Curcumin attenuates hypoxia/reoxygenation-induced cardiomyocyte injury by downregulating Notch signaling

PENG ZHU[1,2*], MANLI YANG[3*], HAO HE[2], ZHIBIN KUANG[2], MU LIANG[2], ANXIAO LIN[2], SONG LIANG[2], QIYUN WEN[2], ZHIQIN CHENG[2] and CHAOFENG SUN[1]

[1]Department of Cardiovascular Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061; [2]Department of Cardiovascular Medicine, The Fifth Affiliated Hospital of Zunyi Medical University; [3]Department of Respiratory Medicine, The Fifth Affiliated Hospital of Zunyi Medical University, Zhuhai, Guangdong 519100, P.R. China

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Abstract. Recovery of the blood supply is the most effective treatment against ischemic heart disease; however, it is also a major cause of myocardial ischemia/reperfusion injury in clinical therapy. Curcumin has been reported to possess beneficial effects against hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury by regulating cell proliferation, apoptosis and antioxidant enzyme activity. The aim of the present study was to investigate the molecular mechanisms underlying the effects of curcumin on H/R-injured cardiomyocytes. H9C2 cardiomyocytes were pretreated with curcumin, and then cultured under H/R conditions. The viability of H9C2 cells was measured using a Cell Counting kit-8 assay, and the levels of intracellular lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) were measured to assess cell injury. Levels of reactive oxygen species (ROS) and apoptosis were evaluated by flow cytometry. The expression levels of Notch intracellular domain (NICD) and numerous downstream genes were analyzed via reverse transcription-quantitative polymerase chain reaction and western blotting. The results revealed that curcumin protected H9C2 cells against H/R-induced injury, reversing the H/R-induced increases in LDH and MDA levels, and decreases in SOD levels. ROS levels in H/R-induced cells were also significantly downregulated by curcumin treatment ($P<0.01$), and the apoptotic rate was significantly decreased from 15.13% in the H/R group to 7.7% in the H/R + curcumin group ($P<0.01$). The expression levels of NICD, hairy and enhancer of split (Hes)-1, Hes-5 and hairy/enhancer-of-split related with YRPW motif protein 1 (Hey-1) were significantly decreased in H/R-treated cells following curcumin treatment. Treatment with Jagged1 attenuated the effects of curcumin on cell viability, ROS levels and apoptosis; the Notch pathway was also reactivated. The present study indicated that there was a role for the Notch pathway in the protective effects of curcumin against H/R-induced cardiomyocyte injury, suggesting that downregulation of the Notch pathway may alleviate H/R-induced injury in H9C2 cells.

Correspondence to: Dr Chaofeng Sun, Department of Cardiovascular Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: sunchaofeng_scf@163.com

*Contributed equally

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Introduction

Ischemic heart disease (IHD) is one of the leading causes of mortality worldwide (1). At present, recovering cardiac blood supply is the most effective treatment against IHD; however, it is also a major cause of myocardial ischemia/reperfusion injury (MIRI) in clinical therapy (2). MIRI can result in further myocardial damage, subsequently worsening the therapeutic outcomes of reperfusion therapy. Currently, methods for treating MIRI are insufficiently effective (3,4). Cardiomyocyte apoptosis is an important characteristic of MIRI that is observed during early phases of reperfusion. Therefore, further investigation of the mechanisms underlying reperfusion-induced cardiomyocyte injury is required to develop treatments for IHD.

Jagged1 belongs to the Delta/Serrate/LAG-2 (DSL) family of ligands for Notch receptors, which regulate cell proliferation and differentiation in mammals (5). Following ligand-mediated activation of the Notch pathway, the Notch intracellular domain (NICD) is released and binds with the transcription factor CBF-1/suppressor of hairless/lag to form a transcription complex, and then induces downstream gene transcription; for example, hairy and enhancer of split (Hes)-1, Hes-5 and hairy/enhancer-of-split related with YRPW motif protein 1 (Hey-1) (6,7). The Notch pathway is an evolutionarily conserved pathway that is widely involved in cell proliferation, differentiation and apoptosis (8). Jagged1, a DSL ligand for the mammalian Notch receptor, activates gene expression and the suppression of differentiation by binding to Notch and inducing the proteolytic release of NICD (9). Activation of the Notch

pathway induces downstream proteins involved in the cell cycle and apoptosis, including NICD, Hes-1, Hes-5 and Hey-1 (5,9). Previous studies have reported that the Notch signaling pathway is involved in hypoxia/reoxygenation (H/R)-induced cardiomyocyte injury (10-12). Additionally, a previous study indicated that inhibition of microRNA-449a protects H9C2 cells against H/R-induced damage by silencing the Notch1 signaling pathway (12).

Curcumin, or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural phenolic substance present in the rhizome of *Curcumae longae* (13,14). Curcumin has received increasing scientific attention due to its range of reported biological effects, including anti-inflammatory, antioxidant, anticarcinogenic and cardioprotective effects (15,16). Previous studies have reported that by regulating cell proliferation, apoptosis and antioxidant enzymes, curcumin induces positive effects on ischemia/reperfusion (I/R) injury in various organs (17,18). Additionally, a number of studies have demonstrated that curcumin attenuates I/R injury by regulating various signaling pathways. In 2017, Liu *et al* (19) demonstrated that curcumin inhibits nitric oxide (NO) signaling to protect kidney tubules against renal I/R injury. Similarly, curcumin also exhibits positive effects on hepatic I/R injury by suppressing the Toll-like receptor (TLR)4 pathway (20). Furthermore, Kim *et al* (21) suggested that curcumin modulates the TLR2/NF-κB signaling pathway to mitigate cardiomyocyte I/R-induced injury. Additional studies have reported that curcumin acts as a G-quadruplex-specific ligand to regulate telomerase activity, thereby regulating apoptosis (22-24). However, the protective mechanisms underlying the protective effects of curcumin against I/R injury are yet to be fully determined.

Focusing on the regulation of apoptosis, the present study aimed to determine the underlying mechanisms of curcumin on H/R-induced cardiomyocyte injury. Additionally, the role of the Notch signaling pathway in the actions of curcumin on cardiomyocyte injury were investigated.

Materials and methods

Cell culture. H9C2 cells (ATCC® CRL-1446™; American Type Culture Collection) were cultured in 6-well plates ($2x10^4$ cells/well) with Dulbecco's modified Eagle's medium (DMEM; cat. no. D5030; Sigma-Aldrich; Merck KGaA) containing 10% fetal bovine serum (FBS; cat. no. 10099141; Thermo Fisher Scientific, Inc.); cells were maintained at 37˚C in a humidified incubator containing 5% CO_2.

Establishment of the H/R model. According to a previous study (25), H9C2 cells cultured in phosphate-buffered saline (PBS) alone were exposed to low oxygen (95% N_2 + 5% CO_2/O_2) for 4 h in a humidified hypoxia chamber (Stemcell Technologies, Inc.), followed by reoxygenation (0-12 h) in DMEM supplemented with 0.5% FBS under normal culture conditions. Cells were harvested to measure cell viability at 4, 8 and 12 h. Control cells were maintained under normoxic conditions.

Cell viability assay. The viability of H9C2 cardiomyocytes was evaluated using a Cell Counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Briefly, after cells were treated in the aforementioned way, cells were seeded into 96-well plates ($3x10^5$ cells/well) and incubated at 37˚C with 5% CO_2 for 24 h. Subsequently, CCK-8 reagent was added to each well, and cardiomyocytes were cultured at room temperature for 4 h. Absorbance at 450 nm was detected using a microplate reader (Cany Precision Instruments Co., Ltd.).

Determination of cell injury. H9C2 cells were digested with trypsin and collected by centrifugation after washing with PBS. Following centrifugation at 8,000 x g for 10 min at 4˚C, the supernatant was collected for testing. According to the manufacturer's protocols, intracellular lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) activity levels were detected using LDH (cat. no. BC0680), MDA (cat. no. BC0020) and SOD (cat. no. BC0170) assay kits (all Beijing Solarbio Science & Technology Co., Ltd.), respectively.

Effects of curcumin pretreatment on cardiomyocytes subjected to H/R. Curcumin (purity >98%; cat. no. 08511; Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide. H9C2 cardiomyocytes were pretreated at 37˚C for 2 h in a humidified incubator containing 5% CO_2 with different concentrations of curcumin (0, 5, 10 and 20 μM) for 24 h, as previously described (26,27), to determine a non-toxic concentration. The viability of pretreated H9C2 cells was examined using a CCK-8 assay; 10 μM was then selected for use in subsequent experiments.

To determine the effects of curcumin on H/R-induced cardiomyocyte injury, cells were assigned to the following four groups: i) H9C2 cells cultured under normoxic conditions (Control); ii) H9C2 cells cultured with curcumin (10 μM) at 37˚C for 2 h under normoxic conditions (Cur); iii) H9C2 cells subjected to 4 h of hypoxia followed by 8 h of reoxygenation (H/R); and iv) H9C2 cells pretreated with 10 μM curcumin for 2 h, then subjected to 4 h of hypoxia followed by 8 h of reoxygenation (H/R + Cur).

Cardiomyocyte viability was assessed following the various treatments using CCK-8 assays, and indicators of cardiomyocyte injury (LDH, MDA and SOD activity) were measured using the aforementioned assay kits.

Assessment of reactive oxygen species (ROS) and apoptosis. The levels of intracellular ROS were determined using a Reactive Oxygen Species Assay kit (cat. no. S0033; Beyotime Institute of Biotechnology). Trypsinized cells were first collected by centrifugation in the aforementioned manner, and then washed with PBS. Cells were cultured with 10 mM dichlorodihydrofluorescein diacetate at 37˚C for 30 min. Subsequently, cells were harvested and analyzed using a BD FACScalibur flow cytometer (BD Biosciences) and the data was analyzed using Summit Software V4.3 (Dako; Agilent Technologies, Inc.).

Apoptosis was evaluated using an Annexin V-FITC/propidium iodide (PI) staining kit (Thermo Fisher Scientific, Inc.). Briefly, following incubation for 48 h, the cells were trypsinized and collected by centrifugation in the aforementioned manner. Subsequently, the cells were resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene name	Primer sequence
NICD	F: 5'-ATGACTGCCCAGGAAACAAC-3'
	R: 5'-GTCCAGCCATTGACACACAC-3'
Hes-1	F: 5'-CAACACGACACCGGACAAAC-3'
	R: 5'-CGGAGGTGCTTCACTGTCAT-3'
Hes-5	F: 5'-ACATGGCCTTGGCTGTCTGA-3'
	R: 5'-TGCACCCACCCATACAAAGG-3'
Hey-1	F: 5'-GCCGACGAGACCGAATCAAT-3'
	R: 5'-GGAGACCAGGCGAACACGA-3
GAPDH	F: 5'-GACATGCCGCCTGGAGAAAC-3'
	R: 5'-AGCCCAGGATGCCCTTTAGT-3

Hes, hairy and enhancer of split; Hey-1, hairy/enhancer-of-split related with YRPW motif protein 1; NICD, Notch intracellular domain; F, forward; R, reverse.

$CaCl_2$) and cultured with Annexin V (1:20) for 3 min at room temperature. The cells were then stained with PI (20 μg/ml) in the dark for 15 min at room temperature. Cell apoptosis was determined immediately using flow cytometry and the data were analyzed using BD CellQuest™ Pro Software version 1.2 (BD Biosciences).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from H9C2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and purified with RNase-free DNase (Takara Bio, Inc.). RNA aliquots (1 μg) from each sample were reversed transcribed into cDNA using a PrimeScript™ RT reagent kit (Takara Bio, Inc.). The RT reaction was conducted as follows: 65˚C for 5 min, 30˚C for 6 min and 50˚C for 60 min. The cDNA was then used for qPCR. Relative mRNA expression levels were analyzed using an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR-Green kit (Takara Bio, Inc.). All primer sequences are presented in Table I. qPCR amplification was conducted as follows: Denaturation at 94˚C for 2 min, followed by 35 cycles of denaturation at 94˚C for 30 sec, annealing at 63˚C for 30 sec and 72˚C for 1 min, and a final extension step at 72˚C for 7 min. All experiments were conducted in triplicate, and relative expression levels were determined using the $2^{-\Delta\Delta Cq}$ method, using GAPDH for normalization (28).

Western blot analysis. Total protein was extracted from cells using RIPA Lysis Buffer (Beyotime Institute of Biotechnology) and centrifuged at 12,000 x g for 20 min at 4˚C. Protein quantification was performed using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Subsequently, total protein (20 μg) was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked at 37˚C for 1.5 h with 5% non-fat milk in TBS-Tween-20 buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 0.1% Tween-20) prior to incubation with primary antibodies at 4˚C overnight. Primary antibodies against NICD (1:100; cat. no. 4147) and GAPDH (1:1,000; cat. no. 2118) were purchased from Cell Signaling Technology, Inc. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) at room temperature for 1 h. Protein bands were visualized using Pierce™ ECL substrate (cat. no. 32106; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol, and the results were normalized to GAPDH expression. Quantity One software version 4.6.2 (Bio-Rad Laboratories) was used to quantify western blots.

Effects of Jagged1 co-treatment with curcumin on H/R-induced H9C2 cell injury. The recombinant mouse Jagged1 protein was obtained from Abcam (cat. no. ab109346). H9C2 cells were co-treated with Jagged1 (50 ng/l) and curcumin (10 μM) in a humidified incubator with 5% CO_2 at 37˚C. To investigate the effects of curcumin on H/R-induced cardiomyocyte injury, cells were assigned to the following six groups: i) H9C2 cells cultured under normoxic conditions (Control); ii) H9C2 cells subjected to 4 h of hypoxia followed by 8 h of reoxygenation (H/R); iii) H9C2 cells cultured with Jagged1 for 1 h under normoxic conditions (Jagged1); iv) H9C2 cells pretreated with Jagged1 for 1 h and then subjected to 4 h of hypoxia followed by 8 h of reoxygenation (H/R + Jagged1); v) H9C2 cells pretreated with curcumin for 2 h and then subjected to 4 h of hypoxia, followed by 8 h of reoxygenation (H/R + Cur); and vi) H9C2 cells co-treated with Jagged1 and curcumin for 3 h and then subjected to 4 h of hypoxia, followed by 8 h of reoxygenation (H/R + Cur + Jagged1).

The viability of H9C2 cells from each group was evaluated using the CCK-8 assay as aforementioned. Flow cytometry was used to detect the ROS levels and apoptosis of cardiomyocytes. Transcriptional and post-transcriptional levels of NICD were determined via RT-qPCR and western blot analysis, respectively. Altered mRNA expression of downstream genes in the Notch signaling pathway was also investigated.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 6.0 software (GraphPad Software, Inc.). All data are presented as the mean ± standard deviation. Differences were analyzed using one-way analysis of variance followed by Tukey's multiple comparison post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H/R reduces H9C2 cardiomyocyte viability and contributes to cell injury. As shown in Fig. 1A, H9C2 cell viability was decreased as the duration of reoxygenation increased. The viability of cardiomyocytes subjected to 4 h of hypoxia followed by 8 h of reoxygenation was markedly reduced compared with the control group ($P<0.05$). The levels of LDH, an indicator of cardiomyocyte injury, were significantly increased following 8 h of reoxygenation compared with the control (Fig. 1B). MDA content was significantly increased following 4 h of reoxygenation, (Fig. 1C), whereas SOD levels

Table II. Effects of reoxygenation duration on MDA, LDH and SOD levels.

Reoxygenation duration	0 h	4 h	8 h	12 h
LDH (U/l)	50.1±4.7	73.8±5.6	150.5±48.5[a]	164.6±41.6[a]
MDA (U/ml)	0.8±0.1	1.8±0.3[a]	3.1±0.5[a]	3.4±0.5[a]
SOD (U/ml)	174.3±39.6	149.5±38.4	82.1±19.6[a]	64.5±13.2[a]

[a]P<0.01 vs. 0 h. LDH, lactate dehydrogenase; MDA, malondialdehyde; SOD, superoxide dismutase.

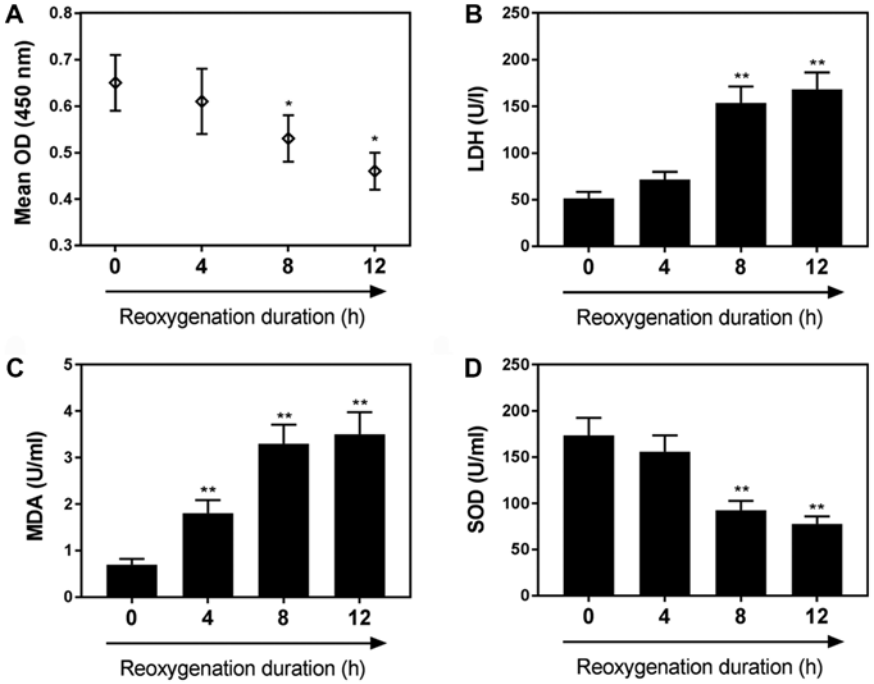

Figure 1. H/R reduces H9C2 cardiomyocyte viability and contributes to cell injury. (A) Effects of H/R on H9C2 cell viability following 0, 4, 8 and 12 h of reoxygenation, as determined by a Cell Counting Kit-8 assay. Effects of H/R on the following markers of cardiomyocyte injury: (B) LDH, (C) MDA and (D) SOD, following 0, 4, 8 and 12 h of reoxygenation. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. 0 h. H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; MDA, malondialdehyde; OD, optical density; SOD, superoxide dismutase.

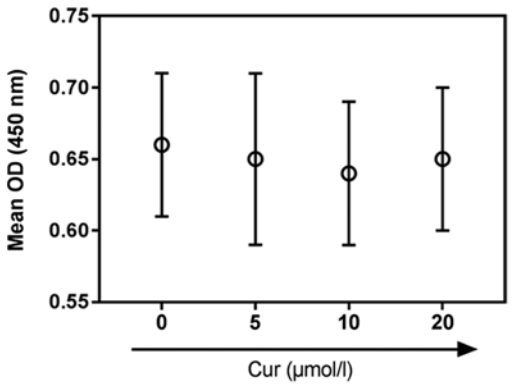

Figure 2. Curcumin does not affect cardiomyocyte viability. Effects of various concentrations of curcumin (0, 5, 10 and 20 μM) on H9C2 cell viability, as determined using a Cell Counting kit-8 assay. Data are presented as the mean ± standard deviation (n=3). Cur, curcumin; OD, optical density.

were significantly decreased following 8 h of reoxygenation, compared with in the control group (P<0.01). Specific values for these assays are presented in Table II.

Curcumin attenuates H/R-induced H9C2 cell apoptosis. Curcumin treatment did not affect H9C2 cell viability under normoxic conditions (Fig. 2). Therefore, 10 μM curcumin was selected for subsequent experiments. As presented in Fig. 3A, curcumin significantly increased the viability of H/R-treated cardiomyocytes compared with H/R treatment alone, whereas it induced no significant effects on the viability of cells cultured under normoxic conditions. Cardiomyocyte injury was induced by H/R treatment, as indicated by the significantly upregulated release of LDH and MDA, and the significantly decreased release of SOD, compared with in the control group. Following curcumin pretreatment, these effects were significantly attenuated compared with in the H/R group (Fig. 3B-D). Additionally, ROS levels were significantly increased in the H/R group compared with in the control group (P<0.01; Fig. 3E). Curcumin significantly reduced the H/R-induced increase in ROS levels (P<0.05). As determined using flow cytometry, the percentage of apoptotic H/R-treated H9C2 cells was 15.13%, which was significantly increased compared with the control group (4.59%; P<0.01); conversely, curcumin pretreatment significantly decreased

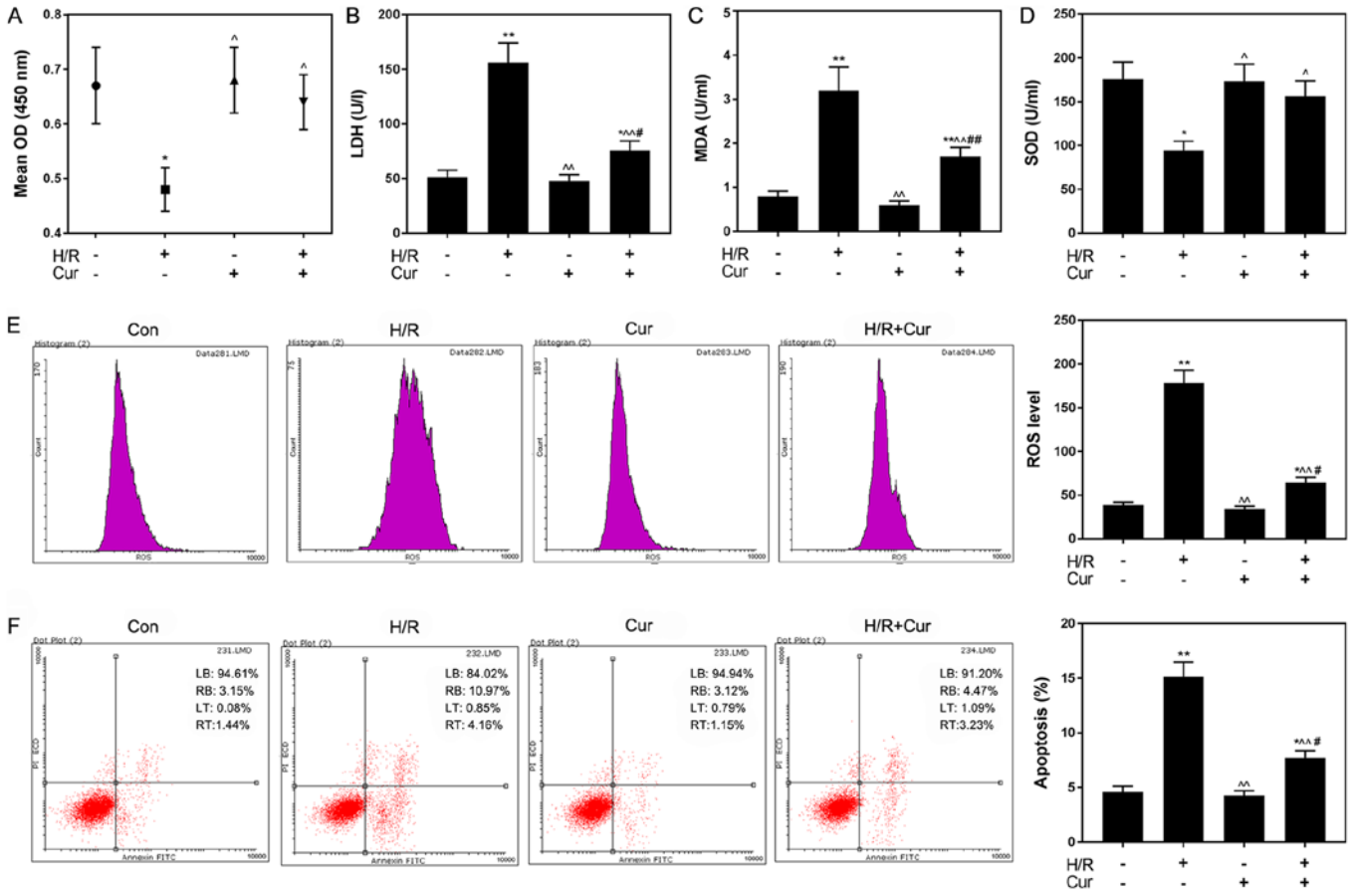

Figure 3. Curcumin attenuates H/R-induced injury and apoptosis in H9C2 cells. (A) Curcumin reversed the inhibitory effects of H/R on H9C2 cell viability. Curcumin pretreatment attenuated the effects of H/R on the levels of (B) LDH, (C) MDA and (D) SOD. Effects of curcumin on H/R-induced increases in (E) ROS levels and (F) apoptosis. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. Con; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. H/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Cur. Con, control (normoxic culture conditions); Cur, curcumin; H/R, hypoxia/reoxygenation; LDH, lactate dehydrogenase; MDA, malondialdehyde; OD, optical density; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase.

the rate of apoptosis in H/R-treated cells to 7.7% (P<0.05; Fig. 3F).

Curcumin pretreatment inhibits the Notch signaling pathway. The levels of NICD, Hes-1, Hes-5 and Hey-1 were measured in H9C2 cells following H/R-induced injury to investigate the effects of curcumin on H/R-induced cardiomyocytes. As presented in Fig. 4A and B, the transcriptional and post-transcriptional levels of NICD were significantly increased following H/R treatment compared with in the control group (P<0.01). Conversely, the expression levels of NICD in the Cur + H/R group were significantly decreased compared with in the H/R group. Additionally, as presented in Fig. 4C, the mRNA expression levels of Hes-1, Hes-5 and Hey-1 following H/R were significantly upregulated compared with in the control group, whereas curcumin pretreatment significantly attenuated this H/R-induced upregulation of genes downstream of the Notch pathway.

Jagged1 suppresses the protective effects of curcumin against H/R-induced H9C2 cell death. Notch signaling was activated using Jagged1 (50 ng/l), and Jagged1 affected the protective effects of curcumin on H/R-induced H9C2 cardiomyocytes. As presented in Fig. 5A, activation of the Notch signaling pathway by Jagged1 partially reversed the protective effects of curcumin on the viability of H/R-damaged cells. Furthermore, it was observed that ROS levels were also significantly increased in the H/R + Cur + Jagged1 group compared with in the H/R + Cur group (P<0.01). Additionally, the apoptotic rate of cells was significantly increased from 7.78% in the H/R + Cur group to 14.21% in the H/R + Cur + Jagged1 group (Fig. 5E).

The increased expression of NICD and downstream genes of the Notch pathway in the Jagged1 group indicated that Jagged1 alone activated Notch signaling (Fig. 6). In addition, NICD, Hes-1, Hes-5 and Hey-1 levels were significantly upregulated in the H/R + Cur + Jagged1 group compared with in the H/R + Cur group (Fig. 6), suggesting that curcumin protected cardiomyocytes from H/R-induced apoptosis by modulating the Notch signaling pathway. A summary of the proposed mechanisms underlying the protective effects of curcumin on H/R injury reported during the present study is presented in Fig. 7.

Discussion

Reperfusion treatment substantially alleviates myocardial ischemia; however, it can lead to irreversible cardiomyocyte apoptosis and even cardiac remodeling, and as a result, MIRI is a leading cause of IHD (29). The prevention of cardiomyocyte apoptosis is hypothesized to be one of the key approaches to

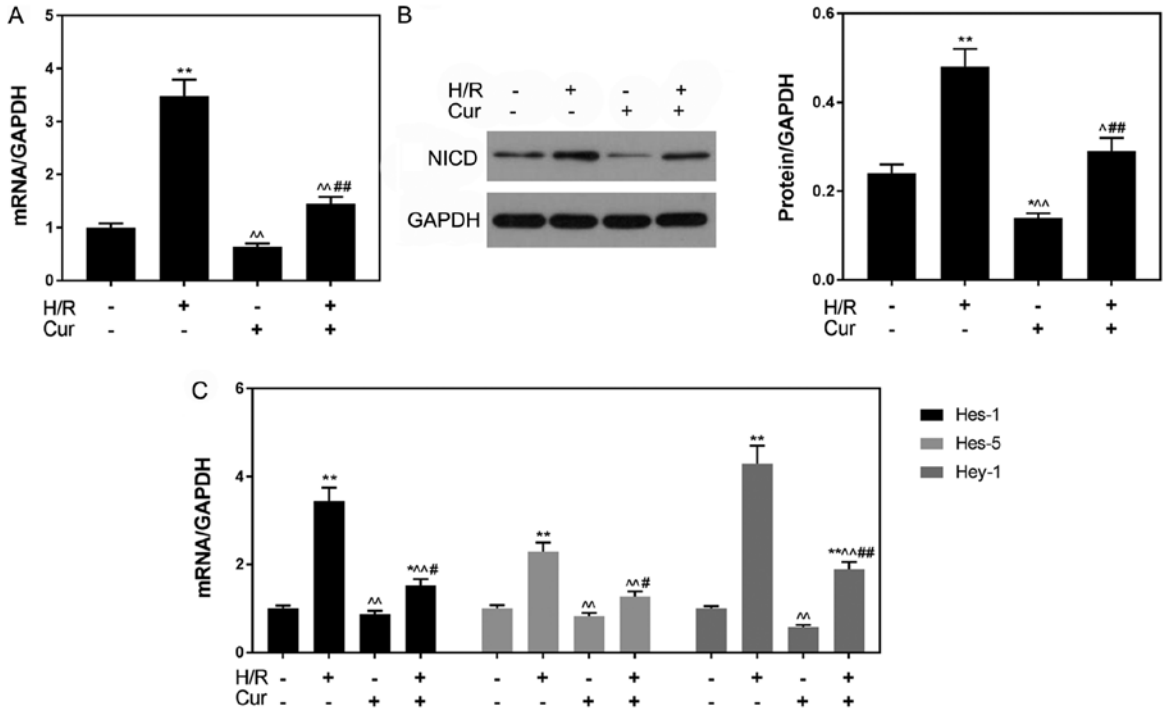

Figure 4. Curcumin pretreatment inhibits the Notch signaling pathway. (A) mRNA and (B) protein expression levels of NICD as determined via RT-qPCR and western blotting, respectively. (C) Expression of downstream genes (Hes-1, Hes-5 and Hey-1) of the Notch pathway as determined via RT-qPCR analysis. GAPDH was used as an internal control. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. Con; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. H/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Cur. Con, control (normoxic culture conditions); Cur, curcumin; H/R, hypoxia/reoxygenation; Hes, hairy and enhancer of split; Hey-1, hairy/enhancer-of-split related with YRPW motif protein 1; NICD, Notch intracellular domain; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

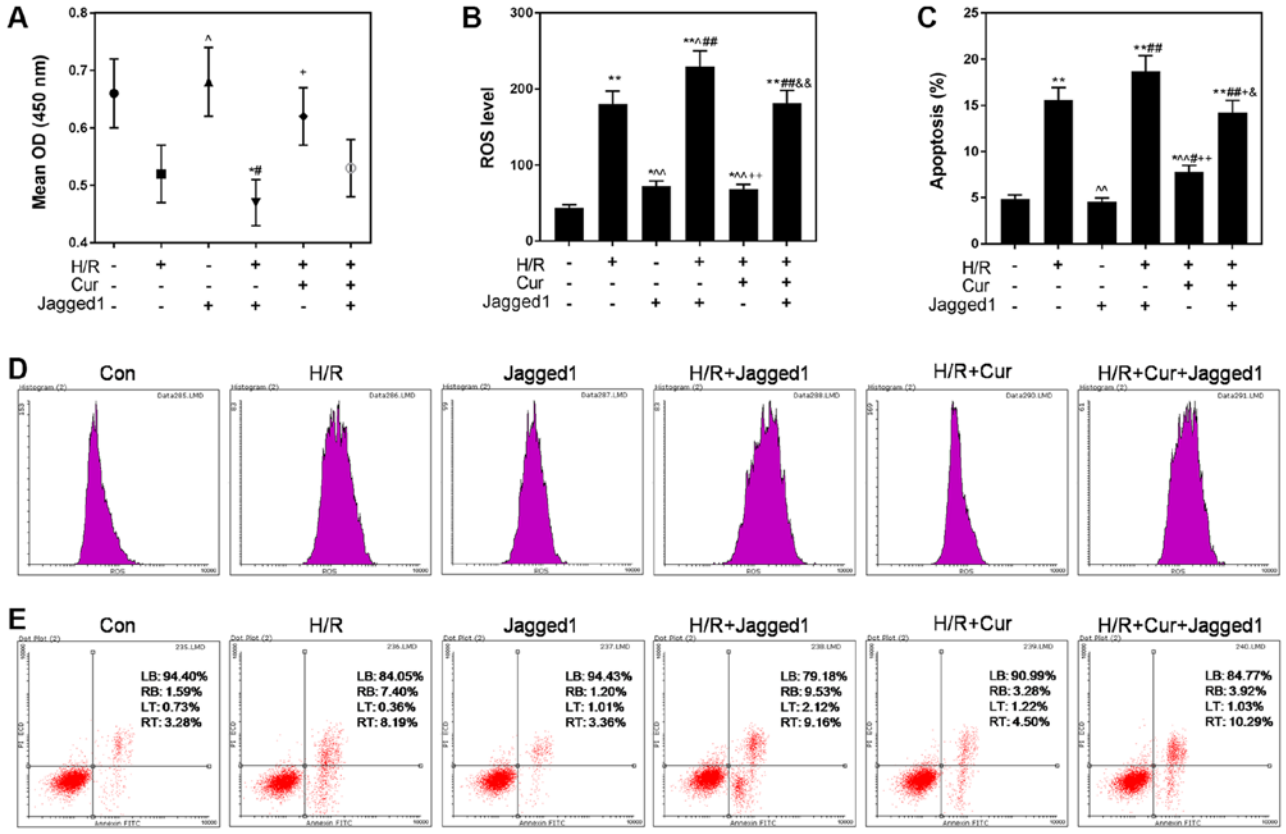

Figure 5. Jagged1 attenuates the protective effects of curcumin against H/R-induced cardiomyocyte cell death. (A) Jagged1 reversed the inhibitory effects of curcumin against H/R-induced reductions in H9C2 cell viability. (B and D) Jagged1 treatment attenuated the downregulation of ROS levels induced by curcumin. (C and E) Jagged1 alleviated the anti-apoptotic effects of curcumin. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. Con; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. H/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Jagged1; $^{+}$P<0.05, $^{++}$P<0.01 vs. H/R + Jagged1; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. H/R + Cur. Con, control (normoxic culture conditions); Cur, curcumin; H/R, hypoxia/reoxygenation; OD, optical density; PI, propidium iodide; ROS, reactive oxygen species.

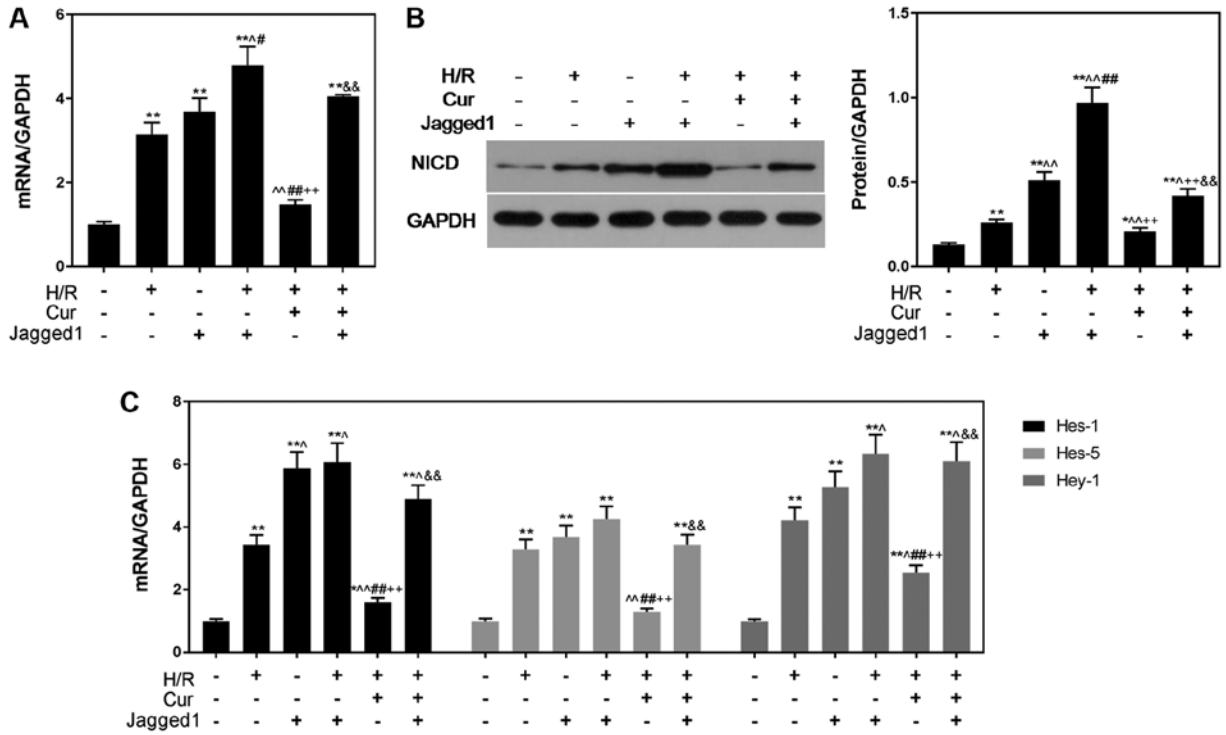

Figure 6. Jagged1 promotes the expression of Notch pathway-associated genes. Jagged1 upregulated the (A) mRNA and (B) protein expression levels of NICD. (C) Expression of downstream genes (Hes-1, Hes-5 and Hey-1) in the Notch pathway was upregulated by Jagged1. GAPDH was used as an internal control. Data are presented as the mean ± standard deviation (n=3). *P<0.05, **P<0.01 vs. Con; $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01 vs. H/R; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Jagged1; $^{++}$P<0.01 vs. H/R + Jagged1; $^{\&\&}$P<0.01 vs. H/R + Cur. Con, control (normoxic culture conditions); Cur, curcumin; H/R, hypoxia/reoxygenation; Hes, hairy and enhancer of split; Hey-1, hairy/enhancer-of-split related with YRPW motif protein 1; NICD, Notch intracellular domain.

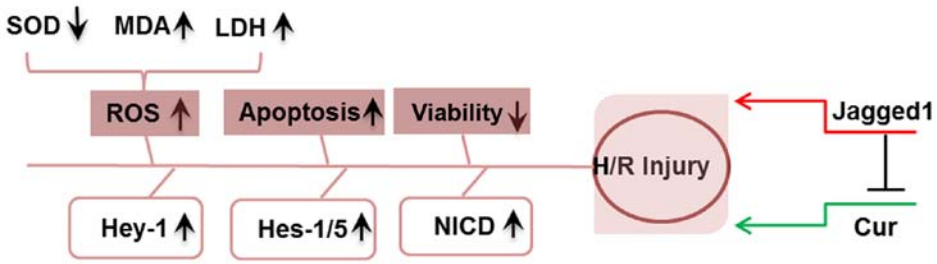

Figure 7. Schematic of the proposed mechanisms underlying the effects of curcumin and Jagged1 reported in the present study. H/R treatment induced cardiomyocyte injury via the elevation of oxidative stress (including ROS production, reduced SOD activity, enhanced MDA content and increased LDH activity), increased apoptosis and decreased cell viability. Curcumin attenuated H/R injury, whereas Jagged1 exacerbated H/R-induced injury and reversed the effects of curcumin. Cur, curcumin; H/R, hypoxia/reoxygenation; Hes, hairy and enhancer of split; Hey-1, hairy/enhancer-of-split related with YRPW motif protein 1; LDH, lactate dehydrogenase; MDA, malondialdehyde; NICD, Notch intracellular domain; ROS, reactive oxygen species; SOD, superoxide dismutase.

preventing MIRI (30). A number of studies have reported that curcumin effectively reduces H/R-induced H9C2 cell injury by modulating various pathways (31-33); however, the mechanisms underlying the protective effects of curcumin on cardiomyocytes remain unclear, and the contribution of the Notch pathway to cardiomyocyte injury induced by H/R is yet to be fully elucidated. In the present study, an important role for the Notch signaling pathway in the protective effects of curcumin against H/R-induced H9C2 cell death was observed. Curcumin significantly decreased cardiomyocyte apoptosis following H/R, potentially by downregulating the Notch pathway.

Firstly, the viability, and the levels of LDH, MDA and SOD were evaluated to determine the effects of curcumin on a cardiomyocyte model of H/R. As biomarkers of lipid peroxidation and oxidative stress, LDH, MDA and SOD levels are closely associated with the degree of cell injury (34). In the present study, LDH and MDA contents progressively increased as the duration of reoxygenation was increased, suggesting that the injury of H9C2 cells, and oxidative stress and lipid peroxidation, were aggravated as the period of reoxygenation was extended. Previous studies have suggested that curcumin protects cardiomyocytes against MIRI by inhibiting the leakage of LDH, enhancing SOD activity and reducing MDA production (33,35). These results were consistent with the present study, in which curcumin modulated the levels of LDH, MDA and SOD to improve H/R-induced oxidative stress and increase antioxidative activity in H9C2 cells. In addition, MIRI promotes the production of ROS, elevated levels of which are sufficiently toxic to damage all cellular components (36); however, the present findings revealed that

curcumin treatment significantly decreased H/R-induced ROS production, reducing the toxic effects of ROS on cardiomyocytes. It has been reported that MIRI induces cardiomyocyte apoptosis via a number of apoptotic pathways, including the Janus kinase 2/STAT3 and TLR4/NF-κB pathways (10,20,37). In the present study, curcumin pretreatment reduced the H/R-induced apoptosis of H9C2 cells. Collectively, the present findings demonstrated the positive effects of curcumin on H9C2 cardiomyocyte apoptosis induced by H/R.

Furthermore, to investigate the potential role of the Notch pathway in MIRI, the expression levels of NICD and certain downstream genes were determined. Previous studies have demonstrated that the Notch signaling pathway serves a positive role in inhibiting MIRI, protecting cardiomyocytes against H/R damage (38-40). Additionally, certain medicines exert inhibitory effects on MIRI via the Notch signaling pathway; for example, relaxin protects myocardial cells against H/R injury by upregulating Notch1 signaling (41). Conversely, opposing findings were reported in the present study, as it was observed that the expression levels of NICD, Hes-1, Hes-5 and Hey-1 were significantly increased in response to H/R treatment. Following curcumin treatment, the expression levels of Notch pathway genes were downregulated, as was H/R-induced cardiomyocyte apoptosis. Therefore, it was hypothesized that Notch pathway activation promoted H/R-induced cardiomyocyte injury. To test this hypothesis, Jagged1 protein, a ligand that interacts with four receptors in the Notch pathway, was used to reactivate the Notch1 receptor and NICD release following curcumin treatment. Notably, it was observed that ROS levels and the apoptotic rate were significantly increased following Jagged1 treatment, and that the mRNA expression levels of Hes-1, Hes-5 and Hey-1 were also significantly unregulated. These results were consistent with the hypothesis that the Notch pathway not only contributed to H/R-induced H9C2 cardiomyocyte apoptosis, but also exacerbated H/R-induced injury. It has been reported that Jagged1 is involved in cell injury (42-44). Therefore, the role of Jagged1 in H/R-induced H9C2 cardiomyocyte injury, and the involvement of Notch signaling in the effects of curcumin were investigated. The results revealed that curcumin possessed the ability to suppress H/R-induced H9C2 cell apoptosis, and that Jagged1 attenuated the inhibitory effects of curcumin by activating the Notch pathway. Therefore, curcumin and Jagged1 induced opposing effects on apoptotic processes in H9C2 cells. Previous studies have also indicated that the ligand Jagged2 exhibits anti-apoptotic effects (45,46). In addition, it was previously reported that inhibition of the Delta1/Notch1 pathway affects apoptosis (47). Pelullo *et al* (48) revealed that Jagged1 potentially contributes to the occurrence of acute lymphoblastic leukemia via Notch3. The exact mechanisms underlying the interaction/competition between the two molecules were not investigated in the present study. In addition, various signaling pathways, including the NO (49), TLR4 (50) and TLR/NF-κB signaling pathways (51) have also been reported to be involved in the regulation of IR injury; however, the involvement of these pathways in the effects of curcumin or Jagged1 were not investigated in the present study.

In conclusion, the present study revealed that H/R injury activated the Notch signaling pathway, and induced H9C2 cardiomyocyte injury and apoptosis; however, *in vivo* experiments are required to validate these findings. Additionally, curcumin not only inhibited the association between H/R injury and Notch signaling by inhibiting NICD expression, but also reduced the H/R-induced apoptosis of H9C2 cells. Furthermore, Jagged1 recombinant protein treatment further suggested that the Notch pathway contributed to H/R damage in cardiomyocytes. These findings provide improved understanding of the mechanisms via which curcumin affects H/R-induced cardiomyocyte apoptosis.

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Availability of data and materials

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

Authors' contributions

PZ and MY made substantial contributions to the conception and design of the study. HH, ML, SL, ZC, CS, ZK, AL and QW performed the acquisition and analysis of data, and interpreted the data. PZ and MY drafted the manuscript. All authors approved of the final version of the manuscript to be published. All authors agreed 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.

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