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ORIGINAL RESEARCH

Mechanisms of Hydromorphone-Mediated Protection Against Myocardial Ischemia-Reperfusion Injury via NLRP3 Inflammasome Inhibition

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Background: Myocardial ischaemia/reperfusion, MI/R injury causes significant cardiac damage, leading to disability and mortality. Although hydromorphone attenuates MI/R injury in rat models, its underlying mechanisms remain unclear.

Objective: This study aimed to investigate the protective effects of hydromorphone against MI/R injury and elucidate its mechanisms.

Methods: A rat MIRI model was established by occluding the left coronary artery for 30 minutes followed by reperfusion for 120 minutes. Infarct size and cardiac function were assessed using hematoxylin-eosin, HE and Masson's staining. An in vitro model was established using H9C2 cells subjected to hypoxia/reoxygenation, and levels of cellular pyroptosis and pyroptosis-related proteins were quantified.

Results: Hydromorphone significantly improved cardiac function in MIRI rats. Pre- and post-treatment with hydromorphone significantly improved cardiomyocyte morphology in MIRI rats. In addition, compared with the model group (IR), the hydromorphone-treated groups (HH+IR, IR+HH) significantly reduced the rna expression of NLRP3, ASC, caspase-1, IL-1β, and IL-18, as well as the protein levels of IL-1β, IL-18. In addition, transmission electron microscopy showed that hydromorphone attenuated CoCl2-induced cardiomyocyte injury.

Conclusion: Pre- or post-treatment with hydromorphone exerts protective effects against MIRI by suppressing NLRP3 inflammasomes, potentially providing a theoretical basis for its use as a therapeutic agent in MIRI patients.

Keywords: myocardial ischaemia/reperfusion, MI/R injury, hydromorphone, hypoxia/reoxygenation, NLRP3 inflammatory vesicles

Introduction

Acute myocardial infarction, AMI is one of the most serious and fatal diseases in internal medicine. Percutaneous coronary intervention, PCI, is effective in improving the symptoms of myocardial ischemia after AMI.[1] PCI can reopen the blocked coronary arteries and thus improve myocardial perfusion. However, rapid restoration of myocardial perfusion exacerbates cardiac dysfunction and structural damage,[2,3] myocardial ischemia-reperfusion injury myocardial ischemia-reperfusion injury, MIRI.MIRI induces oxidative stress and inflammation, which in turn amplify infarct size and exacerbate myocardial injury.[4,5] Currently, there is no definitive treatment to reduce cardiac injury caused by I/R. Therefore, understanding the pathophysiology of I/R and identifying therapeutic strategies to improve outcomes in patients with coronary artery disease are crucial.

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Pyroptosis, a form of programmed necrosis, is morphologically distinct from apoptosis. NLRP3 inflammasomes, one of the most functional members of the NOD-like receptor family and a major mediator of cellular pyroptosis, are composed of caspase-1, apoptosis-associated speck-like protein (ASC), and NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3).[6] They regulate and coordinate immune responses. Inflammasome activation accelerates the production of pro-inflammatory cytokines (primarily IL-1β and IL-18), thereby increasing inflammation. The inflammasome is recognized as a key regulator of inflammation-mediated I/R injury.[7] Duewell et al[8] reported that NLRP3 inflammasome activation in cardiac fibroblasts exacerbates myocardial ischaemic injury. Conversely, Kawaguchi et al observed reduced ischaemia-reperfusion injury in isolated hearts of NLRP3-deficient mice.[9]

Hydromorphone, a potent μ-opioid receptor agonist primarily used to treat postoperative and cancer-related pain,[9] is 5 to 7 times more potent than morphine.[10,11] Morphine, as a classical opioid receptor, has been shown to have a protective effect against MIRI.[12,13] Liu et al [14] found that HH may attenuate MIRI in rats by improving mitochondrial function, reducing oxidative stress, and activating the PI3K/Akt signaling pathway. However, the specific mechanism of HH remains to be elucidated. Therefore, the present study investigated whether hydromorphone protects the heart from I/R-induced cardiac injury and elucidated the potential mechanism of hydromorphone by examining the signaling pathway.

Materials and Methods

Animals

Thirty adult male Sprague-Dawley rats, aged 7 to 8 weeks and weighing 200 to 250 g, were used in this study. The rats were housed under a 12-hour light/dark cycle at a temperature of 22 ± 2°C and a humidity of 50 to 60%. The rats were provided with free access to food and water. All animal experiments were performed in accordance with the "Guidelines for the Care and Use of Laboratory Animals" to ensure animal welfare. This study was reviewed and approved by the Animal Ethics Committee of Central South University.

Animal Grouping and Interventions

After one week of acclimation feeding, the rats were randomly and equally divided into five groups (assessed using randomized groups with blinded): the NC group, the NC+ HH group, the IR group, the HH + IR group, and the IR + HH group. The MIRI rat model was established following the method described in.[15] Rats in the IR, HH + IR, and IR + HH groups were anaesthetised via intraperitoneal injection of 20% urethane at a dose of 0.6 mL/100 g and fixed on an animal operating board in the supine position. The anterior descending branch of the left coronary artery was ligated with a 5–0 surgical suture 2 to 3 mm from its origin. Rats in the NC and NC+HH groups underwent the same procedure but were sutured 2 to 3 mm from the origin of the left anterior descending coronary artery without ligation or compression of the plastic tube. After 30 minutes of ligation-induced ischaemia, the 5–0 surgical suture was gently cut with ophthalmic scissors, and the plastic tube was removed. The rats were subsequently reperfused for 120 minutes to simulate MIRI. Rats in the HH + IR group received a tail vein injection of 0.1 μmol/L HH 10 minutes before ligation, while rats in the IR + HH group received the same dose 10 minutes after reperfusion, as shown in Figure 1.

Enzyme-Linked Immunosorbent Assay (ELISA)

At the end of reperfusion, myocardial tissue and serum samples were collected and serum IL-1β and IL-18 were assessed using Elisa kits (Shanghai Jianglai Company) according to the manufacturer's instructions. Finally, the levels of these parameters were determined using a TECAN enzyme marker at a wavelength of 450 nm.

Hematoxylin-Eosin (HE) and Masson Staining

Once the model was established, all rats were killed and myocardial tissues were collected. Myocardial tissues from rats in each group were fixed in 4% paraformaldehyde for 24 h. The tissues were then dehydrated through a graded ethanol series. The tissues were then cleared with xylene and embedded in paraffin. Paraffin-embedded sections were cut at 5 μm. Sections were deparaffinised and rehydrated in xylene and fractionated ethanol. For HE staining, sections were stained with haematoxylin for 5 minutes and eosin for 2 minutes. For Masson's staining, sections were stained with haematoxylin

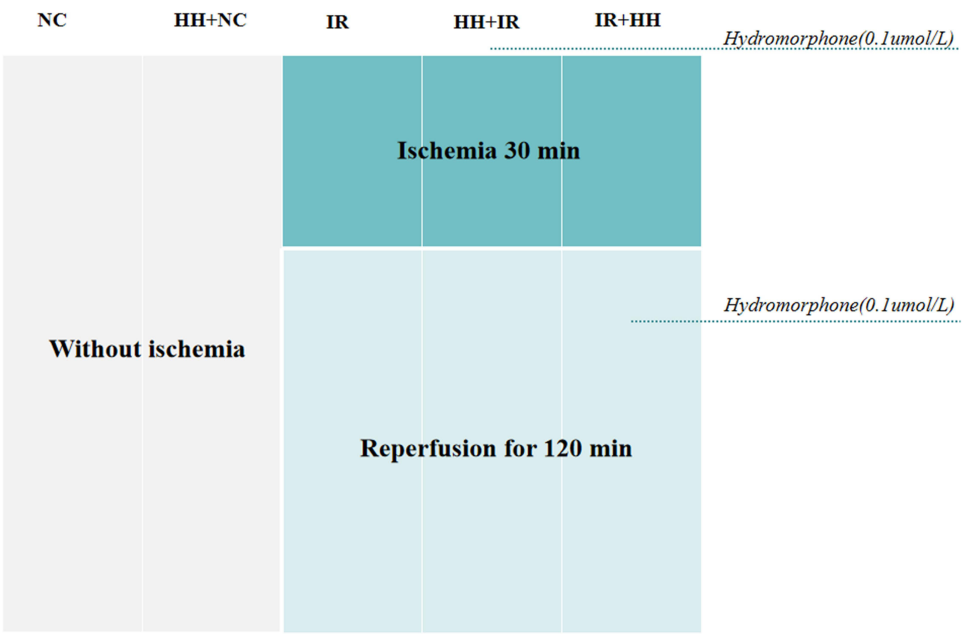

Figure 1 Experimental process diagram. NC: control group, NC + HH: control + hydromorphone group, IR: modeling group, HH + IR: hydromorphone pretreated modeling group, IR + HH: hydromorphone posttreated modeling group.

for 5 minutes, magenta with eosin for 5 minutes and green FCF for 1 minute. Sections were examined under a light microscope.

Cell Viability Assay

Cell viability was determined using CCK-8 (Shanghai Yisheng Technology Co., Ltd., China). After trypsin digestion, H9C2 cells in logarithmic growth phase were inoculated into 96-well plates at 5×103 cells/well. 10 μL CCK-8 working solution was added to each well and incubated at 37°C for 2 h. Cells were then detected using an enzyme marker (TTX). The absorbance was then measured at 450 nm using an enzyme marker (TECAN). The relative cell viability was calculated using cells without any treatment as the control group: relative cell viability = [(A-C)/(B-C)], where A is the optical density (OD) value in the experimental group, B is the OD value in the control group, and C is the OD value in the blank group.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from cultured cells using Trizol according to the manufacturer's protocol, and the extracted RNA was reverse transcribed into complementary DNA (cDNA) using the PrimeScript™ RT Reagent Kit with gDNA Eraser Synthesis Kit (Takara). Real-time quantitative PCR (RT-qPCR) was performed. RT-qPCR primers were designed using Primer 3, the internal reference was standardised to GAPDH, and qPCR primer pairs were synthesised by Shanghai Bioengineering. Three replicate wells were set up for each sample, and 2-ΔΔCt represents the relative expression level of mRNA.

Statistical Analysis

All data were expressed as mean ± standard deviation. GraphPad 7.0 software was applied for statistical analysis. One-way ANOVA test was used to compare differences between groups. $P < 0.05$ was considered statistically significant.

Results

Pathological Structural Changes in Rats with MI/R Infarction

At the end of reperfusion, heart tissue from the rats was collected to observe pathological changes, with the myocardial Masson staining results shown in Figure 2: myocardial cells showed swelling, disrupted tissue structure, atrophied and

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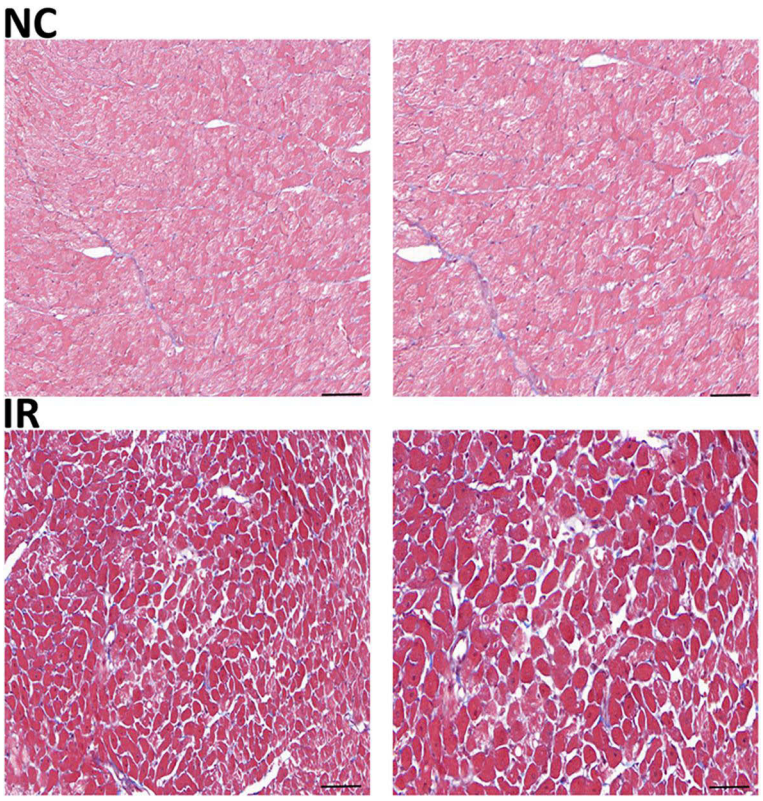

Figure 2 Masson's staining changes in rat pathology. NC: control group, IR: modeling group. Magnification: 100× and 200×.

aggregated nuclei, and increased collagen fibres, indicating that the rat model of myocardial ischaemia/reperfusion injury was successfully established.

Hydromorphone Attenuates Myocardial Tissue Damage in Rats with MIRI

To investigate the effects of hydromorphone on myocardial injury, the MIRI model was treated with hydromorphone.As shown in Figure 3, HE staining showed that myocytes exhibited sparse hypertrophy, collagen fibres were thickened, and the spacing between collagen fibres was increased in myocardial tissue from MIRI rats compared to control rats. Histopathological damage in MIRI rats was attenuated following pre- and post-treatment with hydromorphone. Our results suggest that hydromorphone attenuates myocardial damage in MIRI rats. Myocardial fibrosis is a common pathological phenomenon in damaged heart tissue. To further assess changes in myocardial fibres, we performed Masson's staining (Figure 4), which showed a decrease in smooth muscle fibres and an increase in collagen fibres in MIRI rats compared to control rats. The number of smooth muscle fibres increased, while collagen fibres decreased after hydromorphone pre- and post-treatment. The results showed that hydromorphone attenuated myocardial fibrosis in ischaemia-reperfusion injury.

Hydromorphone Reduces Focal Death in Rats with MI/R

To further investigate the effect of hydromorphone on focal cell death through NLRP3 inflammasomes, RT-qPCR and ELISA were employed to measure the expression levels of factors related to focal cell death in rat myocardial tissue and serum. After I/R injury, the mRNA expression levels of NLRP3, ASC, caspase-1, IL-1β, and IL-18 in myocardial tissue from the model group were significantly higher than those in the normal group ($p < 0.05$). Compared to the model group, the mRNA levels of NLRP3, ASC, caspase-1, IL-1β, and IL-18 were significantly lower in both the hydromorphone pre-treatment and post-treatment groups ($p < 0.05$) (Figure 5A). Subsequently, rat serum was collected to measure the relevant proteins, and it was found that hydromorphone pre-treatment and post-treatment significantly reduced the serum

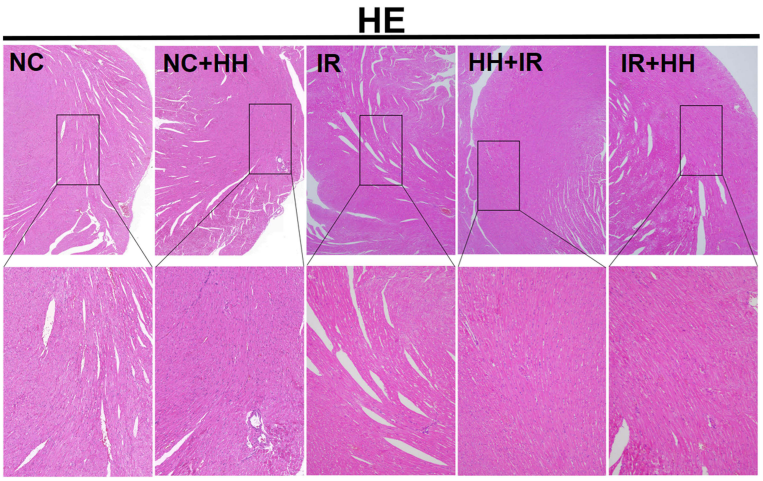

Figure 3 Pre- and post-treatment with hydromorphone attenuates MI/R injury in rats. Histopathological damage was detected by HE staining. Magnification: 40× and 100×.

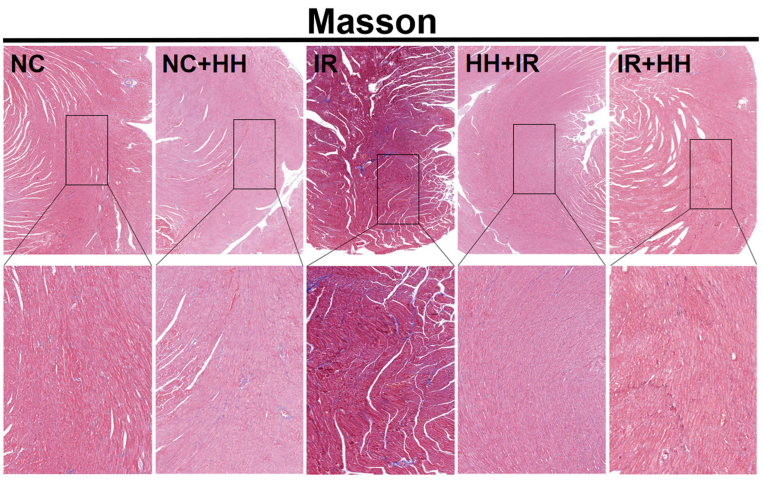

Figure 4 Pre- and post-treatment with hydromorphone attenuates MI/R injury in rats. Histopathological damage was detected by Masson's staining. Magnification: 40× and 100×.

levels of IL-1β and IL-18 (Figure 5B). These results suggest that hydromorphone can downregulate factors related to focal cell death in I/R-induced rat myocardial tissue and serum through NLRP3 inflammasomes.

Effect of Different CoCl2 Concentrations on Cell Viability

The CCK-8 assay was used to analyze how different concentrations of CoCl2 affected cardiomyocyte viability. As shown in Figure 6A and B, CoCl2 significantly inhibited cell viability in a dose-dependent manner compared with the CONT group. In addition, HH pre-treatment resulted in a dose-dependent increase in cell viability up to 1000 nM, followed by a non-linear decrease (Figure 6C). Therefore, 100 m mol/L CoCl2 was chosen to induce hypoxia, and 1000 nM of hydromorphone was used for pre-treatment of H9c2 cardiomyocytes.

Hydromorphone Reduces Hypoxia/Reoxygenation-Induced Pyroptosis in H9C2 Cells

The effect of hydromorphone on OGD/R-induced damage in H9C2 cells under hypoxia/reoxygenation conditions was assessed by measuring H9C2 cell pyroptosis. According to the RT-qPCR assay results, the level of cellular pyroptosis was significantly higher in the IR group compared with the control group, and significantly lower in the HH pre-treated group

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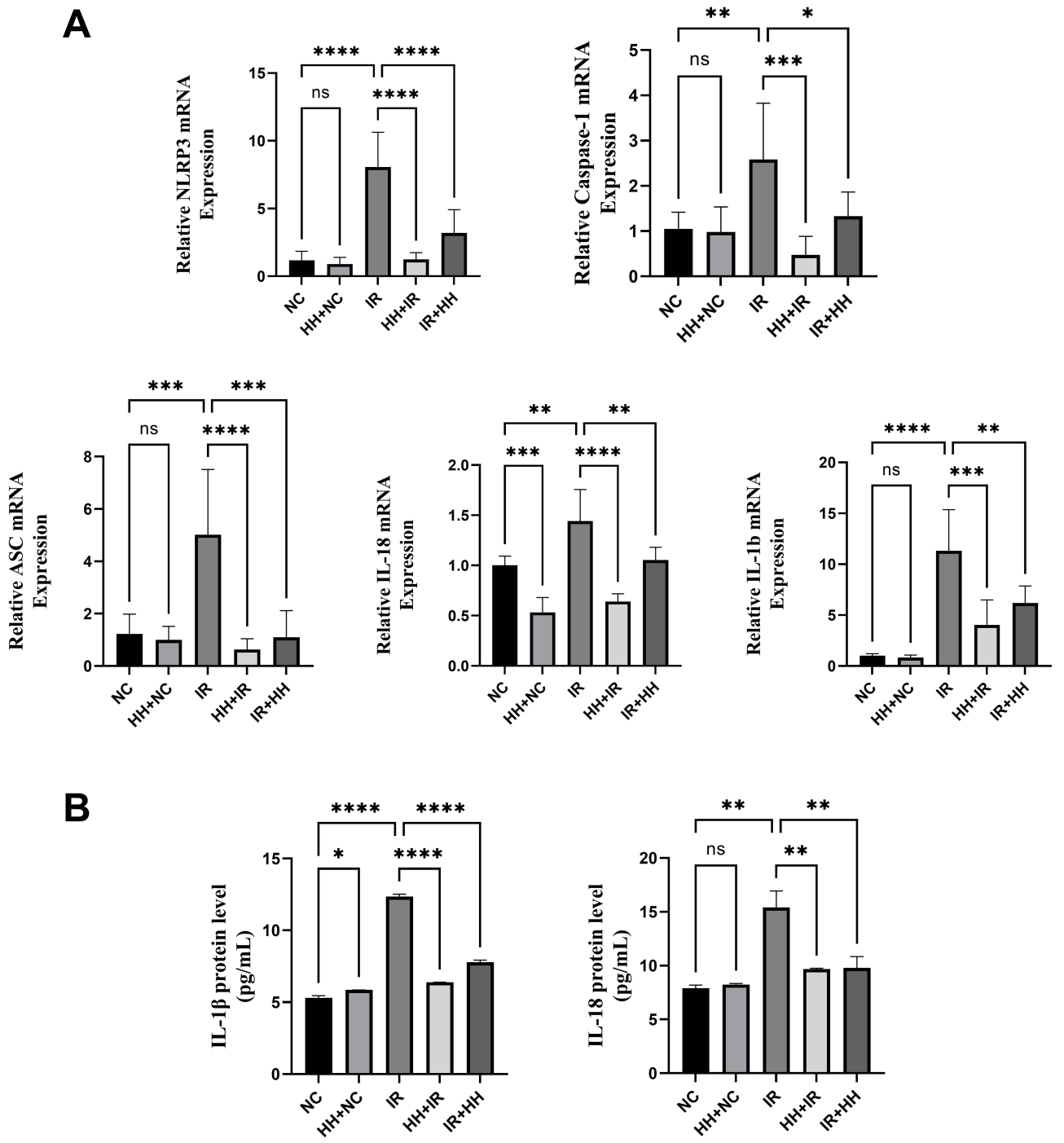

Figure 5 HH inhibits cellular pyroptosis by regulating NLRP3 inflammatory vesicles. (**A**) RT-PCR was used to measure mRNA levels of NLRP3, ASC, caspase-1, IL-1β, IL-18. (**B**) Protein level of IL-1β, IL-18 were measured by ELISA.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Abbreviation: ns, not significant.

compared with the HH post-treated group (Figure 7A). Similarly, according to the ELISA assay results, the level of cellular pyroptosis was significantly reduced in the IR + HH and HH + IR groups compared with the IR group (Figure 7B). To further observe cell morphology, H9C2 cells were examined by transmission electron microscopy, revealing varying degrees of membrane perforation (Figure 8). These results suggest that HH can inhibit H9C2 cell death after hypoxia-reoxygenation.

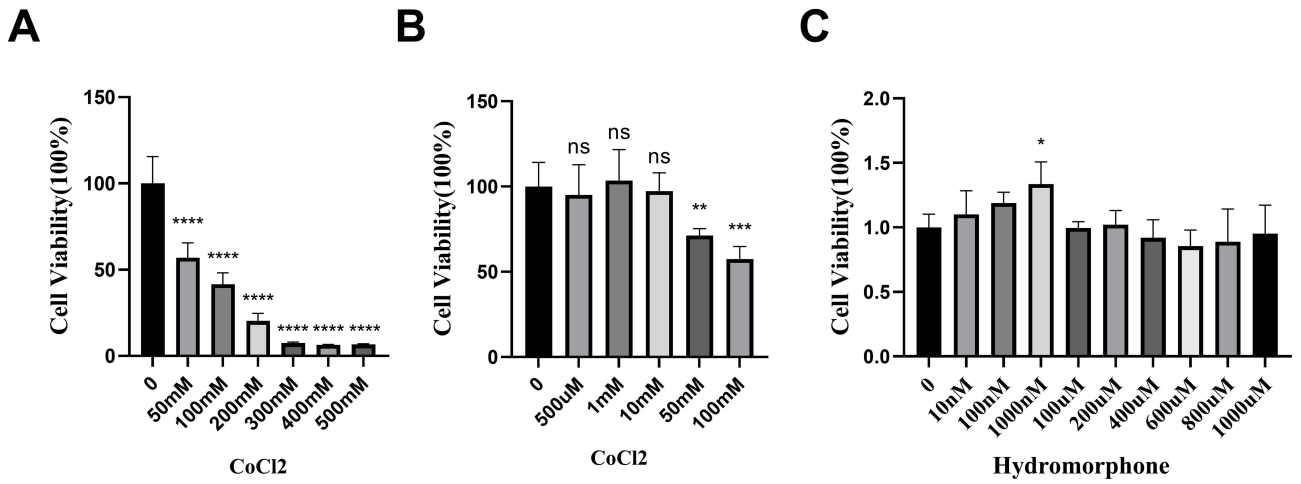

Figure 6 The effects of CoCl2 and HH on the viability of H9c2 cardiomyocytes were investigated by CCK-8 assay. (**A and B**) H9c2 cardiomyocytes were cultured with different concentrations of CoCl2 for 24 hours. (**C**) H9c2 cardiomyocytes were cultured with different concentrations of HH for 24 hours. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviation: ns not significant.

Discussion

The mechanisms of MIRI are complex. To date, several drugs have been shown to effectively mitigate MIRI, but preventive and therapeutic approaches for patients with postoperative myocardial ischaemia-reperfusion injury have been less successful. Opioids such as morphine have been shown to have cardioprotective effects, protecting the heart from ischaemia/reperfusion injury by inhibiting mitochondrial oxidative stress.[16,17] XU et al[18] found that either pretreatment or posttreatment with morphine attenuated MIRI in rats, and in addition, remifentanil has been formally shown to have a protective effect on rat myocardium.[19] Hydrocodone, as a new type of opioid, has gradually become the focus of attention for its protective effects on organ protection, especially ischemia/reperfusion injury. Kim et al[20] found that HH reduced reactive oxygen species (ROS) levels in the rat brain, thereby attenuating cerebral IRI induced by total intravenous anaesthesia. Xie et al[21] found that HH effectively inhibited IRI-induced inflammatory immune response and oxidative stress in the mouse brain, thereby reducing hippocampal neuronal apoptosis and improving cognitive performance. Qiu et al[14] found that HH post-treatment effectively inhibited oxidative stress in MIRI rats and attenuated myocardial injury. However, the specific mechanism of HH remains unclear. In the present study, we used left anterior descending coronary artery ligation and reperfusion to establish a rat model of MIRI and investigated whether HH could improve MIRI in rats with myocardial ischaemia. The results showed that HH attenuated I/R-induced myocardial injury and reduced I/R-induced cardiomyocyte damage in rats. In addition, we showed that HH activated NLRP3 inflammasomes both in vitro and in vivo, and that inhibition of the NLRP3 pathway significantly attenuated I/R-induced cardiomyocyte death. Meanwhile, HH significantly improved structural and morphological changes in cardiomyocytes. The hypoxia/reoxygenation model of CoCl2-induced H9C2 cells was established in vitro. The results also showed that HH effectively inhibited I/R-induced focal cell death in cardiomyocytes. These results suggest that HH has a protective effect against I/R.

Several studies have shown that the expression of the NLRP3 inflammasome is closely related to the severity of MIRI.[22] An inflammasome is an intracellular pattern recognition receptor that triggers inflammatory responses through cellular activation and the release of cytokines and mediators, playing an important role in infection defense and the maintenance of immune homeostasis.Aggregation of the inflammasome induces cleavage of pro-caspase-1 to generate activated caspase-1, promoting the conversion of pro-IL-1β and pro-IL-18 to mature IL-1β and IL-18.Activated caspase-1 induces pro-inflammatory forms of cellular pyroptosis.NLRP3 inflammasome activation, induced by the secretion of pro-inflammatory cytokines IL-1β and IL-18, and cellular pyroptosis, are self-protective measures of the organism.In the

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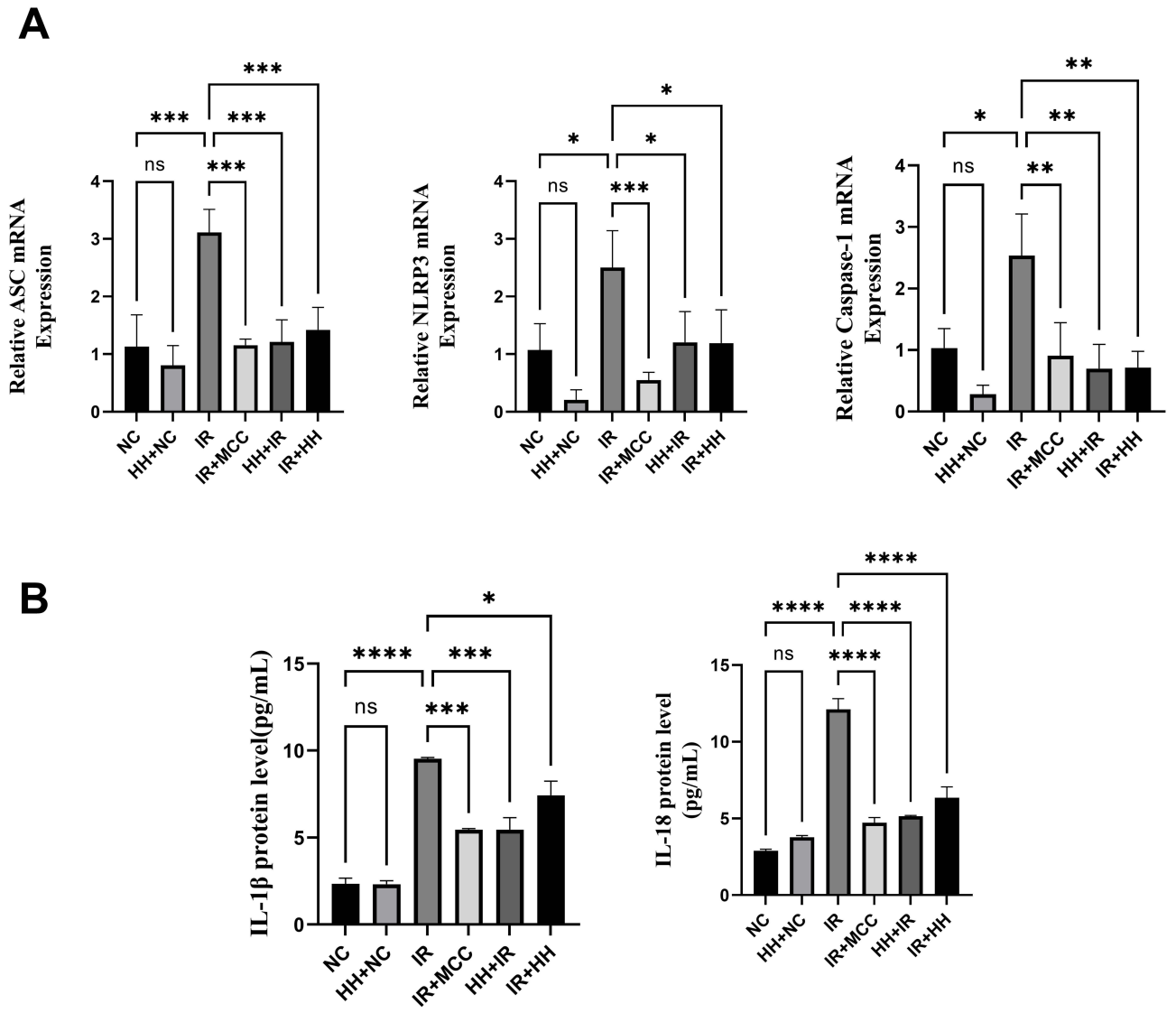

Figure 7 Hydromorphone reduces hypoxia/reoxygenation-induced pyroptosis in H9C2 cells. (**A**) RT-PCR was used to measure the mRNA levels of NLRP3, ASC and caspase-1. (**B**) Protein level of IL-1β, IL-18 were measured by ELISA. NC: control group, NC + HH: control + hydromorphone group, IR: modeling group, IR + MCC: modeling plus MCC950 group, HH + IR: hydromorphone pretreatment modeling group, IR + HH: hydromorphone post-treatment modeling group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Abbreviation: ns not significant.

present study, our results showed that HH downregulated the expression of NLRP3, ASC, caspase-1, IL-1β, and IL-18 in myocardial tissue.This effect was further confirmed in the hypoxia/reoxygenation model of H9C2 cells.Thus, we have demonstrated that HH can influence focal cardiomyocyte injury by modulating NLRP3 inflammasomes in MIRI rats. However, the mechanism by which HH alleviates cardiomyocyte injury needs to be further investigated.

In clinical anesthesia, opioids are often used in combination with other drugs that can have significant effects on the cardiovascular system, such as decreasing cardiac output, which leads to a significant decrease in blood pressure. Several studies have shown that opioids improve cardiac function and reduce infarct size through various mechanisms. However, as anesthetics, opioids have not been shown to reduce intraoperative ischemia, postoperative myocardial infarction, or death.[23] Although the present study demonstrated the potential of HH to attenuate myocardial injury by modulating NLRP3 inflammatory vesicles in MIRI rats, the prospects for clinical translation must be viewed with caution. Experimental animals are often young and healthy individuals, whereas clinical patients often have comorbidities such as hypertension and diabetes mellitus, and a single model does not adequately reflect clinical reality. The focus on

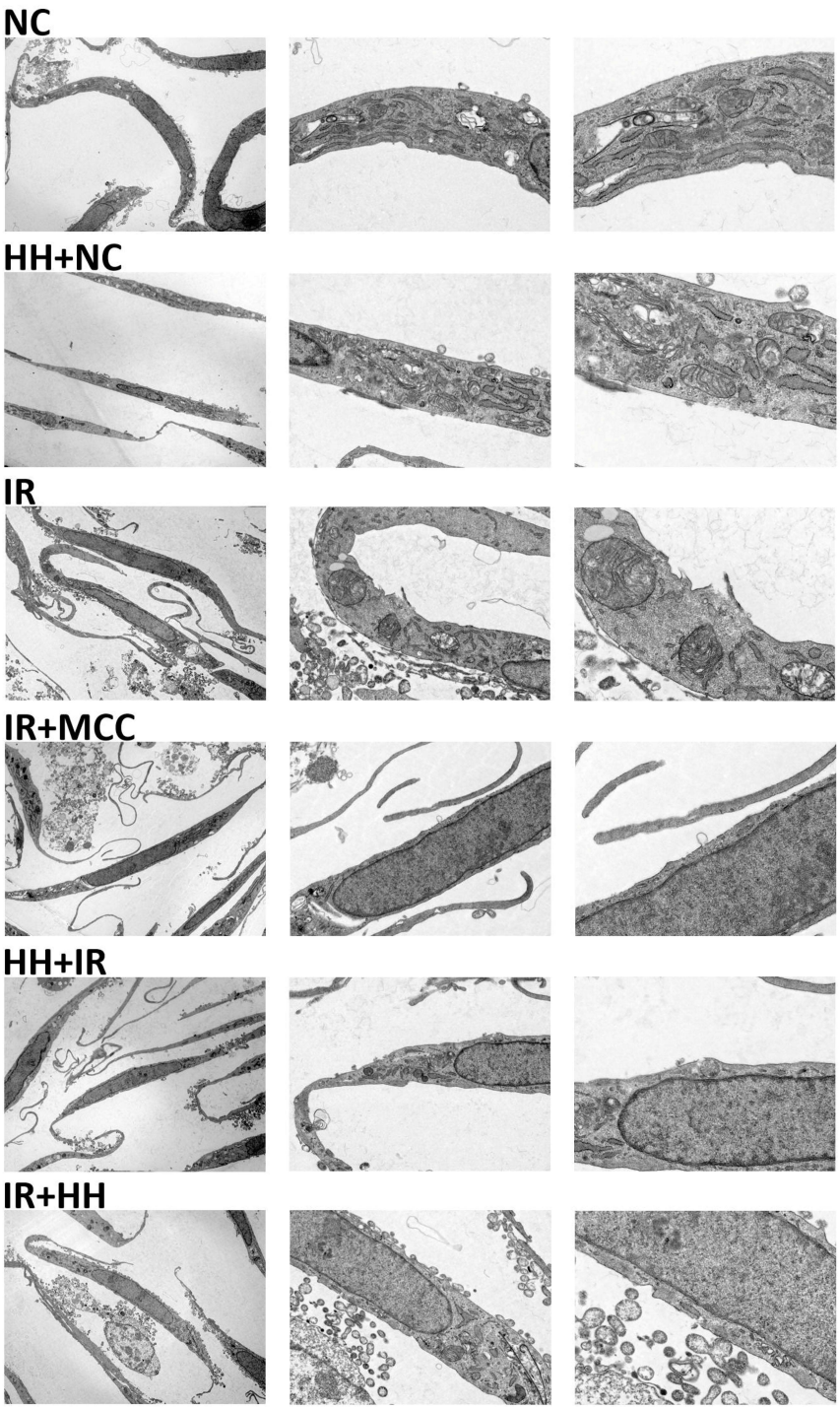

Figure 8 Focal cell death analysed by transmission electron microscopy. Membrane perforation was observed in H9C2 cells.

NLRP3 inflammatory vesicles in this experiment may counteract the cardioprotective effects through other pathways (eg, μ-receptor-mediated respiratory depression, sympathetic activation, etc.) in clinical practice. This study demonstrates that HH alleviates myocardial injury by attenuating cellular pyroptosis, and how HH can be used safely and effectively in the clinic needs further investigation.

Conclusion

Pre- or post-treatment with HH demonstrated a protective effect on MIRI, achieved through the inhibition of NLRP3 inflammasomes. This study not only elucidated the novel pharmacological mechanism of HH but also shed light on its clinical applications and provided new insights into the prevention and treatment of MIRI.

Data Sharing Statement

The data used in this study were provided by Shiyue Zeng and can be obtained from the corresponding corresponding authors, Mingzhi Zheng and Zhujun Huang.

Ethical Approval and Consent to Participate

This study was reviewed and approved by the Animal Ethics Committee of the Animal Center of Central South University.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no known competing financial interests.

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