Curcumin pretreatment attenuates myocardial ischemia/reperfusion injury by inhibiting ferroptosis, autophagy and apoptosis via HES1

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Abstract. The early restoration of hemodynamics/reperfusion in acute myocardial infarction (AMI) is an effective therapeutic strategy to reduce sudden death and improve patient prognosis. However, reperfusion induces additional cardiomyocyte damage and cardiac tissue dysfunction. In this context, turmeric-derived curcumin (Cur) has been shown to exhibit a protective effect against myocardial ischemia/reperfusion injury (I/RI). The molecular mechanism of its activity, however, remains unclear. The current study investigated the protective effect of Cur and its molecular mechanism via *in vitro* experiments. The Cell Counting Kit-8 and lactate dehydrogenase (LDH) assay kit were used to assess the cell viability and cytotoxicity. The contents of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase, glutathione (GSH)/glutathione disulfide (GSSG), total iron, ferrous iron, caspase-3 and reactive oxygen species (ROS) were measured using an appropriate kit. Western blotting was used to detect the expression of relevant proteins. The levels of apoptosis, mitochondrial permeability transition pore (MPTP) opening, and mitochondrial membrane potential (MMP) were detected by flow cytometry. The study findings indicated that anoxia/reoxygenation (A/R) injury significantly decreased cell viability, increased in LDH and caspase-3 activities, induced ferroptosis, increased apoptosis and overactivated autophagy. However, pretreatment with Cur or ferrostatin-1 (Fer-1, a ferroptosis inhibitor) significantly increased A/R-reduced cell viability, SOD, glutathione peroxidase activity, GSH/GSSH ratio and HES1 and glutathione peroxidase 4 protein expression; attenuated A/R-induced LDH, MDA, total iron, ferrous iron, prostaglandin-endoperoxide synthase 2 protein expression and prevented ROS overproduction and MMP loss. In addition, Cur inhibited caspase-3 activity, upregulated the Bcl-2/Bax ratio, reduced apoptotic cell number and inhibited MPTP over-opening. Furthermore, Cur increased P62, LC3II/I, NDUFB8 and UQCRC2 expression and upregulated the p-AMPK/AMPK ratio. However, erastin (a ferroptosis activator), pAD/HES1-short hairpin RNA, rapamycin (an autophagy activator) and Compound C (an AMPK inhibitor) blocked the protective effect of Cur. In conclusion, Cur pretreatment inhibited ferroptosis, autophagy overactivation and oxidative stress; improved mitochondrial dysfunction; maintained energy homeostasis; attenuated apoptosis; and ultimately protected the myocardium from A/R injury via increased HES1 expression.

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Introduction

Acute myocardial infarction (AMI), a cardiovascular ailment of severe its disabling and life-threatening implications, is associated with high morbidity and sudden death rates (1,2). Recent global prevalence statistics underscore its prevalence at 3.8% among individuals aged <60 years (sample size, 2,982,6717), and escalating to 9.5% (sample size, 5,071,185) in those aged >60 years (3). Amidst the rapid aging of the Chinese population, Chinese healthcare professionals will face new challenges in managing AMI. Early therapeutic interventions such as pharmacological thrombolysis,

percutaneous coronary intervention, and surgical bypass grafting effectively restore hemodynamics/reperfusion, ultimately mitigating sudden death rates and improving patient prognosis. However, reperfusion itself precipitates additional cardiomyocytes damage, exacerbating cardiac dysfunction, which is termed ischemia/reperfusion injury (I/RI) (4). Current understanding of the pathophysiological mechanisms underlying I/RI encompasses inflammatory responses, apoptosis, oxidative stress, autophagy, ferroptosis (5-7), and others. Of note, multiple moderated mortality patterns may occur independently, or there may be simultaneous crosstalk or overlap between them (8). Meanwhile, autophagy may play the role of a 'double-edged sword' in the different stages of ischemia and reperfusion. During ischemic, ATP decreases, which activates the mTOR/ULK1/PI3K pathway, thereby forming autophagic vesicles and promoting ATP synthesis. However, upon the restoration of reperfusion, increased ROS and Beclin1 overexpression can lead to autophagy overactivation and cardiomyocyte death promotion (9,10). ATP is the basis of several life activities, including growth, proliferation, bio-metabolism, stress and others. The activation of AMPK, which acts as the gatekeeper of energy metabolism and mitochondrial homeostasis, restores energy balance by promoting ATP-producing catabolic pathways and inhibiting energy-consuming processes. However, mitochondrial dysfunction can lead to reduced ATP production and cellular dysfunction (11). Thus, mitochondria play a critical role in cellular metabolism as the powerhouses of mammalian energy. However, whether energy stress regulates other non-apoptotic forms of regulatory cell death (RCD) in myocardial I/RI is not known. Therefore, there is an urgent need to evaluate the mechanisms of I/RI and find new therapeutic approaches.

Ferroptosis, a novel iron-dependent phospholipid peroxidation-driven mode of unique cell death, was first reported by Dixon in 2012 (12). Over the past decades, a preponderance of research has corroborated the view that ferroptosis is the primary form of RCD in myocardial I/RI (7,13,14). Recent investigation revealed that pretreatment with tanshinone IIA effectively attenuated A/R injury in H9c2 cardiomyocytes by modulating VDAC1-mediated ferroptosis and apoptosis (15). Additionally, puerarin has been shown to safeguard against I/RI in pressure overload-induced heart failure by inhibiting ferroptosis (16). These studies indicated that traditional Chinese medicine (TCM) may offer a new approach for treating cardiovascular disease via ferroptosis modulation.

TCM, a time-honored treasure of China for 5,000 years, has significantly contributed to combating various ailments, including the coronavirus disease 2019 pandemic, malaria and cardiovascular diseases (17-19). Nevertheless, the precise role of TCM in the prevention and treatment of certain diseases remains unclear. Consequently, the search for safe and effective active ingredients of TCM with clear molecular targets and low toxicity and side effects is an important issue that needs to be addressed urgently. Curcumin (Cur), a prominent TCM constituent, mainly extracted from turmeric, has been implicated in modulating apoptosis, oxidative stress, inflammatory responses and autophagy, thereby safeguarding against I/RI across various organs (10). Studies have shown that Cur opposed I/RI by activating the JAK2/STAT3 signaling (20), and attenuated I/R-induced lung injury via the Notch2/Hairy and enhancer of split 1 (Hes1) signaling (21). A prior study revealed astragaloside IV modulation of HES1 and its ligand protein VDAC1 to protect against myocardial I/RI, inhibiting apoptosis through the Notch1/HES1 signaling pathway, thereby exerting myocardial protection (22). Notably, HES1 is an important target gene downstream of the Notch1 signaling pathway that acts as a transcriptional repressor encoding the repressive basic helix-loop-helix (bHLH). A recent study has shown that the Notch1/HES1 signaling pathway can activate the PTEN/Akt signaling pathway through HES1 to reduce ROS generation, stabilize $\Delta\Psi m$ and ultimately decrease apoptosis, thereby protecting the myocardium (23). Nonetheless, to the best of our knowledge, previous studies focused only on inflammatory responses, oxidative stress level and apoptosis, and it is unclear whether HES1 participates in autophagy and ferroptosis after Cur treatment. The present study is the first to suggest that Cur pretreatment attenuates myocardial I/RI by inhibiting ferroptosis and autophagy via HES1. In addition, the present study provides new insights into the crosstalk among ferroptosis, autophagy and apoptosis in myocardial I/RI.

Therefore, in the present study, the H9c2 cardiomyocyte I/R model was established to i) confirm whether myocardial I/R injury induces ferroptosis and excessive autophagy, thereby damaging the myocardium; ii) investigate whether Cur inhibits ferroptosis and excessive autophagy induced by myocardial I/R injury; iii) evaluate whether HES1 mediates Cur inhibition of myocardial ferroptosis and excessive autophagy; and iv) assess whether the myocardial protective effect of Cur is related to the inhibition of oxidative stress, maintenance of energy metabolism homeostasis and maintenance of mitochondrial functional homeostasis.

Materials and methods

Materials and chemicals. Cur, (purity ≥98%) was purchased from Chengdu Must Bio-Technology Co., Ltd. Deferiprone (DFO, iron chelator), ferrostatin-1 (Fer-1, ferroptosis suppressor), erastin, 3-methyladenine (3MA, autophagy inhibitor) and rapamycin (RA, autophagy activator) were purchased from MedChemExpress. Compound C (AMPK inhibitor) was purchased from MilliporeSigma. Adenoviral pAD/HES1-short hairpin (sh) RNA and negative control (NC) were acquired from Cyagen Biologicals Co., Ltd.

Culture of rat H9c2 cardiomyocytes and development of an A/R injury model. A rat H9c2 cardiomyocyte cell line was obtained from the Cell Bank/Stem Cell Bank of the Chinese Academy of Sciences. Under standard conditions (95% humidity, 21% O_2, and 5% CO_2), cells were cultured in a high-glucose Dulbecco's modified Eagle's medium (H-DMEM; HyClone; Cytiva) enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (PSG; 100X) (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. A previous research protocol was used to establish the *in vitro* A/R model using the cellular A/R fluid method (15).

Preparation and transduction of Adenovirus. The adenoviral vectors pAD/HES1, shRNA and NC were prepared using a previously established method (15). The target sequences of pAD/ HES1-shRNA and NC are CAGACATTCTGGAAA

TGACAGTGAA and TTCTCCGAACGTGTCACGT, separately. In brief, the pAD/HES1, shRNA, or NC was introduced into rat H9c2 cells, which were cultured in fresh H-DMEM enriched with 10% FBS ('multiplicative infection' of 80) and incubated at 37˚C, 95% O_2, and 5% CO_2 for 48 h for the subsequent experiments. Non-adenovirus transduced H9c2 cardiomyocytes were used as control. The successful transfection of rat H9c2 cardiomyocytes with HES1 adenovirus was first validated at the protein molecular level as illustrated in Fig. S1.

Treatment of rat H9c2 cardiomyocytes and experimental design. Firstly, to assess weather prior treatment with Cur can mitigate ferroptosis and autophagy, thereby safeguarding rat H9c2 cardiomyocytes against injury caused by A/R, rat H9c2 cardiomyocytes were randomly divided into 9 groups: i) Control, incubated in normal culture medium for 48 h; ii) erastin, rat H9c2 cardiomyocytes exposed to 10 μM erastin in 10% FBS for 24 h; iii): Cur, H9c2 cardiomyocytes treated with 10 μM Cur in 10% FBS for 48 h; iv) erastin + Cur, H9c2 cardiomyocytes pre-treated with Cur in a 10% FBS for 24 h, followed by 24-h co-incubation with 10 μM erastin; v) A/R, H9c2 cardiomyocytes incubated for 48 h in normal culture medium and then exposure to A/R for 3 h/2 h; vi): A/R + Cur, rat H9c2 cardiomyocytes pre-treated with 10 μM Cur under 10% FBS for 48 h; vii): A/R + Cur + erastin, Cur pretreatment for 24 h and then co-incubation with 10 μM erastin for 24 h; viii): A/R + DFO, DFO pretreatment for 24 h; and ix): A/R + Fer-1, Fer-1 pretreatment for 2 h and then exposure to A/R for 3 h/2 h.

Secondly, to investigate whether HES1 is involved in ferroptosis and evaluate its mechanism of action in A/R, rat H9c2 cardiomyocytes were randomly divided into 8 groups: i) Control, incubated for 48 h in normal culture medium; ii) erastin, rat H9c2 cardiomyocytes exposed to 10 μM erastin in 10% FBS for 24 h; iii) erastin + pAD/HES1; iv) erastin + pAD/HES1-shRNA, rat H9c2 cardiomyocytes pretreated with pAD/HES1 or pAD/HES1-shRNA in 10% FBS for 24 h and then co-treated with 10 μM erastin for 24 h; v) A/R, rat H9c2 cardiomyocytes cultured for 48 h and then exposure to A/R for 3 h/2 h; vi) A/R + pAD/HES1; vii) A/R + pAD/HES1-shRNA; and viii): A/R + NC; (rat H9c2 cardiomyocytes pretreated with pAD/HES1, pAD/HES1-RNA, or NC in 10% FBS for 48 h and exposed to A/R for 3 h/2 h).

Thirdly, to evaluate changes in autophagy after A/R injury and the effect of Cur pretreatment on it, rat H9c2 cardiomyocytes were randomly divided into the groups below: I): control; II): A/R; III): A/R + Cur. IV): A/R + Cur + RA; and V): A/R + 3MA; (rat H9c2 cardiomyocytes pre-treated with 10 μM Cur in 10% FBS for 48 h prior to A/R; 10 μM Cur +200 nM RA co-incubated for 48 h; 5 mM 3MA pretreatment for 24 h and then exposure to A/R for 3 h/2 h). The control, A/R, and A/R + Cur groups were treated as per the aforementioned protocols.

Furthermore, to investigate the role of ferroptosis and autophagy regulation during myocardial A/R injury as well as of HES1 and Cur pretreatment, rat H9c2 cardiomyocytes were randomly divided into the following groups: i) control; ii): A/R; iii): A/R + Cur; (the control, A/R and A/R + Cur groups were treated as per aforementioned protocols); iv) A/R + Cur + pAD/HES1-shRNA; v) A/R + pAD/HES1-shRNA; and vi) A/R + Cur + NC; (rat H9c2 cardiomyocytes were pretreated with 10 μM Cur + pAD/HES1-shRNA, pAD/HES1-shRNA, or NC in 10% FBS for 48 h prior to A/R and then exposure to A/R for 3 h/2 h).

Ultimately, to explore how Cur pretreatment enhances and maintains mitochondrial function and energy metabolism in rat H9c2 cardiomyocytes induced by A/R injury, H9c2 cells were randomly grouped as follows: i) Control; ii) A/R; iii) A/R + Cur; (the control, A/R and A/R + Cur groups were treated as per aforementioned methods; and iv): A/R + Cur + Compound C, H9c2 cardiomyocytes were co-incubated with 10 μM Cur + 5 μM Compound C in 10% FBS for 48 h prior to A/R and then exposure to A/R for 3 h/2 h.

Assessment of cell viability and cytotoxicity. Cell survival was measured using the Cell Counting Kit-8 (CCK-8) colorimetric assay (cat. no. GK10001; GlpBio), following the manufacturer's protocol. In brief, rat H9c2 cardiomyocytes were inoculated in the center of a 96-well plate at a density of $1x10^4$ cells/well along with per 100 μl culture medium/10 μl CCK-8 and incubated at 37˚C for 1.5 h. Then, absorbance levels were gauged at 450 nm utilizing a microplate reader (Thermo Fisher Scientific, Inc.).

The supernatant of the treated cells from each group was collected and the lactate dehydrogenase (LDH) level was measured using an LDH assay kit (cat. no. C0017; Beyotime Biotechnology) according to the manufacturer's protocol. Briefly, 60 μl LDH assay working solution was added to per 120 μl of supernatant, thoroughly mixed, and then incubated at 25˚C for 30 min in the absence of light. Absorbance was measured at 490 nm using the aforementioned apparatus.

Quantification of malondialdehyde (MDA), superoxide dismutase (SOD), total iron, glutathione peroxidase (GSH-Px) and glutathione (GSH)/glutathione disulfide (GSSG). After various treatments, cell lysate supernatants were collected. An MDA assay kit (cat. no. S0131M), SOD assay kit (cat no. S0101M), GSH and GSSG assay kit (cat no. S0053; all from Beyotime Biotechnology), GSH-Px Assay kit (cat. no. A005-1-2; Nanjing Jiancheng Bioengineering Institute) and total iron ion colorimetric assay kit (cat. no. E1042-100; Applygen Technologies, Inc.) were used for measuring MDA, SOD, GSH/GSSG, GPX and total iron ion levels, respectively.

Determination of Caspase-3 activity. After various treatments, cell lysate supernatants were collected. The Caspase-3 activity was quantified using a caspase-3 assay kit (cat. no. C1115; Beyotime Biotechnology), following the manufacturer's protocol.

Western blot analysis. After the treatment of rat cardiomyocytes, total protein was extracted from the cells of each group using western and IP cell lysates (cat. no. P0013; Beyotime Biotechnology) following the manufacturer's protocol, followed by bicinchoninic acid protein assay kit (cat. no. P0012; Beyotime Biotechnology) for protein concentration quantification. Proteins were denatured by adding an appropriate amount of sodium dodecyl-sulfate-polyacrylamide gel electrophoresis

(SDS-PAGE) protein sampling buffer (6X; cat. no. P0015F; Beyotime Biotechnology) and boiling at 100˚C for 10 min in a metal heater. Then, a 40-μg protein sample was added to each lane for separation using 10 or 12% SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride membranes, which were sealed with 5% non-fat dry milk at room temperature for 2 h in a three-buffer brine containing 0.1% Tween-20. The membranes were then incubated overnight in a low-speed shaker maintained at 4˚C with primary antibodies against the following: HES1 (1:500; cat. no. sc-5392; Santa Cruz Biotechnology, Inc.), PTGS2 (1:1,000; cat. no. 12375-1-AP; Proteintech Group, Inc.), GPX4 (1:800; cat. no. 381958; ZEN-BIO), P62 (1:5,000; cat. no. 18420-1-AP; Proteintech Group, Inc.), LC3B (1:800; cat. no. 381544; ZEN-BIO), NDUFB8 (1:800; cat. no. 383060; ZEN-BIO), UQCRC2 (1:800; cat. no. 382096; ZEN-BIO), Bcl-2 (1:800; cat. no. 381702; ZEN-BIO), Bax (1:800; cat. no. 380709; ZEN-BIO), phosphorylated (p-) AMPK (1:800; cat. no. 381164; ZEN-BIO), AMPKα (1:1,000; cat. no. AF6195; Beyotime Biotechnology) and β-actin (1:2,000; cat. no. 20536-1-AP; ProteinTech Group, Inc.). On the next day, the membranes underwent five washes, each lasting 6 min, followed by incubation with a secondary Goat Anti-Rabbit/Mouse IgG H&L-conjugated antibody (1:5,000; cat. nos. 511203 and 511103; ZEN-BIO) for 1.5 h at room temperature. Finally, the membranes were washed three times for 6 min/each before being visualized using the BeyoECL Plus kit (cat. no. P0018S; Beyotime Biotechnology). The β-actin was used as a loading control for normalization. The intensity of gray values in the protein lanes was measured using the ImageJ software 1.8.0 (National Institutes of Health).

Quantification of intracellular ferrous iron content. Intracellular $Fe2^{+}$ levels were determined using the FerroOrange kit (cat. no. F374; Dojindo Laboratories, Inc.) according to the manufacturer's protocol. Briefly, following the H9c2 cardiomyocyte treatment, the cells were incubated with 1 μM FerroOrange for 30 min at 37˚C under light protection. Excess FerroOrange was removed by rinsing once with HBSS (cat. no. G4204; Servicebio), and ferrous iron content was assessed under an inverted fluorescence microscope (Olympus Corporation; magnification, x200).

Detection of intracellular ROS. Intracellular ROS level was measured using a DCFH-DA kit (cat. no. S0033S; Beyotime Biotechnology) as described in the manufacturer's protocol.

Evaluation of lysosomes. The reagents were incubated with the LysoTracker Red fluorescent dye (cat. no. C1046; Beyotime Biotechnology) for 45 min at 37˚C in the dark following the reagent manufacturer's protocol. The relevant changes were observed under an inverted fluorescence microscope (Olympus Corporation; magnification, x200).

Assessment of apoptosis. Apoptosis was detected using the Annexin V-FITC/PI apoptosis detection kit (cat. no. BB-4101; BestBio) as per the manufacturer's protocol. Briefly, $1x10^6$ cells/tube were collected after treatment, and resuspended in 400 μl 1X Annexin binding buffer, gently vortexed with 5 μl Annexin V-FITC and 8 μl PI in succession and incubated for 15 and 5 min, respectively, at 6˚C in the dark, followed by immediately flow cytometric analysis using Agilent NovoCyte Advanteon flow cytometer (NovoCyte; Agilent Technologies, Inc.) to detect apoptosis (Agilent; excitation 488 nm; emission 578 nm). The total apoptotic rate of positive cells was calculated as the rate of early apoptotic cells Q2 + rate of late apoptotic cells Q3. Flow cytometric data were analyzed using the FlowJo software (V.10; Tree Star, Inc.).

Evaluation of mitochondrial permeability transition pore (MPTP) opening and mitochondrial membrane potential (MMP). Cellular MPTP and MMP were assessed using the MPTP assay kit (cat. no. BB-48122; BestBio) and MMP assay kit (cat no. BB-4105; BestBio), respectively, adhering to the manufacturer's protocol. In brief, $1x10^6$ cells/tube were collected after treatment and subjected to BBcellProbe M61 assay (which involved the addition of BBcellProbe M61 working solution and quencher, followed by incubation at 37˚C for 15 min in the dark, centrifugation at 600 x g to remove the supernatant, and cell resuspension in 600 μl HBSS) and the JC-1 method (which included the addition of JC-1 staining working solution, followed by incubation at 37˚C for 20 min protected from light, washing twice with PBS, and resuspending the cells in 600 μl PBS), respectively. These cells were then immediately analyzed using Agilent NovoCyte Advanteon flow cytometer for in real time. The FlowJo software was used to analyze the flow cytometric data.

Assessment of mitochondrial ultrastructure using transmission electron microscopy (TEM). Briefly, after completion of the experimental model, H9c2 cardiomyocytes were collected from each group, fixed (incubated with 2.5% glutaraldehyde at 25˚C for 2 h), washed, dehydrated with ascending ethanol, embedded with Epon 812 at 65˚C for 48 h, ultrathin sectioned (50 nm), stained with 2% uranyl acetate and 2.6% lead citrate at 37˚C for 10 min, and observed via TEM (Hitachi, Ltd.). The degree of damage to mitochondrial ultrastructure was assessed using the Flameng score (24).

Statistical analysis. The data were statistically analyzed using GraphPad Prism 9.0 (Dotmatics). Each experiment was performed in triplicate, and the results are presented as the mean ± standard deviation. Group comparisons, involving groups of three or more, were performed using one-way ANOVA, followed by Tukey's post-hoc test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Cur pretreatment protects against A/R-induced cardiomyocyte injury. To verify whether Cur pretreatment safeguards cardiomyocytes from I/R damage, an A/R model was established using rat H9c2 cardiomyocytes. Initially, the dose-response of Cur protection was investigated. In the A/R model of rat H9c2 cardiomyocytes, CCK-8 and LDH assays were used to measure cell viability and toxicity in cells pretreated with different Cur concentrations (2.5, 5, 10, 20 and 40 μM), and the results revealed that the effect of Cur on cells was concentration-dependent (Fig. 1A and B). In stark contrast to the control group, A/R injury prominently decreased cell

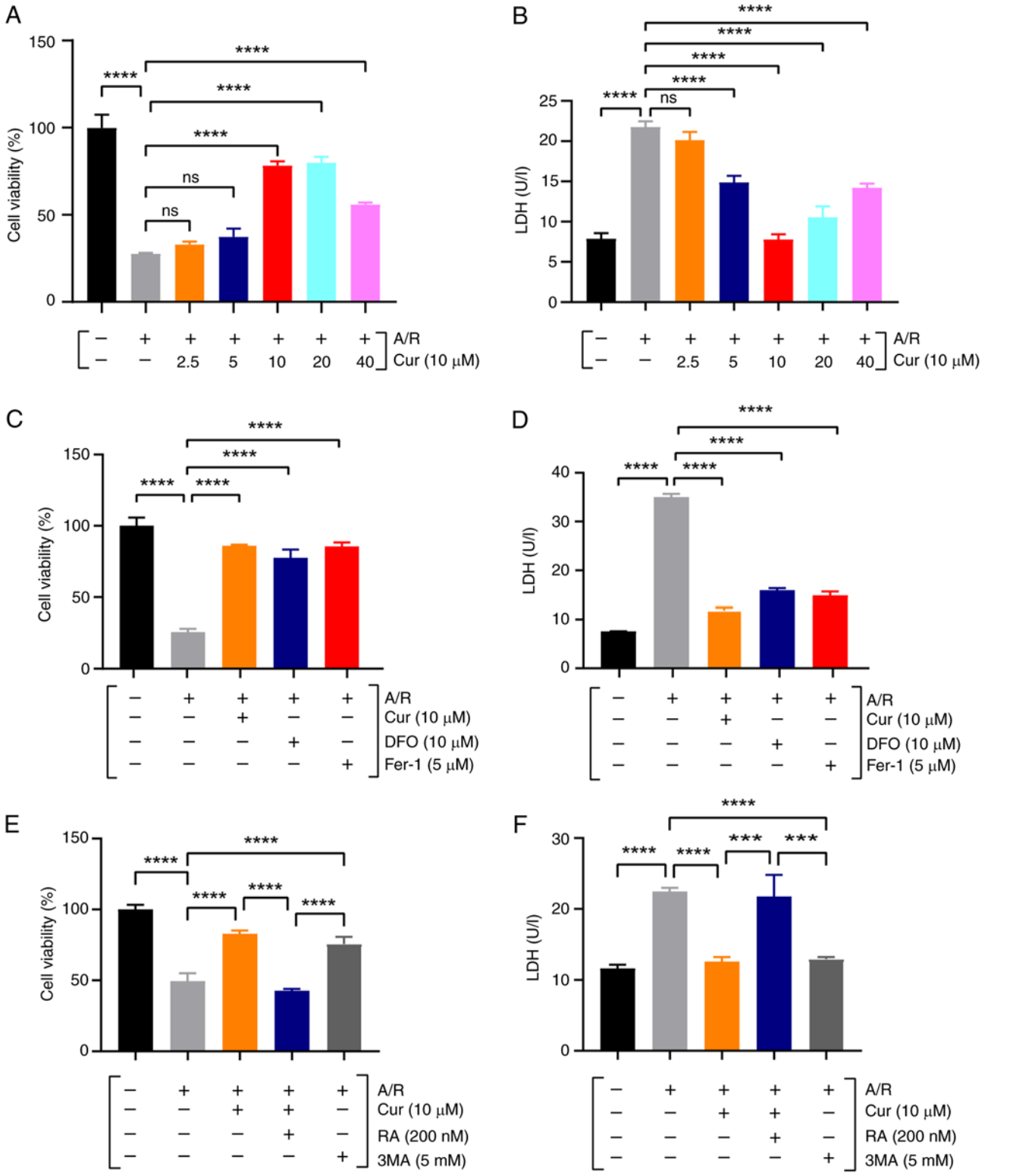

Figure 1. Cur protects rat H9c2 cardiomyocytes from A/R injury. (A, C and E) Cell Counting Kit-8 assay of A/R-triggered H9c2 cardiomyocyte viability after Cur, DFO, Fer-1, RA and 3MA pretreatment. (B, D and F) LDH. Data are presented as the mean ± SD (n=3). ***P<0.001 and ****P<0.0001. Cur, curcumin; A/R, anoxia/reoxygenation; LDH, lactate dehydrogenase; DFO, deferiprone; Fer-1, ferrostatin-1, RA, rapamycin; 3MA, 3-methyladenine, ns, not significant.

viability and elevated LDH activity; whereas compared with A/R, pretreatment with 10 μM Cur significantly bolstered A/R-induced H9c2 cell viability and decreased LDH activity, without eliciting any notable cytotoxicity. Therefore, 10 μM Cur was selected as the optimal concentration for subsequent experiments.

Moreover, the cardioprotective potency of Cur pretreatment was found to be commensurate with that observed with DFO, Fer-1, or 3MA. However, the beneficial effects of 10 μM Cur were notably attenuated by RA (Fig. 1C-F). These findings indicated the protective role of Cur against I/R injury in cardiomyocytes.

Cur pretreatment ameliorates A/R or erastin injury-induced ferroptosis in cardiomyocytes. Iron overload, ROS and lipid peroxidation constitute the triad of factors pivotal to ferroptosis (13). Thus, iron content, ROS and lipid oxidation-related parameters were measured in H9c2 cells. Lipid oxidation metabolites such as ROS, MDA and total and labile iron pools, were significantly increased in the A/R group relative to the control group, whereas these levels were significantly decreased following pretreatment with 10 μM Cur; similar results were obtained with 10 μM DFO and 5 μM Fer-1 pretreatment (Fig. 2A-H). GSH/GPX4 is a component of the classical regulatory mechanism of ferroptosis (25). Following A/R treatment, the activity of antioxidant enzymes such as SOD, GSH/GSSG ratio and GSH-Px was significantly reduced, which were reversed via pretreatment with Cur, DFO and Fer-1 (Fig. 2I-K). The molecular levels of ferroptosis-related proteins were further examined by western blotting, and it was found that pretreatment with Cur, DFO and Fer-1 significantly inhibited PTGS2 protein expression and upregulated GPX4 protein expression, respectively (Fig. 2L and M). These results suggested that Cur may be involved in ferroptosis and oxidative stress after A/R injury.

To corroborate that Cur mitigates A/R-associated injury by inhibiting ferroptosis and oxidative stress, the present study further investigated the protective effect of Cur on erastin-related injury. Upon erastin treatment, a significant reduction in cell viability, SOD activity and the GSH/GSSG ratio was observed, accompanied by an elevation in LDH, MDA levels and MPTP opening. Intriguingly, pretreatment with 10 μM Cur significantly attenuated these erastin-mediated injuries (Fig. S2A-E and L). However, in the A/R model, when Cur and erastin co-treatment were performed in cardiomyocytes, erastin counteracted the protective effect of Cur (Fig. S2F-J and M). Western blot analysis illuminated that Cur pretreatment robustly upregulated HES1 and GPX4 protein expression levels after erastin-induced injury (Fig. S2K). These results suggested that Cur ameliorates A/R injury-induced ferroptosis by inhibiting oxidative stress and that HES1 may be involved in ferroptosis after A/R injury.

In addition, to investigate whether HES1 is involved in erastin or A/R-induced ferroptosis, H9c2 cardiomyocytes were transfected with high and low HES1-expressing adenoviruses before A/R or erastin treatment. Unlike the control group, the erastin or A/R group exhibited significantly reduced cell viability and elevated LDH activity, whereas adenoviral transfection along with pAD/HES1 overexpression attenuated cell viability reduction and LDH elevation due to erastin or A/R injury. However, adenoviral transfection along with pAD/HES1-shRNA further significantly aggravated the erastin- or A/R injury-induced reduced cell viability, elevated LDH and MDA level. Moreover, relative to the control, SOD activity and GSH/GSSG ratio were significantly decreased and MPTP was over-opened after erastin or A/R treatment. Of note, although pAD/HES1-shRNA aggravated erastin or A/R injury, pAD/HES1 pretreatment reversed the changes. Meanwhile, the detection of relevant proteins in cell lysates showed that HES1 expression was reduced in the erastin or A/R group compared with the control group and that it was further significantly reduced after pAD/HES1-shRNA pretreatment. However, HES1 expression was significantly increased after pAD/HES1 pretreatment. In addition, PTGS2 expression was significantly increased in the erastin or A/R group and further increased after pAD/HES1-shRNA pretreatment, whereas GPX4 expression was reduced in the erastin or A/R group and further significantly reduced after pAD/HES1-shRNA pretreatment. However, pAD/HES1 pretreatment reversed these changes (Fig. S3A-N). These findings indicated the potential role of HES1 in ferroptosis triggered by erastin or A/R. However, additional research is required to clarify the exact mechanism.

Cur pretreatment attenuates A/R-induced excessive autophagy in cardiomyocytes. Autophagy, a lysosome-dependent process, is pivotal in maintaining intracellular homeostasis and survival by degrading abnormal or damaged macromolecules and organelles. When cardiomyocytes are exposed to certain external stimuli, such as ischemia and inflammation, ATP depletion inhibits the mTOR pathway and induces ULK1 activation, which stimulates autophagic vesicle formation and promotes ATP synthesis. Upon stimulus exacerbation such as in reperfusion or sepsis, the increased overexpression of Beclin1 and excessive accumulation of ROS can lead to the overactivation of autophagy, which promotes cardiomyocyte death (9,26,27). Fascinatingly, the current study revealed that prior treatment with Cur influenced autophagy-related marker expression, as evidenced by a significant increase in P62 expression and LC3II/LC3I expression ratios in Cur-treated H9c2 cells (Fig. 3A-C). This finding indicated that the aforementioned safeguarding effect of Cur pretreatment might be linked to the suppression of autophagy overstimulation.

To confirm that Cur preconditioning plays a protective role against myocardial A/R injury by affecting autophagy, RA (autophagy activator) and 3MA (autophagy inhibitor) were used to observe their effects on autophagy. Analysis of cell lysates revealed pertinent protein alterations. Compared with the A/R group, there was a significant increase in the HES1 level and P62 level, substantial decrease in LC3II/LC3I ratio, significant reduction in the ferroptosis indicator PTGS2, and significant increase in GPX4 level following pretreatment with 10 μM Cur and 5 mM 3MA; whereas in the A/R + Cur + RA group, RA reversed favorable changes when the myocardium was co-incubated with Cur and RA (Figs. 3D-G and S4A-F). These results indicated that autophagy and ferroptosis probably overlap or crosstalk and that HES1 may be involved in the Cur-based amelioration of A/R injury in cardiomyocytes by inhibiting excessive autophagy activation and ferroptosis.

Cur pretreatment ameliorates A/R-induced apoptosis in cardiomyocytes. In the present investigation of the intricate interplay between A/R-induced ferroptosis, autophagy, and the prophylactic role of Cur pretreatment, a novel aspect was fortuitously uncovered: Cur pretreatment modulated the expression of proteins implicated in apoptosis. Specifically, the BCL2/BAX ratio was significantly reduced in the A/R group compared with the control, whereas Cur pretreatment exerted a restorative influence, reversing this trend (Fig. 4A and B). Additionally, Cur pretreatment significantly attenuated A/R-stimulated caspase 3 levels, further substantiating its anti-apoptotic effect (Fig. 4C). Utilizing flow cytometry, apoptosis, MPTP and MMP were analyzed. The present

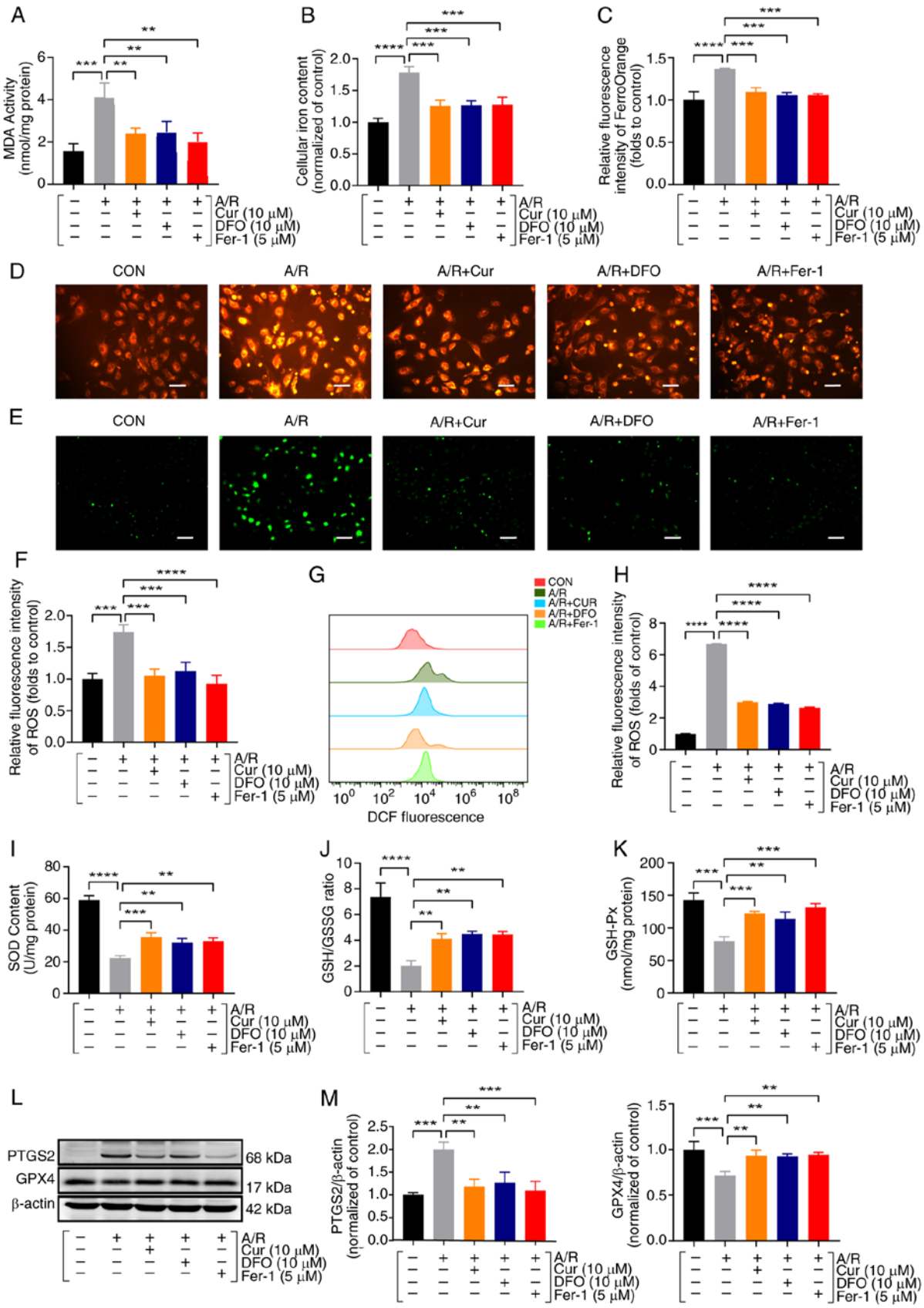

Figure 2. Cur pretreatment ameliorates A/R injury-triggered ferroptosis in cardiomyocytes. (A) MDA assay of A/R-triggered H9c2 cardiomyocyte after Cur, DFO and Fer-1 pretreatment. (B) Total iron content. (C and D) Ferrous ions (magnification, x200; scale bar, 100 μm). (E and F) ROS was detected using a quantification kit in A/R-triggered cells after Cur, DFO, or Fer-1 pretreatment (magnification, x100; scale bar, 200 μm). (G and H) DCFH-DA assay for ROS with flow cytometry. (I) SOD. (J) GSH/GSSG ratio. (K) GSH-Px. (L and M) Protein expression of PTGS2 and GPX4 was determined using western blot analysis in cell lysates after A/R-triggered following pre-treatment with Cur, DFO, or Fer-1. Data are presented as the mean ± SD (n=3). **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; DFO, deferiprone; Fer-1, ferrostatin-1; A/R, anoxia/reoxygenation; MDA, malondialdehyde; GSH/GSSH, glutathione/glutathione disulfide; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; ROS, reactive oxygen species; PTGS2, prostaglandin-endoperoxide synthase 2; GPX4, glutathione peroxidase 4.

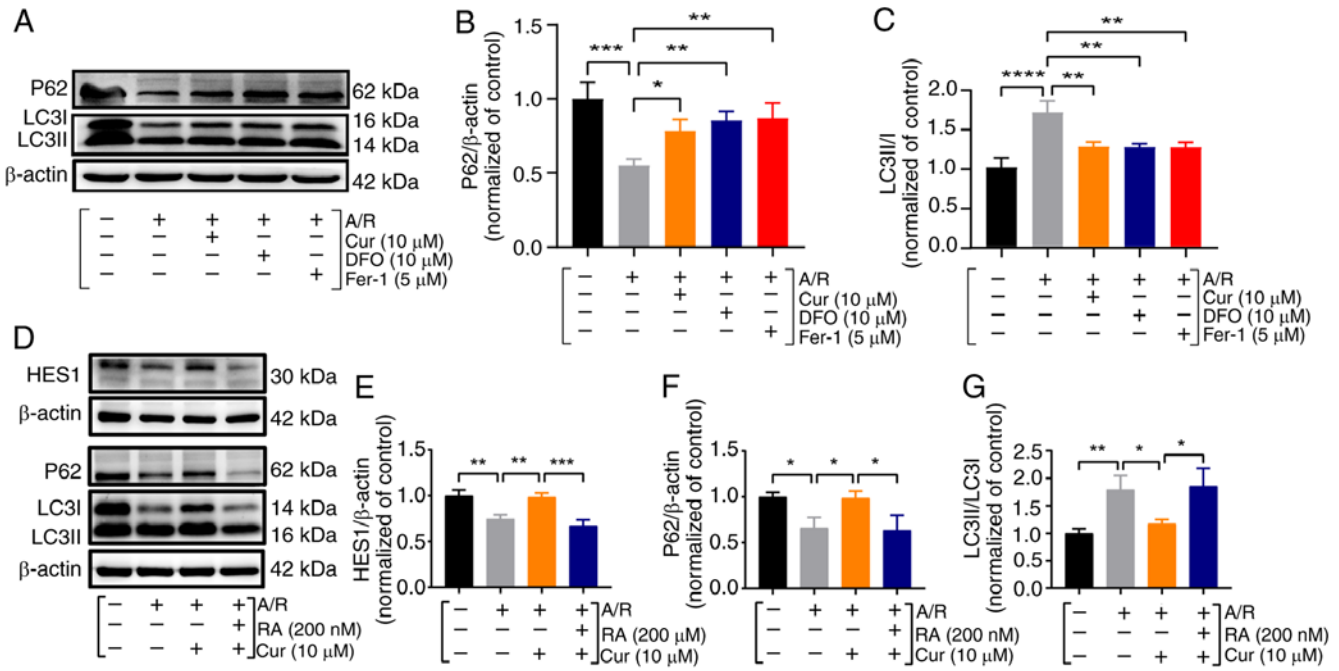

Figure 3. Cur pretreatment inhibits A/R-triggered excessive autophagy in cardiomyocytes. (A-G) Expression and quantification of HES1, P62 and LC3II/I proteins in A/R-triggered cells were determined using western blot analysis after pretreatment with Cur, DFO, Fer-1 and RA. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; DFO, deferiprone; Fer-1, ferrostatin-1; RA, rapamycin; A/R, anoxia/reoxygenation. HES1, hairy and enhancer of split 1; P62, Sequestosome 1; LC3II/I, microtubule-associated protein 1 light chain 3.

findings revealed that A/R injury led to a significant increase in apoptotic rates, accompanied by exaggerated MPTP opening and MMP disruption. Notably, pretreatment with Cur, DFO and Fer-1 effectively alleviated A/R-mediated damage, as evidenced by reduced apoptotic indices and mitigated mitochondrial dysfunction (Fig. 4D-I). These observations underscore the potential of Cur pretreatment to mitigate A/R injury by inhibiting apoptosis, ferroptosis and autophagy. Furthermore, protein analyses demonstrated that Cur pretreatment significantly enhanced the expression of the HES1 protein (Fig. 4J and K). These findings indicated that Cur pretreatment can inhibit ferroptosis, autophagy and apoptosis by upregulating HES1.

Cur pretreatment inhibits A/R-induced ferroptosis in H9c2 cardiomyocytes via mediating HES1. To robustly substantiate the HES1-dependency of Cur pretreatment in safeguarding against myocardial A/R damage through ferroptosis suppression, experiments were conducted using H9c2 cardiomyocytes transfected with an adenovirus (pAD/HES1-shRNA or NC). After A/R treatment, MDA, SOD, GSH/GSSG ratio, GSH-Px and total intracellular iron content were detected in cell lysates. The results revealed that H9c2 cardiomyocytes subjected to A/R injury exhibited heightened MDA and total intracellular iron levels, alongside diminished SOD activity, GSH/GSSG ratio and GSH-Px activity, compared with control. Notably, 10 μM Cur pretreatment significantly ameliorated these detrimental effects. By contrast, pAD/HES1-shRNA treatment abolished the protective effect of Cur pretreatment and increased the susceptibility of cardiomyocytes to A/R injury (Fig. 5A-D). ROS and ferrous iron deposition acted as primary drivers of ferroptosis (14). Thus, these factors were quantified in H9c2 cells using immunofluorescence and flow cytometry, and the obtained results were consistent with the aforementioned findings (Fig. 5E-K). Therefore, it was hypothesized that Cur preconditioning alleviates I/RI-associated ferroptosis by regulating HES1. Additionally, the relevant protein molecules of cell lysates were also detected. Compared with the A/R group, a significant reduction in the expression of the ferroptosis marker PTGS2 and a significant increase in GPX4 expression after 10 μM Cur pretreatment were revealed. By contrast, Cur + pAD/HES1-shRNA co-incubation significantly downregulated GPX4 expression and upregulated PTGS2 compared with the Cur pretreatment group alone, and pAD/HES1-shRNA exacerbated A/R-induced damage (Fig. 5L and M), highlighting the counteractive effect of HES1 knockdown.

Collectively, Cur pretreatment effectively ameliorated HES1-mediated I/RI-associated ferroptosis, while pAD/HES1-shRNA blocked the protective effect and exacerbated A/R-induced damage. These results indicated that HES1 may be involved in the Cur-based amelioration of cardiomyocyte A/R via ferroptosis inhibition.

Cur preconditioning inhibits A/R-induced excessive autophagy in H9c2 cardiomyocytes via mediating HES1. The aforementioned experimental observations illuminated that Cur upregulated HES1 expression after A/R injury, thereby mitigating excessive autophagy activation. To confirm that Cur preconditioning depends on active HES1 expression by inhibiting autophagic hyperactivation, based on the upregulation of HES1 expression, HES1 expression was silenced the present study by pAD/HES1-shRNA. The results demonstrated that compared with the A/R group, HES1 expression was significantly increased, the LC3 II/LC3 I expression ratio was significantly decreased, and P62 expression was significantly

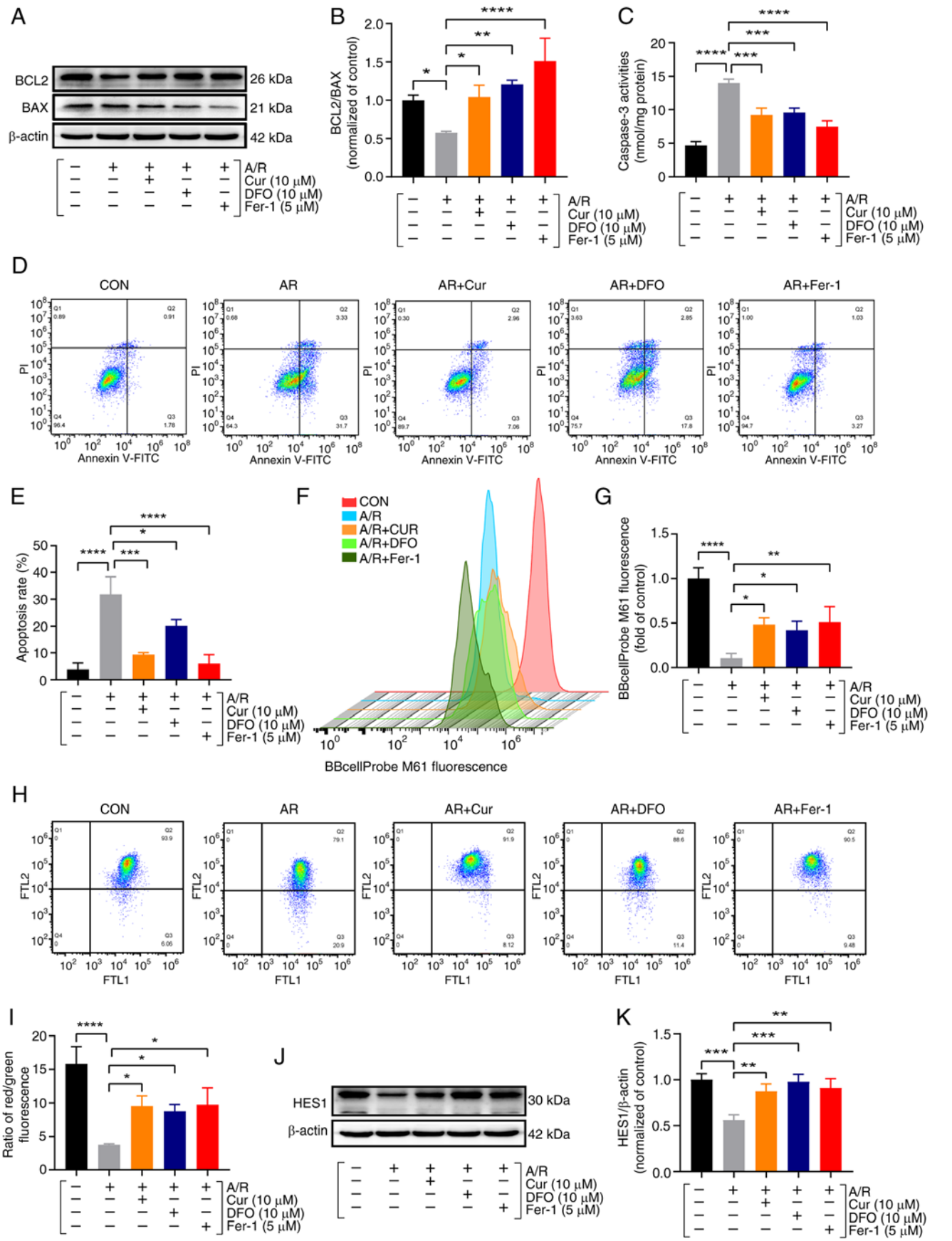

Figure 4. Cur pretreatment ameliorates A/R-triggered apoptosis in cardiomyocytes. (A and B) Protein expression of BCL2 and BAX was determined using western blot analysis in cell lysates after A/R-triggered following pre-treatment with Cur, DFO, or Fer-1. (C) Caspase-3 activity. (D and E) Annexin V-FITC/PI assay for apoptosis with flow cytometry. (F and G) BBcellProbe M61 assay for cellular MPTP. (H and I) JC-1 assay for cellular MMP. (J and K) Protein expression of HES1. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; DFO, deferiprone; Fer-1, ferrostatin-1; A/R, anoxia/reoxygenation; BCL2, B-cell lymphoma2; BAX, BCL2-associated X protein; MPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential.

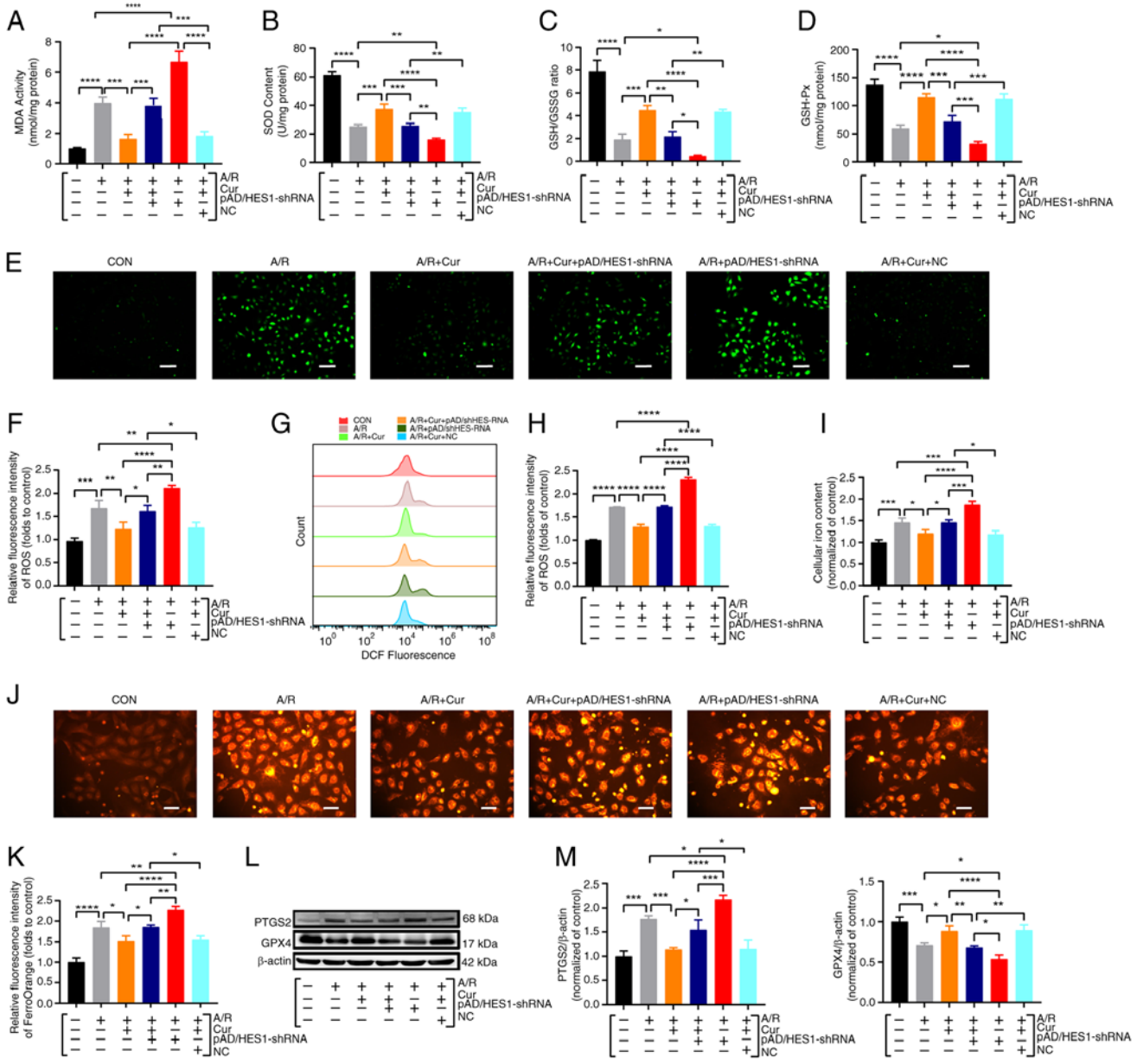

Figure 5. Cur pretreatment inhibits A/R-triggered ferroptosis in H9c2 cardiomyocytes via mediating HES1. (A) MDA. (B) SOD. (C) GSH/GSSG ratio. (D) GSH-Px. (E and F) Immunofluorescence for the detection and quantitative analysis of ROS (magnification, x100; scale bar, 200 μm), (G and H) DCFH-DA assay for ROS with flow cytometry. (I) Total iron content. (J and K) Immunofluorescence for the detection of ferrous ions (magnification, x200; scale bar, 100 μm). (L and M) Western blot analysis for the detection of protein expression and quantitative analysis of PTGS2 and GPX4 in cell lysates after treatment. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; A/R, anoxia/reoxygenation; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; GSH/GSSH, glutathione/glutathione disulfide; GSH-Px, glutathione peroxidase; GPX4, glutathione peroxidase 4; NC, negative control; shRNA, short hairpin RNA.

upregulated in the 10 μM Cur pretreatment group. Conversely, co-incubation with Cur + pAD/HES1-shRNA significantly decreased HES1 and P62 expression while upregulating the LC3 II/LC3 I ratio. Notably, pAD/HES1-shRNA exacerbated A/R injury (Fig. 6A-D). The LysoTracker Red dye is used to observe autophagy lysosomes. In A/R-injured cardiomyocytes, the red fluorescent spot was more intense, which was significantly reduced by Cur pretreatment. However, co-incubation with Cur + pAD/HES1-shRNA significantly increased the red fluorescence intensity (Fig. 6E-H). Although Cur pretreatment effectively attenuated HES1-mediated autophagy hyperactivation-associated cell death, pAD/HES1-shRNA counteracted this protective effect. These results suggested that HES1 is involved in the Cur-based amelioration of cardiomyocyte I/RI by inhibiting autophagic hyperactivation.

Cur preconditioning inhibits A/R-induced apoptosis in H9c2 cardiomyocytes via mediating HES1. In addition, interestingly, alterations in apoptosis-related indices were also observed. Specifically, A/R injury significantly downregulated BCL2/BAX ratio, augmented Caspase 3 levels, and increased the total apoptotic rate (early + late apoptosis). Cur pretreatment reversed A/R injury-induced apoptosis, yet pAD/HES1-shRNA blocked this protective effect (Fig. 7A-E). These results indicated that in I/RI, there may be a mutual overlap and crosstalk among ferroptosis, apoptosis and

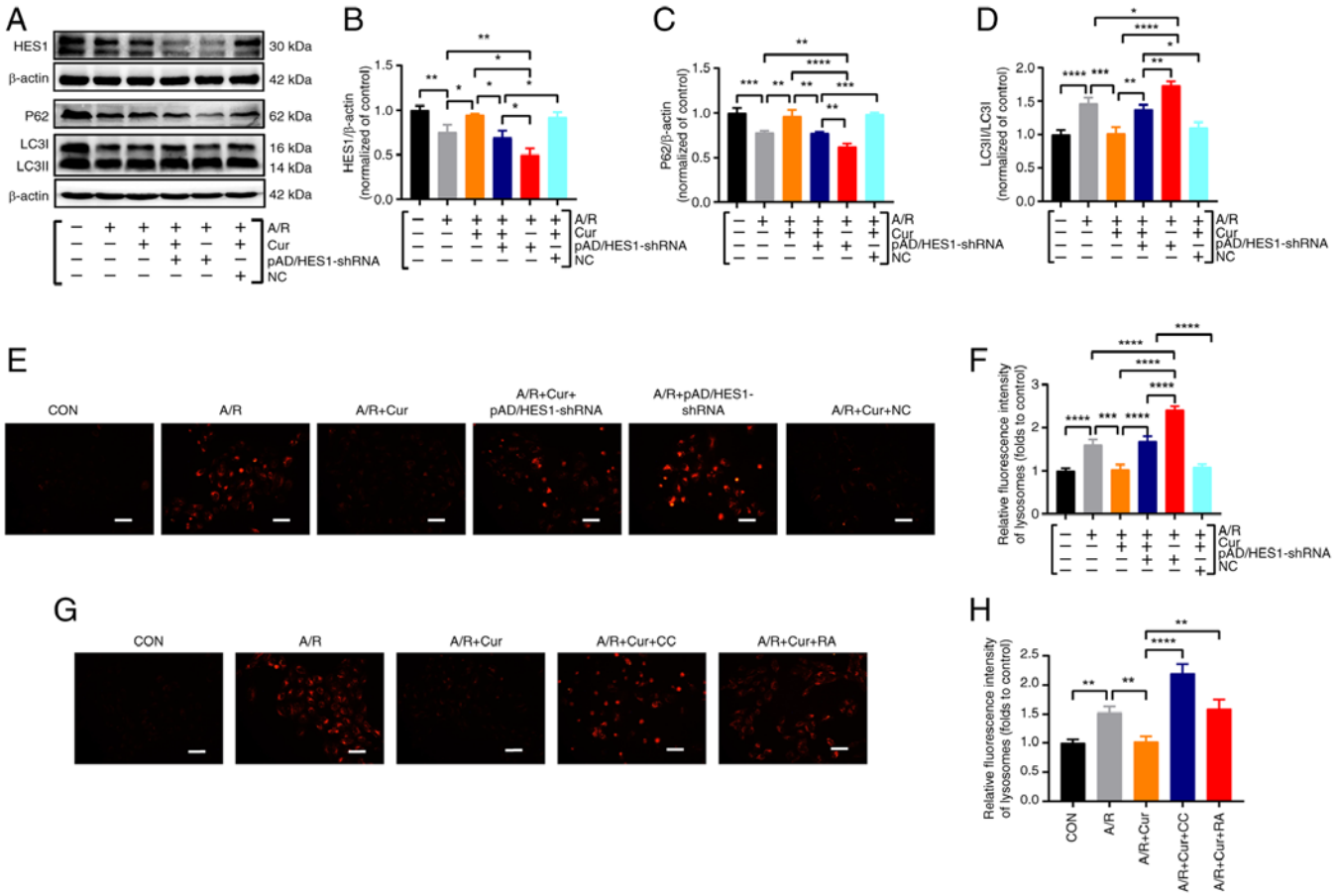

Figure 6. Cur pretreatment inhibits excessive autophagy of A/R-triggered cardiomyocytes via adjusting HES1. (A-D) Western blot analysis for the detection of protein expression and quantitative analysis of P62 and LC3II/I in cell lysates after treatment. (E-H) Fluorescent probe LysoTracker Red for detecting lysosomes and quantitative analysis following treatment (magnification, x200; scale bar, 100 μm). Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; A/R, anoxia/reoxygenation; P62, Sequestosome 1; LC3II/I, microtubule-associated protein 1 light chain 3; CC, compound C; RA, rapamycin; NC, negative control; shRNA, short hairpin RNA.

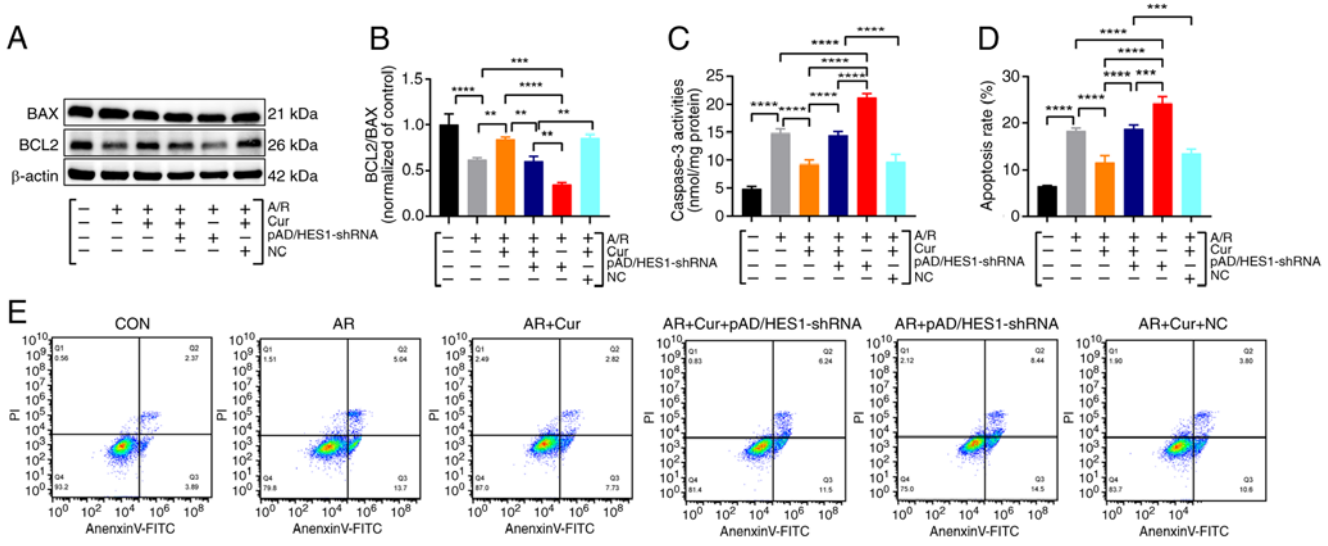

Figure 7. Cur pretreatment ameliorates apoptosis of A/R-triggered cardiomyocytes via mediating HES1. (A and B) The expression of BCL2 and BAX protein in cell lysates after treatment. (C) Caspase-3 activity. (D and E) Annexin V-FITC/PI assay for apoptotic rate. Data are presented as the mean ± SD (n=3). **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; A/R, anoxia/reoxygenation; BCL2, B-cell lymphoma2; BAX, BCL2-associated X protein; NC, negative control; shRNA, short hairpin RNA.

autophagy, thereby together affecting the homeostasis of the internal environment and final state of cardiomyocytes. However, this requires an in-depth investigation of the pathways and mechanisms among them.

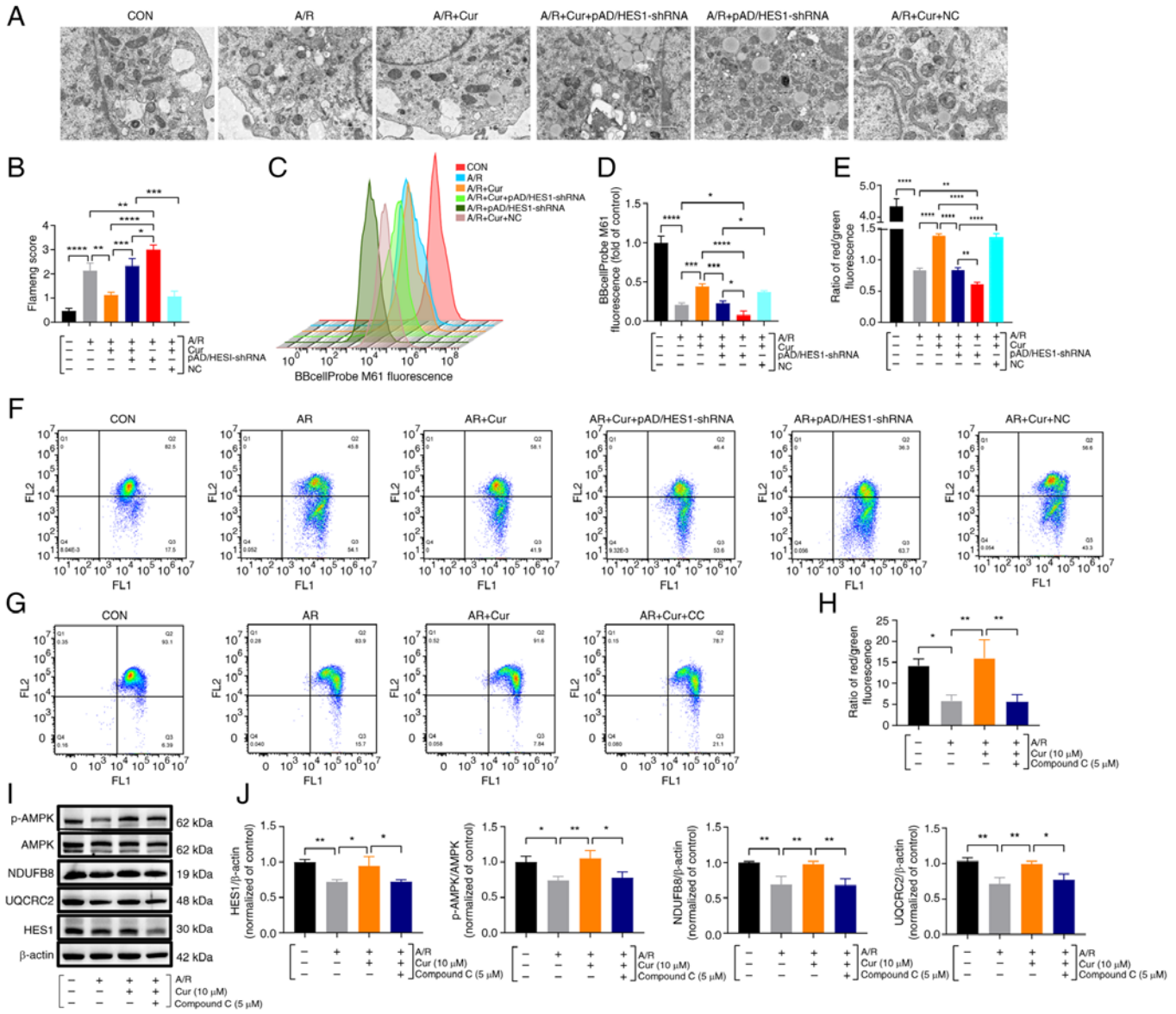

Figure 8. Cur preconditioning ameliorates mitochondrial dysfunction in A/R injured cardiomyocytes via adjusting HES1 and upregulates AMPK, maintaining energy metabolism homeostasis. (A and B) Transmission electron microscopy for assessing mitochondrial ultrastructure and Flameng scoring (magnification, x8000; scale bar, 1 μm). (C and D) BBcellProbe M61 assay for cellular MPTP by Flow cytometry. (E-H) JC-1 assay for cellular MMP. (I and J) Protein expression and quantification of HES1, p-AMPK, AMPK, NDUFB8 and UQCRC2 in the lysates of exposed A/R cells after Cur or Compound C pretreatment using western blotting. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Cur, curcumin; A/R, anoxia/reoxygenation; MPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential; NDUFB8, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8; UQCRRC2, cytochrome b-c1 complex subunit 2; NC, negative control; shRNA, short hairpin RNA; p-, phosphorylated.

Cur pretreatment ameliorates mitochondrial dysfunction via adjusting HES1 and upregulates AMPK maintained homeostasis of energy metabolism in A/R injured cardiomyocytes. MPTP and MMP, one of the essential components of mitochondria, may be involved in the exchange of mitochondrial components during cell death. They play a significant part in cell survival, apoptosis and ferroptosis, which are associated with several functions, such as tumor progression and ischemia/reperfusion (15,28). Based on the upregulation of HES1 expression, the present study aimed to investigate the effect of Cur pretreatment on mitochondrial function and morphological changes in H9c2 cardiomyocytes with A/R injury. For a visual assessment of mitochondrial morphological changes, TEM was used to observe the changes among the different groups. Mitochondria from A/R-treated H9c2 cells were largely wrinkled, with an obvious distortion of the internal structure, reduced or broken cristae, and a significantly higher Flameng score. By contrast, 10 μM Cur pretreatment attenuated the A/R-induced effects, whereas pAD/HES1-shRNA reversed the effects, blocked the protective effect of Cur, and increased the sensitivity of H9c2 to A/R injury (Fig. 8A and B). Furthermore, the results revealed that Cur or Cur + NC pretreatment significantly inhibited MPTP opening as well as elevated MMP in H9c2 cardiomyocytes after A/R injury, thereby maintaining mitochondrial function and homeostasis. However, pAD/HES1-shRNA pretreatment blocked the protective effect of Cur (Fig. 8C-F).

Energy is the basis of several life activities, including growth, proliferation, bio-metabolism and stress, among others.

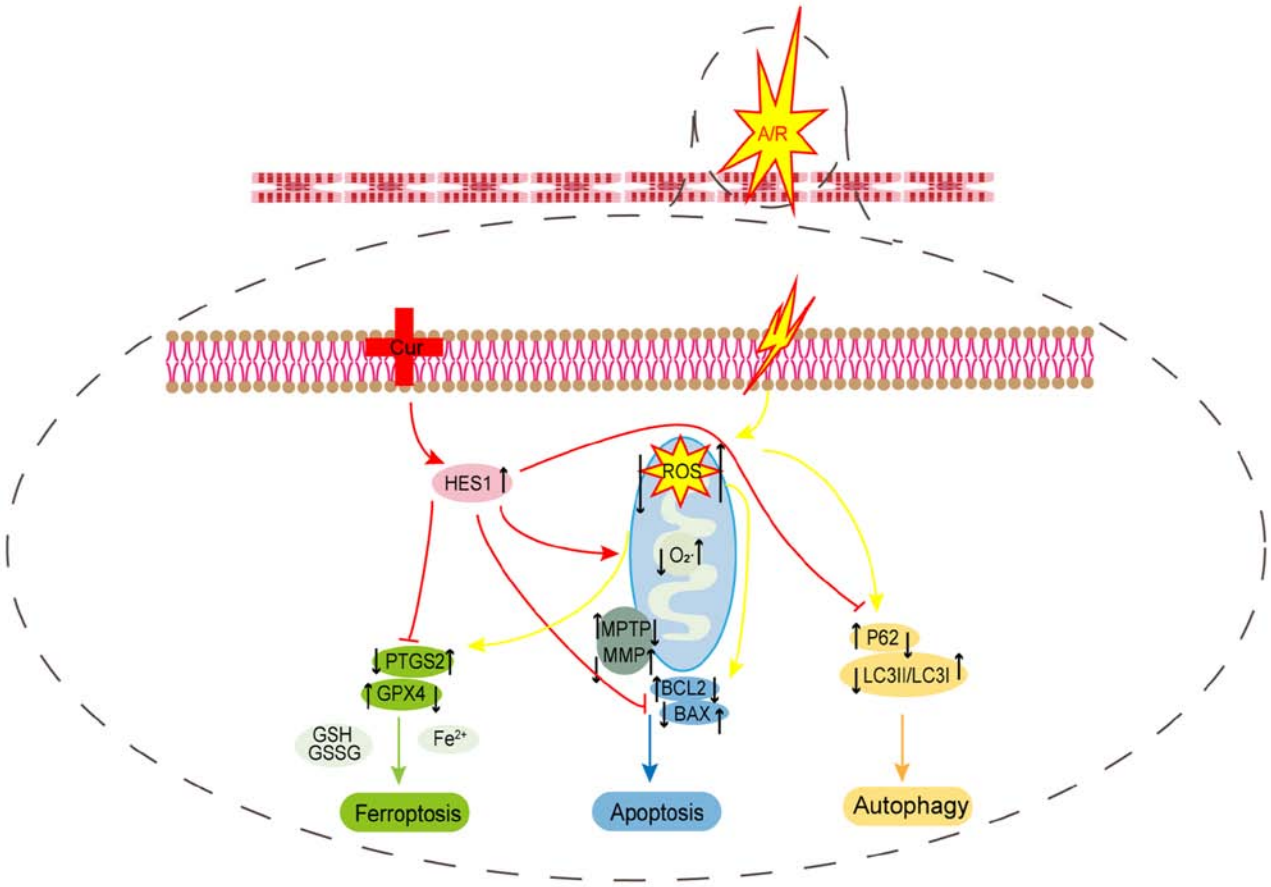

Figure 9. Potential mechanism of Cur in A/R-triggered injury. Pre-treatment with Cur could inhibit oxidative stress, ferroptosis, apoptosis and autophagy while ameliorating mitochondrial function to protect cardiomyocytes from A/R injury by adjusting HES1. Cur, curcumin; A/R, anoxia/reoxygenation; HES1, hairy and enhancer of split 1; PTGS2, prostaglandin-endoperoxide synthase 2; GPX4, glutathione peroxidase 4; P62, Sequestosome 1; LC3II/I, microtubule-associated protein 1 light chain 3; ROS, reactive oxygen species; GSH/GSSH, glutathione/glutathione disulfide; BCL2, B-cell lymphoma2; BAX, BCL2-associated X protein; MPTP, mitochondrial permeability transition pore; MMP, mitochondrial membrane potential.

The activation of AMPK, the gatekeeper of energy metabolism and mitochondrial homeostasis, restores energy balance by stimulating ATP-generating catabolic routes and suppressing energy-consuming processes (29). Compared with the control, A/R injury significantly decreased the red/green fluorescence ratio and decreased MMP, Cur pretreatment significantly increased MMP and maintained MMP homeostasis, and Compound C (AMPK inhibitor) reversed these changes (Fig. 8G and H). Protein detection in cell lysates revealed that Cur pretreatment significantly upregulated the p-AMPK/AMPK ratio and NDUFB8, UQCRC2 and HES1 expression compared with the A/R group and that Compound C blocked the protective effect of Cur (Fig. 8I and J). Thus, it was hypothesized that HES1 is involved in the Cur-based regulation of the maintenance of cellular energy metabolism homeostasis via AMPK to ameliorate I/RI-induced mitochondrial dysfunction.

Discussion

Owing to the rapidly aging society of China, the count of patients suffering from cardiovascular conditions, especially AMI, has been escalating annually (30). Consequently, this poses formidable challenges for medical personnel while presenting a unique, once-in-a-century opportunity. Preventing and treating patients with AMI in an improved and faster manner and reducing morbidity, death and disability have become important issues to be solved. While early hematopoiesis/reperfusion therapy represents an effective therapeutic strategy to reduce the rate of sudden death and improve the prognosis of patients, reperfusion itself aggravates cardiomyocyte damage and cardiac tissue dysfunction in patients with AMI, that is, I/RI (4). The pathophysiological mechanisms of I/RI are complex and involve multiple forms of RCD, encompassing inflammatory responses, oxidative stress, apoptosis, pyroptosis, ferroptosis and autophagy, among others. These may occur independently or in cross-talk with each other, occasionally overlapping (5-8). Therefore, the exploration and elucidation of the underlying pathophysiological mechanisms is critical for the discovery and development of more effective molecular drug targets against I/RI. The present study results revealed that after A/R injury, LDH levels were significantly elevated and cell viability was suppressed; MDA, total iron and free iron levels were enhanced; and SOD activity and GSH/GSSG ratio were inhibited compared with the control group, indicating that H9c2 cardiomyocytes were significantly injured in the I/RI model *in vitro*. Furthermore, the protein expression of BCL2, BAX, PTGS2, GPX4, LC3II/I and P62 was altered, suggesting that apoptosis-, ferroptosis- and autophagy-based regulatory mechanisms are involved in I/RI *in vitro* (Fig. 9).

Hypoxic/ischemic preconditioning, as its nomenclature suggests, entails the alleviation of I/RI through the employment of diverse salutary preconditioning protocols before hypoxia/ischemia. In recent years, there has been a surge of research interest in various forms of preconditioning, notably cardiac ischemic preconditioning, remote ischemic preconditioning and pharmacological preconditioning (31). In particular, pharmacological preconditioning to improve I/RI is of great clinical utility owing to the simplicity of protocols and their ease of implementation. Illustrative instances include dihydrotanshinone I preconditioning protects the myocardium from ischemic injury via PKM2 glutathione sialylation; naringenin improves mitochondrial dysfunction in I/RI via the AMPK-SIRT3 pathway; and HHQ16, a Flavin IV derivative, ameliorates myocardial infarction by degrading lncRNA4012/9456 (32-34). Thus, it was hypothesized that Cur is a candidate phytochemical for treating I/RI.

Cur, a class of naturally occurring polyphenolic compounds primarily extracted from turmeric rhizomes within TCM, has emerged as a pivotal agent exhibiting protective function against sepsis, Alzheimer's disease and I/RI, as evidenced by studies (21,35,36). Hu *et al* showed that Cur could prevent and treat myocardial infarction via the Epac1-Akt pathway to alleviate A/R injury (37). Additionally, Kim *et al* (38) revealed that Cur could prevent and treat myocardial infarction through TLR2 inhibition. Furthermore, Cur has been shown to regulate the Notch2/HES1 pathway, leading to reduced I/R-induced lung injury (39). HES1, a vital gene downstream of the Notch signaling pathway, encodes a repressive bHLH that acts as a transcriptional repressor. HES1 proteins regulate numerous biological processes in the organism, including cell proliferation, apoptosis and stress (40). Previous studies revealed that the upregulation of HES1 protein contributed to the alleviation of I/RI and improvement of AMI (41,42). In our recent study, it has been revealed that ischemic preconditioning/post-ischemic treatment attenuates A/R damage via the Notch1/HES1/VDAC1 axis (43). However, whether Cur can improve I/RI by regulating HES1 expression remains unknown. Therefore, further in-depth studies are required to investigate the regulatory mechanism of Cur. The present study delves into this knowledge gap and demonstrated that Cur pretreatment significantly enhanced cell survival rate, decreased LDH activity, inhibited oxidative stress, reduced iron level, and ameliorated the *in vitro* I/RI injury mimicry by upregulating HES1 expression. Of note, Cur may have effects similar to those of the small molecule drugs DFO, Fer-1 and 3MA, highlighting its therapeutic potential.

Ferroptosis is an iron-dependent form of non-apoptotic cell death that involves lipid hydroperoxide accumulation, which distinguishes it from other RCDs (12,13). Over the past decade, ferroptosis has emerged as a pivotal mechanism underlying the initiation and progression of various cardiovascular disease subtypes, encompassing actinomycin- or sepsis-induced cardiomyopathy, atherosclerosis, myocardial I/RI, arrhythmias and diabetic cardiomyopathy (7,44,45). Iron, an essential trace element, plays pivotal roles in numerous biological processes, spanning growth, development and energy metabolism across life forms. Notably, iron serves as a central player in the occurrence of ferroptosis, emphasizing the significance of maintaining iron homeostasis for preserving cardiac physiological function (46). Deviations from iron homeostasis, such as deficiency, can precipitate heart failure in humans (47). Iron overload, either secondary or primary, particularly unstable ferrous ions, can lead to cardiac damage through the action of oxidative stress (48,49). However, the mechanisms regulating these phenomena are currently unknown. In addition to altering iron homeostasis, excessive ROS accumulation directly damages cardiomyocytes via oxidative lipid metabolism (50). Apart from iron metabolism, ROS and lipid metabolism, the glutathione-dependent antioxidant system, which is the most classical anti-ferroptosis pathway, has been shown to prevent and treat cardiovascular disease. Meanwhile, cysteine deficiency, glutathione depletion and inactivation of the phospholipid hydroperoxide GPX4 have been identified to promote ferroptosis (51). In the present study, Cur pretreatment significantly decreased the overall iron level inside cells and ferrous ion level in the unstable iron pool, decreased lipid metabolism indices (such as MDA), increased SOD, and GSH/GSSG ratios, and inhibited ROS overproduction. In addition, Cur pretreatment significantly reduced PTGS2 expression and increased GPX4 and HES1 expression. Of note, pAD/HES1-shRNA counteracted the effect of Cur pretreatment on A/R injury. Therefore, it was hypothesized that Cur inhibits ferroptosis against A/R injury by upregulating HES1.

Autophagy, a pivotal mechanism, enables organisms to respond to various external stimuli, maintain homeostasis of the internal environment, and adapt for survival through the phagocytosis of abnormal molecules or organelles (52). However, research focusing on the impact of autophagy on I/RI has yielded contrasting findings. Certain studies posit that augmenting autophagy mitigates I/RI, whereas others contend that inhibiting excessive autophagy shields the myocardium from I/RI (53-56). Notably, in acute myocardial I/RI, autophagy is a 'double-edged sword'. If autophagic homeostasis is disrupted, autophagy overactivation will degrade normal intracellular proteins, subcellular organelles, membranes and other substances, ultimately resulting in cell death. In the current study, P62 expression and LC3II/LC3I ratio were significantly downregulated in simulated I/RI *in vitro*, suggesting excessive autophagy activation after reoxygenation. Notably, Cur pretreatment reversed these alterations, indicating that Cur's protective effect may stem from impeding excessive autophagy activation. To confirm that Cur preconditioning may play a protective role against myocardial A/R injury via autophagy regulation. RA (autophagy activator) and 3MA (autophagy inhibitor) were used to observe their effects on autophagy. The results showed that 3-MA inhibited autophagy to attenuate I/RI, whereas RA activated excessive autophagy to exacerbate I/RI, suggesting that Cur has a similar effect to 3MA. Of note, with the autophagy tool drug, P62 expression and LC3II/LC3I ratio were affected similarly to PTGS2 and GPX4 expression, suggesting a possible overlap or crosstalk between ferroptosis and autophagy. Nevertheless, pAD/HES1-shRNA blocked the myocardial protective effect of Cur preconditioning against A/R injury. The findings indicated that Cur may play a role in mitigating A/R injury in cardiomyocytes by inhibiting excessive autophagy activation and ferroptosis via mediating HES1.

The excessive autophagy triggered by I/R linked to myocardial mitochondrial homeostasis and energy metabolism. It has been revealed that during cardiomyocyte ischemia, ATP depletion activates the mTOR/ULK1/PI3K pathway, fostering autophagic vesicle formation and ATP synthesis. Conversely, upon reperfusion, ROS surplus elicits autophagy overactivation, exacerbating cardiomyocyte demise (57). Notably, mitochondrion is the main site of ATP production in mammals, and ATP serves as the energy source for most life activities, including growth and development, proliferation, metabolism and stress, among others. In addition, the activation of AMPK, which serves as a gatekeeper of energy metabolism and mitochondrial homeostasis, helps restore energy balance by facilitating catabolic routes for ATP production while inhibiting energy overconsumption. However, mitochondrial dysfunction can lead to reduced ATP production, ROS overproduction and cellular dysfunction (11,29). Moreover, MMP and MPTP, one of the key components of the mitochondrial membrane, may be engaged in the exchange of mitochondrial and cytoplasmic intercellular components during cell proliferation, apoptosis, ferroptosis and autophagy in several diseases, such as septic cardiomyopathy, tumors, and myocardial ischemia/reperfusion (28,58,59). In the present study, Cur preconditioning increased NDUFB8 and UQCRC2 protein expression and p-AMPK/AMPK protein ratio, whereas Compound C (an AMPK inhibitor) inhibited the defensive impact of Cur preconditioning against A/R damage. In addition, Cur pretreatment prevented A/R-induced MPTP over-opening and MMP reduction, whereas pAD/HES1-shRNA blocked this effect. Thus, it was hypothesized that Cur preconditioning attenuates A/R injury by participating in the maintenance of mitochondrial functional homeostasis and energy biogenesis.

The findings of present study indicated that I/R causes significant harm to the myocardium by triggering ferroptosis, apoptosis and excessive autophagy. Notably, Cur can regulate ferroptosis and apoptosis-related protein expression by upregulating HES1 expression. Furthermore, the current results demonstrated that Cur pretreatment increased the expression of P62 and the LC3II/I ratio, reduced ROS generation, stopped excessive MPTP opening, stabilized MMP levels, upregulated pAMPK/AMPK ratio, and maintained mitochondrial function. Collectively, HES1-mediated Cur preconditioning protected the myocardium from I/RI by inhibiting ferroptosis, apoptosis, excessive autophagy and oxidative stress; ameliorating mitochondrial dysfunction; and maintaining energy homeostasis.

In the present study, the molecular protective mechanism of Cur was explored using only ferroptosis inhibitor/activator and autophagy inhibitor/activator in an *in vitro* A/R model of H9c2 cells. To understand the potential mechanism of action, transgenic or knockdown-based HES1-overexpressing I/RI animal models need to be established. In addition, although these results suggested that ferroptosis, apoptosis, autophagy crosstalk, or overlap may exist in A/R, clear hub molecular targets need to be identified using more in-depth experiments.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SQL and JCL conceived and designed the study. YY and HH performed cell experiments, data analysis and visualization. TH and CCZ performed cell experiments and data curation. YMQ and MF developed methodology. All authors wrote the original draft. All authors read and approved the final version of the manuscript. SQL and JCL revised the manuscript. SQL and HH supervised the study and confirm the authenticity of all the raw data.

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.

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