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# Curcumin attenuates myocardial ischemia and reperfusion-induced proinflammatory response through activation of the Nrf-2/HO-1 signaling pathway

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## Abstract

Acute myocardial infarction (MI) is a global issue with high incidence and mortality. Rapid recovery of the blocked coronary flow represents the most effective strategy to reduce the size of myocardial infarction and improve the cardiac function. The aim of the study is to investigate the effects of curcumin in attenuates myocardial ischemia and reperfusion-induced proinflammatory response through activation of the Nrf-2/HO-1 signaling pathway. Wild-type C57BL/6J mice (male, 8–10 weeks old) were used and murine myocardial ischemia and reperfusion injury (IRI) model was conducted, cardiac function was evaluated by echocardiography. Infarct size measurement, cardiac troponin-I assays, oxidative stress analysis and histopathological analyzed. The resulted data showed that curcumin alleviates myocardial inflammatory responses and oxidative stress during myocardial IRI. Also, curcumin reduces cell apoptosis induced by myocardial IRI, curcumin reduces cell apoptosis induced by myocardial IRI and activates the Nrf-2/HO-1 signaling pathway during myocardial. Inconclusion, this study demonstrates that curcumin attenuates myocardial IRI by inhibiting proinflammatory cytokines through a mechanism that may be related to activation of the Nrf-2/HO-1 signaling pathway.

**Keywords**: Myocardial IRI, Nrf-2/HO-1, Curcumin

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## Introduction

Acute myocardial infarction (MI) is a global issue with high incidence and mortality [1]. Rapid recovery of the blocked coronary flow represents the most effective strategy to reduce the size of myocardial infarction and improve the cardiac function [2]. However, reperfusion led to irreversible myocardial injury [3], which is known as myocardial ischemia-reperfusion (IR) injury [4]. Myocardial IR exacerbates the cardiac dysfunction and increases the incidence of

adverse prognosis. However, the mechanism of IR injury remains elusive, and effective treatments are still lacking [5].

Transcardiac inflammation has already been described in some clinical situations of reperfusion, particularly balloon angioplasty under stable [5] or post-infarction conditions [6]. There even seems to be a causal link between acute inflammatory responses during reperfusion (formation of leukocyte–platelet aggregates) and the occurrence of restenosis [7].

Curcumin (diferuloylmethane), a yellow pigment from Curcuma longa, is a major component of turmeric (also called curry powder) and is commonly used as a spice and food-coloring agent. It has been known to mitigate I/R or cytotoxic injury in various tissues including the heart [8], through its anti-inflammatory [9], and antioxidative properties. Curcumin has also been shown to reduce the phosphorylation of JNK, p38-MAPKs, signal transducer and activator of transcription 3 (STAT-3), and subsequent downstream signals in human endothelial cells in vitro [10]. Nevertheless, the activity of the antiapoptotic kinases and its balance with proapoptotic proteins under curcumin have not been determined [11]. Therefore, the present study was aimed to determine the effect of curcumin in regional myocardial I/R injury and, if any, its underlying mechanisms involving the role of prosurvival kinases such as PI3K-Akt, extracellular signal-regulated kinase (ERK1/2) and subsequent downstream signaling pathway glycogen synthase kinase (GSK-3β), and apoptotic kinases such as p38 and JNK [12].

Heme oxygenase-1 (HMOX1, HO-1) is a target gene of nuclear factor erythroid 2-related factor 2 (Nrf2), which has been shown to protect against a variety of pathologies including sepsis, hypertension, and atherosclerosis [13]. HO-1 is further responsible for catalyzing the disintegration of heme into the antioxidant biliverdin, which is an anti-inflammatory agent, carbon monoxide, and iron [14]. Metabolites such as ferric iron, CO, and bilirubin are known to have antioxidative, anti-inflammatory, and anti-atherogenic properties [15]. The HO-1 deficiency model is related to endothelial cell injury, weakness, stress, ischemia, and growth retardation. In other studies, experimental HO-1 gene delivery has been shown to alleviate atherosclerosis [16], vascular neointima formation [17], ischemic heart injury [18], and vascular dysfunction [19].

## Materials and Methods

### Animals

Wild-type C57BL/6J mice (male, 8–10 weeks old) were purchased from SLAC Laboratory Animal co., Ltd. (Shanghai, China). Mice were kept in the specific-pathogen-free (SPF) room with constant temperature (23-24°C), humidity (55 ± 5%) and light (12 h light-dark cycle). All

the mice had free access to food and water. The in vivo manipulations were approved by the Institutional Animal Care and Use Committee. Computer-generated random numbers were used for random grouping in the present study.

Myocardial IR model

Mice myocardial IR model was conducted according to our previous study [20]. Mice in GW4869 group received intraperitoneal injection of GW4869 (2.5 mg/kg, Sigma-Aldrich) 1 h before IR surgery. After anesthetizing with 1.5% pentobarbital (50 mg/kg body weight, Sigma-Aldrich), chest cavity was opened at the 4th intercostal space under mechanical ventilation. The left anterior descending (LAD) coronary artery was ligated for 45 min with an 8-0 silk suture. Once removing the silk suture, intracardiac injection of 50μL IR-EVs (2×108/μL) or PBS was performed through the left ventricle. After closing thoracic incision, the mice were placed on a heated blanket until recovery from anesthesia.

The experimental mice were divided into 4 groups: (1) control group without any drugs; (2) curcumin group received curcumin 100 mg/kg; (3) group received ML-385 (inhibitor of Nrf-2/HO-1) 1 mg/kg; (4) ML-385 (inhibitor of Nrf-2/HO-1) 1 mg/kg plus curcumin; All study drugs or vehicle were administered 20 minutes before LAD occlusion.

The dosage of curcumin (Sigma, St Louis, Missouri; 100 mg/kg) was adopted, since it was shown to attenuate myocardial I/R in the mice, and the time point of its administration was adopted, since it reaches its peak plasma level within 15 minutes after IP administration. Curcumin was dissolved in dimethyl sulfoxide (DMSO) and administered IP.

Echocardiography

Cardiac function was evaluated by echocardiography (VisualSonics, Canada). The mice were anesthetized with isoflurane after depilation. The left ventricular end ditroponinolic diameter (LVEDD) and left ventricular end systolic diameter (LVESD) were measured in two-dimensional long axis views. The left ventricular ejection fraction (EF), fraction shortening (FS), left ventricular mass, and left ventricular contraction volume were calculated for cardiac function assessment.

Infarct size measurement

C57BL/6J mice were anesthetized, intubated, and ventilated with a respirator (Harvard apparatus, Holliston, MA) as described [21-25]. After left lateral thoracotomy, the left anterior descending coronary artery (LAD) was occluded for 20 min with 8-0 nylon suture and polyethylene tubing to prevent arterial injury and reperfused for up to 3h. Vehicle (Hepes buffer) or AT was administered via the tail vein injection 5 min before reperfusion. The ECGs confirmed the ischemic hallmark ST-segment elevation during coronary occlusion (AD

Instruments, Colorado Springs, CO). Cardiectomy was performed at the conclusion of reperfusion. Left ventricular ischemic regions were isolated prior to freeze clamping in liquid nitrogen. Sham refers to same surgical procedures without occlusion.

For infarct size measurement, hearts were reperfused for 3h, and then excised for dual staining. The non-necrotic tissue in the ischemic region was stained red with 2, 3, 5-triphenyltetrazolium (TTC), and non-ischemic regions were stained blue with Evans blue dye. The hearts were fixed and sectioned into 1 mm slices, photographed utilizing a Lexica MZ95 microscope, and analyzed by NIH Image analysis software [26]. The myocardial infarct size was calculated as the ratio of the percentage of myocardial necrosis to the ischemic area at risk (AAR).

Cardiac troponin-I assays

Mice were completely exsanguinated after 20 min of ischemia and 3h of reperfusion. Serum levels of the cardiac-specific isoform of troponin-I were assessed using an ELISA kit (Life Diagnostics (West Chester, PA) according to the manufacturer's

Cytokine analysis

ELISA kits provided by Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) were employed to measure serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels according to the instructions provided by the manufacturer of the kits.

Oxidative stress analysis

A colorimetric assay kit (Nanjing Jiancheng Biology Research Institute, Nanjing, China) was used to measure the levels of superoxide dismutase (SOD) and malondialdehyde (MDA) in cardiac tissue according to the instructions provided with the kit.

Histopathological analysis

Paraformaldehyde (4%) was used to fix heart samples for paraffin embedding. The embedded tissues were cut into 5-μm-thick sections that were then stained with hematoxylin and eosin (H&E). Suzuki's score was adopted to evaluate the level of cardiac tissue damage [27].

Western blotting analysis

Total protein was extracted from heart tissues in RIPA lysis buffer (Thermo Scientific, Rockford, USA) containing protease inhibitors, and a Bradford assay was used to determine the protein concentration. Equal amounts of total protein were separated by 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by transfer to

polyvinylidene difluoride membranes. After 2 h of blocking at room temperature with 5% skimmed milk powder in TBST, the membranes were incubated overnight with primary antibodies at 4°C. The following rabbit polyclonal antibodies were used: anti-Nrf-2 (1:1000), anti-HO-1 (1:500), anti-Bcl-2 (1:1000), anti-Bax (1:5000), anti-caspase-3 (1:500) and anti-GAPDH (1:5000). After three washes in TBST, the membranes were incubated for 2 h with a horseradish peroxidase-conjugated secondary antibody (1:5000) at room temperature. All the above antibodies were purchased from Proteintech Group (Manchester, UK). An ECL chemiluminescence system (Bio–Rad, USA) was used to visualize the protein expression levels, which were then measured by densitometry using ImageJ software (NIH, Bethesda, MD, USA).

Immunofluorescence staining

Immunofluorescence was performed as previously described [28]. Briefly, the sections were incubated with an antibody against MPO (1:200, Servicebio, Wuhan, China), washed, and incubated with Alexa Fluor 555-conjugated secondary antibody (1:200, Servicebio, Wuhan, China). Finally, the slides were counterstained with DAPI and analyzed by fluorescence microscopy (Olympus, Japan).

Histological evaluation

Following I/R, the hearts were perfused with relaxation buffer (25 mM KCl and 5% dextrose in PBS) with heparin to wash out blood. The hearts were removed, fixed in 10% formalin, and embedded in paraffin. Paraffin-embedded myocardial sections (5 μm), stained with hematoxylin and eosin, were examined by light microscopy. To demonstrate neutrophils within the myocardium of the I/R region, paraffin-embedded myocardial sections from vehicle, I/R and I/R plus AT mice were treated with Leder stain (fuchsin acid, sodium nitrite, and naphthol AS-D chloroacetate esterase in PBS), which identifies chloroacetate esterase within neutrophils, and examined by light microscopy [29].

## Results

### Curcumin attenuates myocardial IRI

To determine whether pretreatment with Curcumin protects against cardiac IRI, the serum levels of cytokine and troponin were measured. As shown in Figures 1(b) and (c), the IRI group showed significantly higher cytokine and troponin levels than the sham group. The animals that were pretreated with Curcumin showed a marked attenuation of the increase in cardiac enzymes that was observed in the animals in the IRI group. Consistent with the biochemical markers, H&E staining showed that compared with the control group, the heart

in mice in the IRI group displayed severe sinusoidal congestion related to marked cardiac vacuolation and degeneration and obvious increases in Suzuki scores. Furthermore, compared to the IRI group, Curcumin pretreatment obviously reduced cardiac injury and Suzuki score (Figures 1(d) and (e)). These results indicate that Curcumin protects against cardiac IRI.

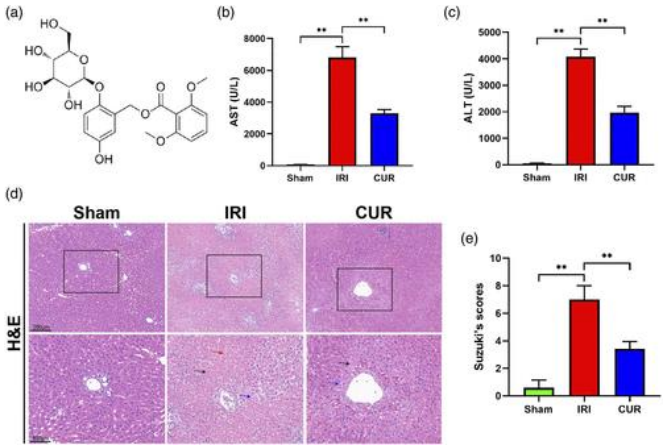


**Figure 1.**

Curcumin exhibits protective effects against cardiac IRI. Representative images of H&E-stained heart tissues. Scale bar = 200 μm (upper panels) and 100 μm (lower panels). All data are presented as mean ± SD (n = 8 per group). *p < .05 and **p <.01.

**Curcumin alleviates cardiac oxidative stress and inflammatory response induced by IRI**

During cardiac IRI, oxidative stress and the inflammatory response are two primary detrimental events that contribute to myocadiac cell death. We sought to assess whether Curcumin affects this process. As shown in Figures 2(a) and (b), the SOD level in the cardiac tissue of the animals in the IRI group was greatly decreased, and the MDA concentration was greatly increased, compared to that in the sham group. The CUR group showed a lower level of SOD and a higher MDA concentration than the IRI group. In addition, the IRI group showed greatly increased levels of expression of TNF-α and IL-6 compared with the sham group, whereas both of these proinflammatory cytokines were expressed at lower levels in the CUR group than in the IRI group (Figures 2(c) and (d)). Cardiac neutrophil infiltration was detected by MPO staining assays. As shown in Figures 2(e) and (f), mice pretreated with Curcumin showed greatly reduced cardiac infiltration by neutrophils compared with IRI controls. These outcomes indicate that oxidative stress and the inflammatory response are alleviated by Curcumin during cardiac IRI.

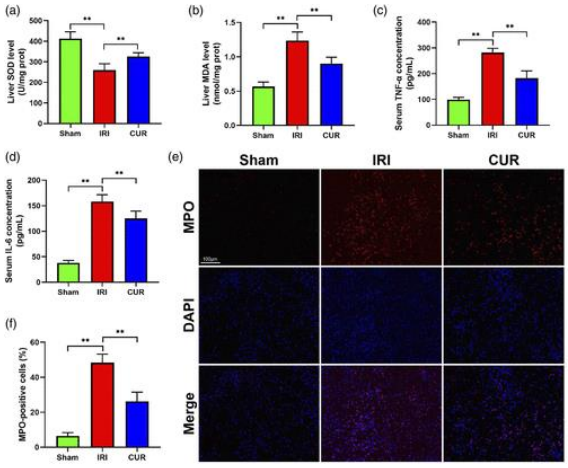


**Figure 2.**

Curcumin alleviates myocardial inflammatory responses and oxidative stress during myocardial IRI. (a–b) Concentration of SOD (a) and MDA (b) in heart tissue. (c–d) Levels of the proinflammatory factors TNF-α (c) and IL-6 (d) in serum. (e) Representative images showing immunofluorescence staining (MPO staining) in heart sections. Scale bar=100 μm. (f) Quantitation of MPO-positive cells. All data are presented as mean ± SD ($n$ = 8 per group). $^{*}p < .05$ and $^{**}p < .01$.

**Curcumin protects the heart from cell apoptosis induced by IRI**

Apoptosis induces cardiac death as a vital mechanism during heart IRI. myocardial apoptosis was examined by measuring the levels of TUNEL, cleaved caspase-3, Bax, and Bcl-2 to investigate whether Curcumin protects against IRI-induced cardiac apoptosis.

As shown in Figures 3(a)-(d), cardiac IRI significantly increased the expression of the pro-cell death proteins cleaved caspase-3 and Bax and decreased the expression of the anti-apoptosis protein Bcl-2 in heart tissue compared to the sham group, and these effects were sharply reversed after Curcumin pretreatment. Moreover, the number of TUNEL-positive cells was greatly increased in the IRI group compared with the sham group; nevertheless, the number of TUNEL-positive cells (Figures 3(e) and (f)) was markedly reduced after Curcumin pretreatment. These findings indicate that Curcumin reduces cell apoptosis resulting from cardiac IRI.

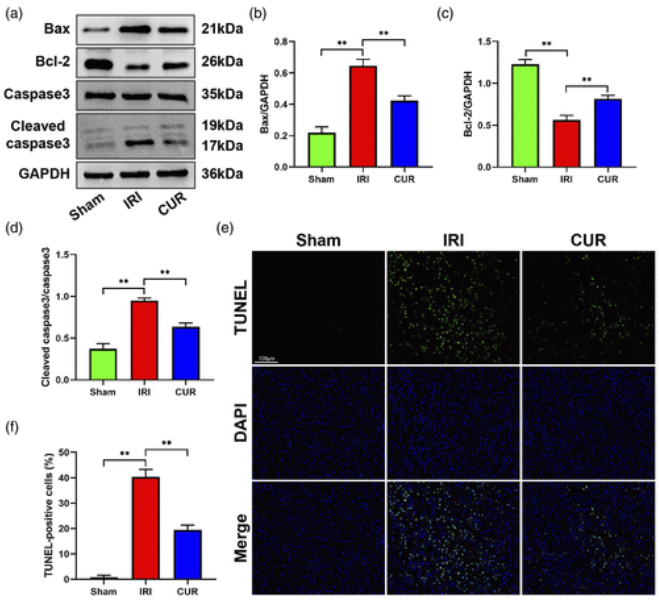


**Figure 3.**

Curcumin reduces cell apoptosis induced by cardiac IRI. (a) Western blot analysis of Bax, Bcl-2, caspase-3, and cleaved caspase-3 expression (GAPDH served as an internal control). (b–d) Quantification of Bax (b), Bcl-2 (c), and cleaved caspase-3 (d) expression. (e) Representative images of TUNEL staining in heart sections. Scale bar=100 μm. (f) Quantitation of TUNEL-positive cells. The experiments were repeated three times. All data are presented as mean ± SD ($n$ = 8 per group). $^{*}p <$ .05 and $^{**}p <$ .01.

### Curcumin induces Nrf-2/HO-1 activation during cardiac IRI

The finding that Curcumin plays a significant protective role against cardiac IRI led us to investigate the mechanism underlying this effect. Previous studies have shown that the Nrf-2/HO-1 signaling pathway exerts a significant regulatory effect on both inflammatory responses and cell apoptosis during cardiac IRI. Therefore, we analyzed the activation of Nrf-2 and HO-1 at the protein and mRNA levels. As shown in Figures 4(a)-(e), Nrf-2 and HO-1 expression was decreased in heart tissues after cardiac IRI, and Curcumin pretreatment greatly increased the expression of Nrf-2 and HO-1. Moreover, ML-385, a specific Nrf-2 inhibitor, significantly decreased the expression of Nrf-2 and HO-1, and its inhibitory effect was partially neutralized by pretreatment with Curcumin. These results suggest that activation of the Nrf-2/HO-1 signaling pathway may be necessary for Curcumin to function.

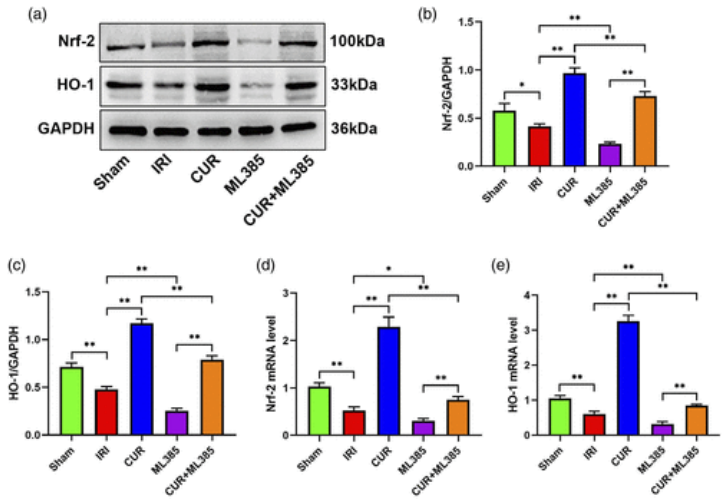


**Figure 4.**

Curcumin activates the Nrf-2/HO-1 signaling pathway during cardiac IRI. (a) Western blot analysis of Nrf-2 and HO-1 expression (GAPDH served as an internal control). (b–c) Quantification of Nrf-2 (b) and HO-1 (c) expression. (d–e) Relative mRNA expression of Nrf-2 (d) and HO-1 (e). The experiments were repeated at three times. All data are presented as mean ± SD ($n$ = 8 per group). $^{*}p < .05$ and $^{**}p < .01$.

**Curcumin attenuates cardiac IRI through activation of the Nrf-2/HO-1 pathway**

Based on the results presented above, it was speculated that Curcumin acts in a protective manner in cardiac IRI by activating the Nrf-2/HO-1 signaling pathway. To test this speculation, we blocked Nrf-2/HO-1 signaling using ML-385. Figures 5(a)-(f) show that heart injury was greatly aggravated in the CUR+ML-385 group compared with the CUR group, as evidenced by higher troponin, cytokine, and TUNEL-positive cell levels and Suzuki scores in the former. These results demonstrate that ML-385 pretreatment counteracts the protective effects of Curcumin against heart damage. Moreover, the Curcumin-induced increase in SOD levels and the decrease in MDA levels were reversed by ML-385 (Figures 5(g) and (h)), and inhibition of the release of TNF-α and IL-6 and neutrophil recruitment by Curcumin was nullified by ML-385 (Figures 5(i-l)). Collectively, these results indicate that Curcumin plays a protective role in cardiac IRI by activating the Nrf-2/HO-1 pathway.

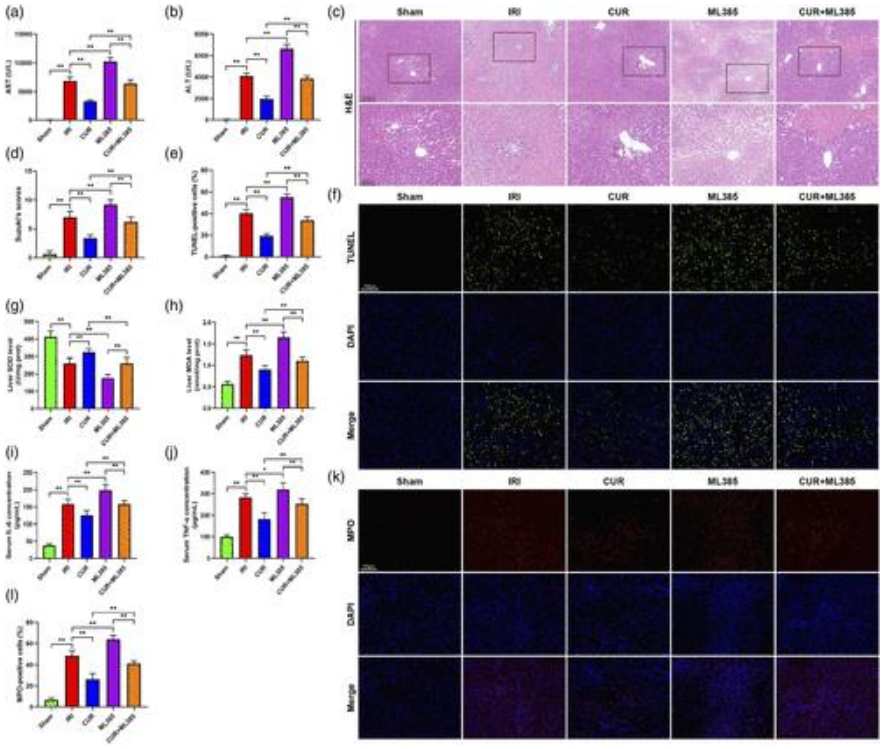


**Figure 5.**

Curcumin protects the heart from IRI by activating the Nrf-2/HO-1 pathway. (a–b) Serum troponin (a) and cytokine (b) levels in each group. (c) Typical images used in the histological analysis of H&E-stained heart tissues. Scale bar= 200 μm (upper panels) and 100 μm (lower panels). (d) The degree of cardiac injury was scored based on Suzuki scores. (e) TUNEL-positive cells were quantified. (f) Representative images showing TUNEL staining in heart sections. Scale bar=100 μm. (g–h) Concentrations of SOD (g) and MDA (h) in heart tissue. (i–j) Levels of the proinflammatory factors IL-6 (i) and TNF-α (j) in serum. (k) Representative images obtained by immunofluorescence staining (MPO staining) of heart sections. Scale bar= 100 μm. (L) Quantification of MPO-positive cells. All data are presented as mean ± SD ($n$ = 8 per group). $^{*}p < .05$ and $^{**}p < .01$.

## Discussion

Inflammation and heart diseases are strongly connected and mutually reinforce each other. Ischemic heart injury provokes sterile inflammation in the heart itself. Long-term or excessive inflammatory response aggravates heart injury after acute MI [28]. This points out the need to control the inflammatory process at an early stage to avoid the persistent inflammation and heart injury. Release of damage associated molecular patterns (DAMPs) by stressed, malfunctioning, or necrotic cells during IR is widely known to provoke sterile inflammation and recruitment of immune cells [29].

The present study demonstrates that Curcumin exerts a protective effect on myocardial ischemia and reperfusion by reducing heart necrosis, alleviating oxidative stress, and

inhibiting inflammation and that the mechanism underlying these effects may be activation of the Nrf-2/HO-1 signaling pathway [30].

During myocardial ischemia and reperfusion, oxidative stress occurs because of a disturbed balance between the antioxidant defense system and ROS generation. Lipid peroxidation and the production of extremely aggressive oxidants such as MDA are promoted by increased levels of ROS, finally resulting in myocytes damage and even death. SOD is the major enzyme in the mitochondrial matrix that defends cells against damage caused by superoxide anions; by catalyzing the oxidation of ROS into inactive substances and oxygen, it protects cells against damage caused by IRI-induced ROS generation [31].

Protection against myocardial ischemia and reperfusion by treatment with antioxidants might regulate the overall protective mechanism that acts to reduce the initiation and progression of myocardial ischemia and reperfusion-induced effects. Previous studies have shown that Curcumin acts as a powerful antioxidant through its ability to scavenge superoxide radicals in cell-free systems by decreasing the amount of LDH and MDA and by increasing nitrite levels and GSH-Px activity in H2O2-treated human umbilical vein endothelial cells (HUVECs) [32].

Consistent with previous findings, the results of our experiments suggest that Curcumin pretreatment increases SOD activity and reduces MDA concentrations during myocardial ischemia and reperfusion. Thus, Curcumin could alleviate oxidative stress-mediated injury during myocardial ischemia and reperfusion [33].

Cytokine though the mounting of a moderate inflammatory response can defend the body against alien invaders, an excessive inflammatory response may cause much more damage than the injury itself [34]. The increased production of cytokines, including TNF-α and IL-6, is a major complication in the inflammatory cascade during myocardial ischemia and reperfusion, and these cytokines are thought to be the main mediators of inflammation that causes cardiac injury by promoting the recruitment of neutrophils [35]. Curcumin protects against osteoporosis by inhibiting the expression of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and COX-2 [36]. Moreover, Curcumin exerts a neuroprotective effect by abrogating HMGB1 release and NF-κB activation activated by TNF-α and ameliorates MCAO-induced cerebral IRI [37].

The current research demonstrates that pretreatment with Curcumin markedly decreases the release of TNF-α and IL-6 and the neutrophil recruitment induced by myocardial ischemia and reperfusion, indicating that Curcumin attenuates the inflammatory responses that normally occur during myocardial ischemia and reperfusion [38].

As a highly regulated process of programmed cell death, apoptosis is mediated by various proteins, including members of the caspase and Bcl-2 protein families [39]. During cardiac

IRI, some myocardial cells experience cell death in the form of apoptosis after ischemic insult, and pharmacological interventions that block apoptosis have been shown to be protective against reperfusion injury [40]. It has been reported that Curcumin protects neurons from N-methyl-D-aspartate-induced excitotoxicity by inhibiting cell death and apoptosis.38 Moreover, Curcumin protects HUVECs from H2O2-induced apoptosis by decreasing caspase-3 activation [41]. Similarly, the results of the present work showed that treatment with Curcumin effectively reduced the expression of Bax, the activation of cleaved caspase-3, and the number of TUNEL-positive cells during myocardial ischemia and reperfusion while increasing the expression of Bcl-2, indicating that Curcumin reduced cell apoptosis resulting from myocardial IRI [42].

To investigate the possible mechanisms underlying the protective effects of Curcumin against myocardial IRI, the expression levels of Nrf-2 and HO-1 in heart tissues were measured [43]. It is generally known that the Nrf-2/HO-1 pathway enhances the cellular defense system that counteracts oxidative injury and inflammation. There is increasing evidence that the Nrf-2/HO-1 pathway has a protective effect against myocardial ischemia and reperfusion. Other showed that inflammation, oxidative stress, and cell apoptosis during myocardial ischemia and reperfusion were aggravated by depletion of Nrf-2 in mice, while activation of Nrf-2 greatly alleviated cardiac damage [44]. Moreover, other reported that sulforaphane alleviates myocardial ischemia and reperfusion by boosting the activation of Nrf-2/HO-1 signaling. Importantly, Curcumin mitigates oxidative stress by activating Nrf-2 and inhibiting the NF-κB pathway. The current research shows that Nrf-2 and HO-1 were downregulated in myocardial ischemia and reperfusion model mice and that pretreatment of the animals with Curcumin rescued the decreased levels of Nrf-2 and HO-1.

## Conclusion

This study demonstrates that Curcumin attenuates myocardial ischemia and reperfusion by inhibiting proinflammatory cytokines through a mechanism that may be related to activation of the Nrf-2/HO-1 signaling pathway.

## Ethical Approval

The study was approved by the Ethical Committee.

## Conflicts of Interest

The authors declare that they have no competing interests.

**Authors' Contributions**

All authors shared in conception, design of the study, acquisition of data, and manuscript writing, the critical revising and final approval of the version to be published.

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