LDH, MDA and SOD were reversed by Cur, compared with those in the H/R group (Fig. 4B-D). Similarly, H/R injury-induced apoptosis (Fig. 4E and G) and ROS (Fig. 4F and H) response were inhibited by Cur. The results suggested that Cur protected the damage caused by H/R injury.

*ER stress and the MAPK signaling pathway may be involved in the protective effect of Cur in H9c2 cells.* In the present study, the expression levels of GRP78 and CHOP were found to be significantly increased by H/R at the mRNA and protein levels, indicating that the activation of ER stress and ER stress-related apoptotic signals were caused by H/R injury (Fig. 5A-D). Pretreatment with Cur (10  $\mu$ M) for 2 h before exposure to H/R

injury effectively ameliorated these changes (Fig. 5A-D). We then further determined the effect of Cur on MAPK, and found that MAPK-related protein expression levels of p-ERK1/2, p-38 and p-JNK were suppressed by Cur, when compared to these levels in the H/R group (Fig. 5E and F).

## Discussion

In the hypoxia/reoxygenation (H/R) injury cell model, we observed that the cellular morphology was obviously altered and the levels of LDH and MDA and cell viability were significantly decreased. Meanwhile, we found that the accumulation of ROS during the development of reperfusion injury effectively decreased SOD activity. We observed that apoptosis also occurred following H/R injury, and that Cur strengthened the

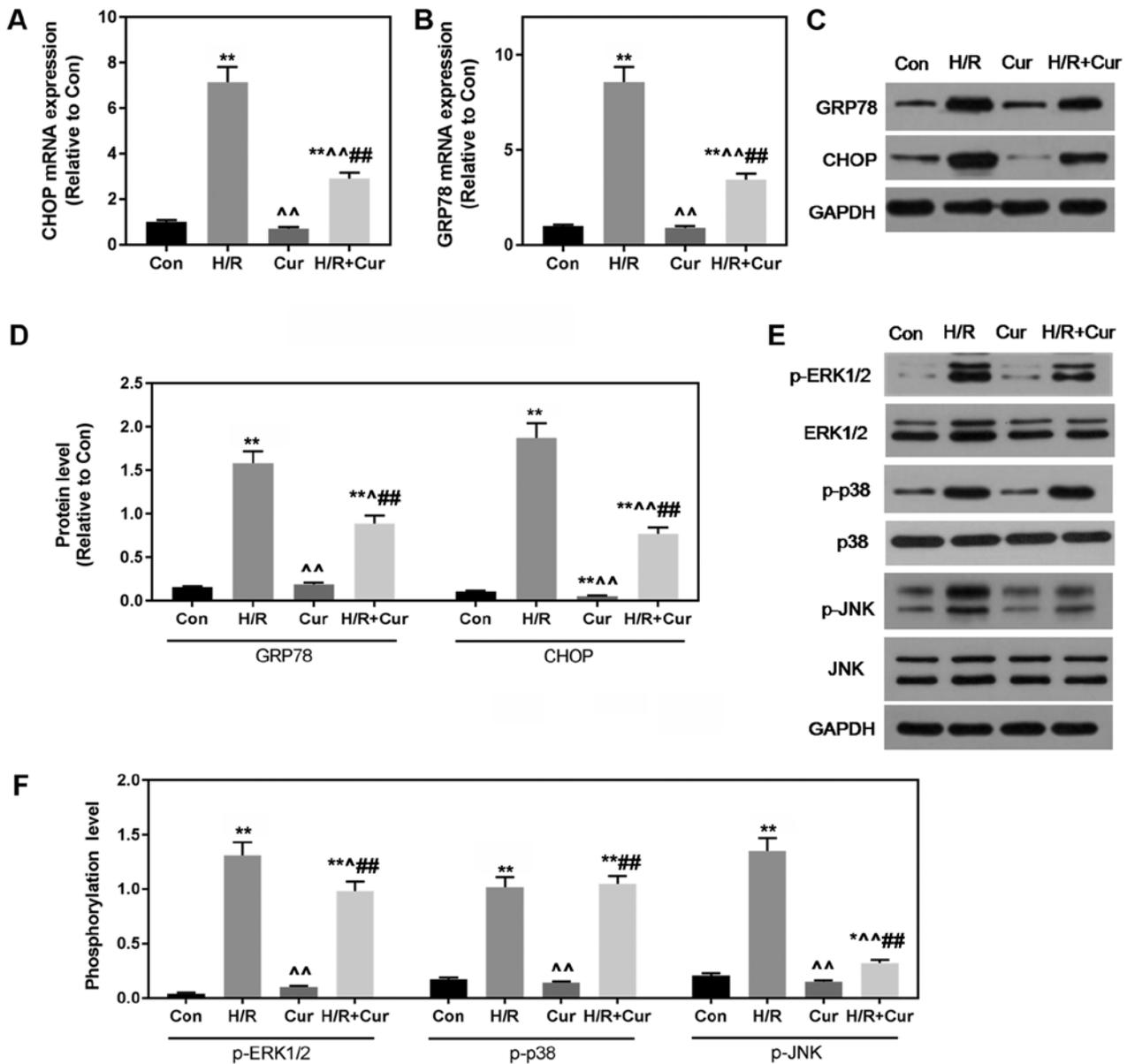


Figure 5. Cur-mediated protection of H9c2 cardiomyocytes from H/R-induced cell death may involve ER stress and the MAPK signal pathway. (A and B) ER stress-related mRNA expression levels of CHOP and GRP78 were determined by reverse transcription-quantitative PCR. (C and D) The protein expression levels of GRP78 and CHOP were determined by western blot analysis and quantified. GAPDH was used as a loading control. (E and F) MAPK-related protein expression levels of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK and JNK were determined by western blot analysis and quantified. GAPDH was used as a loading control. n=3 wells/group. All data were expressed as the mean  $\pm$  SEM. \*P<0.05, \*\*P<0.01 vs. Con; ^P<0.05, ^^P<0.01 vs. H/R; ##P<0.01 vs. Cur. CHOP, C/EBP homologous protein; GRP78, 78-kDa glucose-regulated protein; MAPK, mitogen-activated protein kinase; p-, phosphorylated; H/R, hypoxia/reoxygenation; Cur, Curcumin.

function of cardiomyocytes under H/R injury and downregulated the activities of LDH and MDA, therefore, it protected cardiomyocytes from injury. We also revealed that the inhibitory effect of Cur on the accumulation of ROS may be through the upregulation of SOD in H/R injury, and Cur attenuated apoptosis. Cur also inhibited the expression of GRP78 and CHOP, which are markers of ER stress and apoptosis. Furthermore, Cur significantly decreased phosphorylation of MAPK (ERK1/2 and JNK) in the H/R + Cur group, compared with that noted in the H/R group. Taken together, these data showed that Cur treatment produced a strong protective effect on H9c2 cardiomyocytes during H/R injury.

H9c2 cells exhibit a similar morphology to immature embryonic myocardial cell morphology, which retain the features

of adult cardiac muscle cells (30). Many studies have also shown that H9c2 cells have been widely used to study the pathological processes of enlarged heart and apoptosis (31-33). Thus, we used H9c2 cells to set up a cardiomyocyte ischemia-reperfusion injury model.

Cell viability is a vital indicator to cell survival, and LDH is a marker of cardiac cell damage which leaks into the bloodstream when the cell membrane becomes permeable or ruptured (34). In the present study, the cell viability and LDH levels were obviously decreased in the H/R injury group and Cur pretreatment mitigated increases of the LDH level and also increased H9c2 cell viability during H/R, which suggested that Cur has protective effects on cardiomyocytes against H/R-mediated damage.

Promoted by H/R injury, oxidative stress accumulation is one of the causes of myocardial cell death (35). ROS scavenger has been shown to reduce the myocardial cell injury induced by H/R (36). Clinical studies and vector animal experiments have shown that ischemia-reperfusion results in a significant accumulation of oxidative stress (37,38). Our experiment found that Cur reduced cell ROS production, decreased cardiomyocyte MDA concentrations and increased SOD activity, indicating that Cur relieved over-oxidation caused by H/R. Studies have shown that apoptosis is induced by myocardial ischemia/reperfusion in humans and rats (39,40), and that ROS may activate apoptosis (1,41,42). It is known that apoptosis is usually a passage to death after cells have been damaged. Our experimental results also confirmed that apoptosis was promoted by H/R injury and pretreatment with Cur relieved apoptosis. These data indicate that Cur protects against myocardial damage caused by H/R. Studies have shown that ER stress is an important pathway in cardiac myocyte apoptosis during the progression of H/R (43). ER is considered to be an important organelle involved in the apoptotic signaling pathway and a common feature of mediated destruction, especially in apoptosis (44,45). Activation of ER partner proteins is an early adoption of ER stress response, and is also a sign indicating stress severity (46). In the present study, we found that Cur downregulated ER chaperone protein GRP78, induced by H/R injury. Transcription factor CHOP is one of the important factors that promotes the ER stress-mediated apoptosis factor, playing an important role in myocardial cell apoptosis (11,47). ER stress can trigger pro-apoptotic signals including CHOP-dependent pathways (48). We discovered that the expression of CHOP was decreased by Cur, and therefore, it could reasonably be assumed that the decrease in ER chaperon proteins was attributed to ER stress inhibition. Thus, these data suggest that ER stress relief was responsible for the antiapoptotic effect of Cur.

The cell response to external stimuli is primarily realized by modulating signaling pathways within the cell. Studies have shown that MAPK plays a vital role in cardiomyocytes. The dominant inactivation or constitutive activation of the p38 MAPK and ERK1/2 signaling pathways indicate that activation of p38 MAPK or the inhibition of ERK1/2 is essential for many cells (including cardiomyocytes) to induce apoptosis (49). Studies have shown that the inhibition of ERK1/2 could enhance apoptosis induced by ischemia/reoxygenation and norepinephrine-induced hypertrophy (50). In the present study, we found that p-ERK1/2 in the H/R groups was suppressed by Cur at the protein level. The suppression of p38 MAPK and JNK is effective at reducing heart remodeling (51). However, p-JNK rather than p-p38 was observed to be inhibited by Cur in the H/R groups. Under physiological conditions, the ERK1/2 signaling pathway is a signaling pathway for survival, while p-38 and JNK are signaling pathways for promoting apoptosis and the balance between the two is important in deciding the fate or survival of the cell (15,49,52). Cur significantly inhibited H/R injury-induced phosphorylation of ERK1/2 and JNK rather than p-38, showing a better response of Cur to ERK1/2 and JNK. Therefore, the suppression of apoptosis by Cur may be related to inhibition of the ERK1/2 and JNK signaling pathways.

In summary, we found that Cur exerted significant effects, such as protecting cardiomyocytes against H/R injury through the reduction of LDH and MDA activity, by decreasing ROS

production, and by increasing antioxidant activity and mitigating ER stress and apoptosis induced by hypoxia/reoxygenation in H9c2 cells. Additionally, the anti-apoptotic effects of Cur may be related to the reduction in the proteins levels of p-ERK1/2 and p-JNK. Thus, Cur may be used as an effective therapeutic agent for clinical cardiac ischemia-reperfusion injury.

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#### Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

#### Authors' contributions

WW made substantial contributions to the conception and design of the research study. JP and JL conducted all data acquisition, data analysis and interpretation. WW performed the drafting of the manuscript and critical revision for important intellectual content. All authors approved the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

#### References

1. Murphy E and Steenbergen C: Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* 88: 581-609, 2008.
2. Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M and Eckle T: Myocardial ischemia reperfusion injury: From basic science to clinical bedside. *Semin Cardiothorac Vasc Anesth* 16: 123-132, 2012.
3. Heusch G and Gersh BJ: The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: A continual challenge. *Eur Heart J* 38: 774-784, 2017.
4. Hausenloy DJ and Yellon DM: Ischaemic conditioning and reperfusion injury. *Nat Rev Cardiol* 13: 193-209, 2016.
5. Heusch G: Critical issues for the translation of cardioprotection. *Circ Res* 120: 1477-1486, 2017.
6. Zhuo XZ, Wu Y, Ni YJ, Liu JH, Gong M, Wang XH, Wei F, Wang TZ, Yuan Z, Ma AQ and Song P: Isoproterenol instigates cardiomyocyte apoptosis and heart failure via AMPK inactivation-mediated endoplasmic reticulum stress. *Apoptosis* 18: 800-810, 2013.

7. Dalal S, Foster CR, Das BC, Singh M and Singh K: B-adrenergic receptor stimulation induces endoplasmic reticulum stress in adult cardiac myocytes: Role in apoptosis. *Mol Cell Biochem* 364: 59-70, 2012.
8. Wu T, Dong Z, Geng J, Sun Y, Liu G, Kang W, Zhang Y and Ge Z: Valsartan protects against ER stress-induced myocardial apoptosis via CHOP/Puma signaling pathway in streptozotocin-induced diabetic rats. *Eur J Pharm Sci* 42: 496-502, 2011.
9. Fu HY, Okada K, Liao Y, Tsukamoto O, Isomura T, Asai M, Sawada T, Okuda K, Asano Y, Sanada S, *et al*: Ablation of C/EBP homologous protein attenuates endoplasmic reticulum-mediated apoptosis and cardiac dysfunction induced by pressure overload. *Circulation* 122: 361-369, 2010.
10. Wu XD, Zhang ZY, Sun S, Li YZ, Wang XR, Zhu XQ, Li WH and Liu XH: Hypoxic preconditioning protects microvascular endothelial cells against hypoxia/reoxygenation injury by attenuating endoplasmic reticulum stress. *Apoptosis* 18: 85-98, 2013.
11. Sari FR, Widyantoro B, Thandavarayan RA, Harima M, Lakshmanan AP, Zhang S, Muslin AJ, Suzuki K, Kodama M and Watanabe K: Attenuation of CHOP-mediated myocardial apoptosis in pressure-overloaded dominant negative p38 $\alpha$  mitogen-activated protein kinase mice. *Cell Physiol Biochem* 27: 487-496, 2011.
12. Okada K, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, Hirata A, Fujita M, Nagamachi Y, Nakatani T, *et al*: Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: Possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. *Circulation* 110: 705-712, 2004.
13. Arumugam S, Thandavarayan RA, Arozal W, Sari FR, Giridharan VV, Soetikno V, Palaniyandi SS, Harima M, Suzuki K, Nagata M, *et al*: Quercetin offers cardioprotection against progression of experimental autoimmune myocarditis by suppression of oxidative and endoplasmic reticulum stress via endothelin-1/MAPK signalling. *Free Radic Res* 46: 154-163, 2012.
14. Muslin AJ: MAPK signalling in cardiovascular health and disease: Molecular mechanisms and therapeutic targets. *Clin Sci (Lond)* 115: 203-218, 2008.
15. Chen S, Yang B, Xu Y, Rong Y and Qiu Y: Protection of Luteolin-7-O-glucoside against apoptosis induced by hypoxia/reoxygenation through the MAPK pathways in H9c2 cells. *Mol Med Rep* 17: 7156-7162, 2018.
16. Martin JL, Avkiran M, Quinlan RA, Cohen P and Marber MS: Antiischemic effects of SB203580 are mediated through the inhibition of p38 $\alpha$  mitogen-activated protein kinase: Evidence from ectopic expression of an inhibition-resistant kinase. *Circ Res* 89: 750-752, 2001.
17. Nagarkatti DS and Sha'afi RI: Role of p38 MAP kinase in myocardial stress. *J Mol Cell Cardiol* 30: 1651-1664, 1998.
18. Maulik A, Davidson SM, Piotrowska I, Walker M and Yellon DM: Ischaemic preconditioning protects cardiomyocytes from anthracycline-induced toxicity via the PI3K pathway. *Cardiovasc Drugs Ther* 32: 245-253, 2018.
19. Manolova Y, Deneva V, Antonov L, Drakalska E, Momekova D and Lambov N: The effect of the water on the curcumin tautomerism: A quantitative approach. *Spectrochim Acta A Mol Biomol Spectrosc* 132: 815-820, 2014.
20. Sikora E, Scapagnini G and Barbagallo M: Curcumin, inflammation, ageing and age-related diseases. *Immun Ageing* 7: 1, 2010.
21. Koeberle A and Werz O: Multi-target approach for natural products in inflammation. *Drug Discov Today* 19: 1871-1882, 2014.
22. Nakmareong S, Kukongviriyapan U, Pakdeechote P, Donpunha W, Kukongviriyapan V, Kongyingyoes B, Sompamit K and Phisalaphong C: Antioxidant and vascular protective effects of curcumin and tetrahydrocurcumin in rats with L-NAME-induced hypertension. *Naunyn Schmiedebergs Arch Pharmacol* 383: 519-529, 2011.
23. Topcu-Tarladacalisir Y, Akpolat M, Uz YH, Kizilay G, Sapmaz-Metin M, Cerkezayabekir A and Omurlu IK: Effects of curcumin on apoptosis and oxidoinflammatory regulation in a rat model of acetic acid-induced colitis: The roles of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. *J Med Food* 16: 296-305, 2013.
24. Anastopoulos I, Kavou A, Tentis I, Kortsaris A, Panayiotidis M, Lazou A and Pappa A: Silibinin protects H9c2 cardiac cells from oxidative stress and inhibits phenylephrine-induced hypertrophy: Potential mechanisms. *J Nutr Biochem* 24: 586-594, 2013.
25. Jia WQ, Wang ZT, Zou MM, Lin JH, Li YH, Zhang L and Xu RX: Verbasoside inhibits glioblastoma cell proliferation, migration and invasion while promoting apoptosis through upregulation of protein tyrosine phosphatase SHP-1 and inhibition of STAT3 phosphorylation. *Cell Physiol Biochem* 47: 1871-1882, 2018.
26. Al-Obeid O, Vaali-Mohammed MA, Eldehna WM, Al-Khayal K, Mahmood A, Abdel-Aziz HA, Zubaidi A, Alafeefy A, Abdulla M and Ahmad R: Novel quinazoline-based sulfonamide derivative (3D) induces apoptosis in colorectal cancer by inhibiting JAK2-STAT3 pathway. *Oncotargets Ther* 11: 3313-3322, 2018.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
28. Naserzadeh P, Mehr SN, Sadabadi Z, Seydi E, Salimi A and Pourahmad J: Curcumin protects mitochondria and cardiomyocytes from oxidative damage and apoptosis induced by *Hemiscorpius lepturus* venom. *Drug Res (Stuttg)* 68: 113-120, 2018.
29. Lv FH, Yin HL, He YQ, Wu HM, Kong J, Chai XY and Zhang SR: Effects of curcumin on the apoptosis of cardiomyocytes and the expression of NF- $\kappa$ B, PPAR- $\gamma$  and Bcl-2 in rats with myocardial infarction injury. *Exp Ther Med* 12: 3877-3884, 2016.
30. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W and Schultz G: Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* 69: 1476-1486, 1991.
31. Dai D, Yang J, Zhao C, Wu H, Ding J, Sun X and Hu S: Effect of geranylgeranyl pyrophosphate synthase on hypoxia/reoxygenation-induced injury in heart-derived H9c2 cells. *Int Heart J* 59: 821-828, 2018.
32. Cheng J, Wu Q, Lv R, Huang L, Xu B, Wang X, Chen A and He F: MicroRNA-449a inhibition protects H9C2 cells against hypoxia/reoxygenation-induced injury by targeting the Notch-1 signaling pathway. *Cell Physiol Biochem* 46: 2587-2600, 2018.
33. Yang HH, Chen Y, Gao CY, Cui ZT and Yao JM: Protective effects of microRNA-126 on human cardiac microvascular endothelial cells against hypoxia/reoxygenation-induced injury and inflammatory response by activating PI3K/Akt/eNOS signaling pathway. *Cell Physiol Biochem* 42: 506-518, 2017.
34. Cai Y, Hu X, Yi B, Zhang T and Wen Z: Glucagon-like peptide-1 receptor agonist protects against hyperglycemia-induced cardiomyocytes injury by inhibiting high mobility group box 1 expression. *Mol Biol Rep* 39: 10705-10711, 2012.
35. Bartz RR, Suliman HB and Piantadosi CA: Redox mechanisms of cardiomyocyte mitochondrial protection. *Front Physiol* 6: 291, 2015.
36. Granger DN and Kvietys PR: Reperfusion injury and reactive oxygen species: The evolution of a concept. *Redox Biol* 6: 524-551, 2015.
37. Fan Q, Chen M, Fang X, Lau WB, Xue L, Zhao L, Zhang H, Liang YH, Bai X, Niu HY, *et al*: Aging might augment reactive oxygen species (ROS) formation and affect reactive nitrogen species (RNS) level after myocardial ischemia/reperfusion in both humans and rats. *Age (Dordr)* 35: 1017-1026, 2013.
38. Rachmat FD, Rachmat J, Sastroasmoro S and Wanandi SI: Effect of allopurinol on oxidative stress and hypoxic adaptation response during surgical correction of tetralogy of fallot. *Acta Med Indones* 45: 94-100, 2013.
39. Liu M, Zhang P, Chen M, Zhang W, Yu L, Yang XC and Fan Q: Aging might increase myocardial ischemia/reperfusion-induced apoptosis in humans and rats. *Age (Dordr)* 34: 621-632, 2012.
40. Hu Q, Luo W, Huang L, Huang R and Chen R: Apoptosis-related microRNA changes in the right atrium induced by remote ischemic preconditioning during valve replacement surgery. *Sci Rep* 6: 18959, 2016.
41. Li Q, Xiang Y, Chen Y, Tang Y and Zhang Y: Ginsenoside Rg1 protects cardiomyocytes against hypoxia/reoxygenation injury via activation of Nrf2/HO-1 signaling and inhibition of JNK. *Cell Physiol Biochem* 44: 21-37, 2017.
42. Molavi B and Mehta JL: Oxidative stress in cardiovascular disease: Molecular basis of its deleterious effects, its detection, and therapeutic considerations. *Curr Opin Cardiol* 19: 488-493, 2004.
43. Zhang C, Tang Y, Li Y, Xie L, Zhuang W, Liu J and Gong J: Unfolded protein response plays a critical role in heart damage after myocardial ischemia/reperfusion in rats. *PLoS One* 12: e0179042, 2017.
44. Xu J, Hu H, Chen B, Yue R, Zhou Z, Liu Y, Zhang S, Xu L, Wang H and Yu Z: Lycopene protects against hypoxia/reoxygenation injury by alleviating ER stress induced apoptosis in neonatal mouse cardiomyocytes. *PLoS One* 10: e0136443, 2015.

45. Zou XJ, Yang L and Yao SL: Endoplasmic reticulum stress and C/EBP homologous protein-induced Bax translocation are involved in angiotensin II-induced apoptosis in cultured neonatal rat cardiomyocytes. *Exp Biol Med (Maywood)* 237: 1341-1349, 2012.
46. Gupta MK, Tahir FG, Knezevic T, White MK, Gordon J, Cheung JY, Khalili K and Feldman AM: GRP78 interacting partner Bag5 responds to ER stress and protects cardiomyocytes from ER stress-induced apoptosis. *J Cell Biochem* 117: 1813-1821, 2016.
47. Long M, Chen X, Wang N, Wang M, Pan J, Tong J, Li P, Yang S and He J: Proanthocyanidins protect epithelial cells from zearalenone-induced apoptosis via inhibition of endoplasmic reticulum stress-induced apoptosis pathways in mouse small intestines. *Molecules* 23: E1508, 2018.
48. Gorman AM, Healy SJ, Jäger R and Samali A: Stress management at the ER: Regulators of ER stress-induced apoptosis. *Pharmacol Ther* 134: 306-316, 2012.
49. Xia Z, Dickens M, Raingeaud J, Davis RJ and Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270: 1326-1331, 1995.
50. Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B and Ohlstein EH: Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ Res* 86: 692-699, 2000.
51. See F, Thomas W, Way K, Tzanidis A, Kompa A, Lewis D, Itescu S and Krum H: p38 mitogen-activated protein kinase inhibition improves cardiac function and attenuates left ventricular remodeling following myocardial infarction in the rat. *J Am Coll Cardiol* 44: 1679-1689, 2004.
52. Hassanzade A, Mandegary A, Sharif E, Rasooli R, Mohammadnejad R and Masoumi-Ardekani Y: Cyclooxygenase inhibitors combined with deuterium-enriched water augment cytotoxicity in A549 lung cancer cell line via activation of apoptosis and MAPK pathways. *Iran J Basic Med Sci* 21: 508-516, 2018.



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