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Hemin as a protective agent in an in vitro model of hypoxia/reoxygenation-induced injury

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Abstract

Objective: Ischemia-reperfusion injury exacerbates myocardial damage and affects the prognosis of patients with ST-elevation myocardial infarction. This study investigates the potential cytoprotective effects of hemin in an in vitro cardiomyocyte model subjected to hypoxia/reoxygenation, a simulation of ischemia-reperfusion injury, building upon previous evidence of hemin's efficacy in modulating ischemia-reperfusion injuries in various biological tissues.

Methods: H9c2 cardiomyocytes were exposed to a simulated hypoxia/reoxygenation environment. The experimental setup included pretreatment with hemin at varying concentrations, with subsequent assessment in the presence and absence of a heme oxygenase-1 inhibitor (Zinc-Protoporphyrin IX (heme oxygenase-1 inhibitor)).

Results: Pretreatment with 5 μM hemin notably attenuated the oxidative stress and apoptosis in H9c2 cardiomyocytes following hypoxia/reoxygenation exposure, while simultaneously upregulating heme oxygenase-1 expression. This protective effect was found to be heme oxygenase-1 dependent, as evidenced by its attenuation upon the introduction of Zinc-Protoporphyrin IX (heme oxygenase-1 inhibitor), a heme oxygenase-1 inhibitor.

Conclusion: The findings suggest that low-dose, short-term hemin pretreatment can effectively reduce hypoxia/reoxygenation-induced cellular damage in cardiomyocytes through the upregulation of heme oxygenase-1. These results underscore the therapeutic potential of hemin in attenuating myocardial hypoxia/reoxygenation injury.

Keywords

Ischemia-reperfusion injury, ST-elevation myocardial infarction, hemin, heme oxygenase-1, cardiomyocyte

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Introduction

Ischemia-reperfusion injury is a phenomenon observed in various clinical settings, including acute myocardial infarction.[1] This injury can negate the beneficial effects of reperfusion therapies like primary percutaneous coronary intervention, leading to further expansion of the myocardial infarct area[2] and increased mortality rates.[3] The pathophysiology of ischemia-reperfusion injury is intricate, involving oxidative stress,[4] calcium overload, and inflammatory processes,[5,6] all of which can culminate in cellular apoptosis and necrosis.[7]

In this context, hemin, a breakdown product of hemoglobin present in the serum, has garnered attention for its dual biological roles.[8] While serum hemin has been shown to have detrimental effects in cerebral hemorrhage through oxidative stress and pro-inflammatory pathways,[9,10] it has also been suggested to have protective effects in renal ischemia-reperfusion injury models.[11] Intriguingly, hemin is a potent inducer of heme oxygenase-1 (HO-1),[12] a protein known for its protective role against cardiomyocyte ischemia-reperfusion injury.[13]

This investigation aims to elucidate the protective potential of hemin pretreatment in a hypoxia–reoxygenation (H/R) cardiomyocyte model, which serves as an in vitro simulation of the myocardial ischemia–reperfusion process. By exploring the therapeutic efficacy of hemin, this study provides insights that may guide future strategies for managing myocardial ischemia–reperfusion injury.

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Methods

Cell culture and treatment

The neonatal cardiomyocyte cell line H9c2 was purchased from the American Type Culture Collection. These H9c2 cardiomyocytes were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/mL penicillin G and 100 μg/mL streptomycin), in an incubator maintained at 37°C with an atmosphere of 5% CO_2 for 36 h. Subsequently, the cells were categorized into the following groups: In the control group, cells were continuously incubated for 18 h under the initial culture conditions.In the (H/R) group, H9c2 cells at 70%–80% confluence were transferred to a glucose- and serum-free DMEM, and then subjected to hypoxic conditions inside a ProOxC system chamber (BioSpherix, NY, USA) with an oxygen controller that maintained an atmosphere of 5% CO_2 and 95% nitrogen at 37°C for 12 h. Following this, the culture medium was reverted to normal DMEM, and the cells were cultured for an additional 6 h under normoxic conditions (37°C with 5% CO_2). In the H/R + Hemin) group, H9c2 cells were first preconditioned with 5 μmol/L of hemin for 1 h before undergoing the same H/R protocol as described for the H/R group.

Apoptosis determination via flow cytometry

Upon completing the respective treatments, H9c2 cells from each group were washed, trypsinized, resuspended in chilled 1 × PBS, and centrifuged at 2000 rpm for 5–10 min to cleanse the cells. The resulting cell pellet was resuspended in 500 μL of binding buffer to which 5 μL of Annexin V-FITC and 5 μL of PI were added. Following a 15-min incubation at room temperature in the dark, the rate of apoptosis in each group was ascertained using a flow cytometer (BD AccuriTM C6, BD Biosciences, USA). Each experiment was performed in triplicate for each group.

Quantitative measurement of free radical production

Posttreatment, the culture medium was removed, and the cells were washed with PBS three times. The cells were then harvested, resuspended to a density of 1–2 × 107 cells/mL, and incubated at 37°C for 30 min with 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) at a final concentration of 10 μM. This was followed by washing the cells three times with serum-free culture medium to remove any DCFH-DA that had not been internalized by the cells. Finally, intracellular ROS levels were determined using a flow cytometer (BD Accuri™ C6, BD Biosciences, USA).

Assessment of MDA, GSH-Px levels, and SOD activity

Postharvesting, cells were lysed with 0.1 mL of lysis buffer per 1 million cells at 4°C for 40–60 min. The lysate was then centrifuged at 10,000–12,000 *g* for 10 min, and the supernatant was collected for subsequent measurements. The bicinchoninic acid (BCA) protein concentration determination kit was used to assess protein concentration.

The MDA levels were measured using the lipid peroxidation malondialdehyde (MDA) assay kit (Beyotime Institute of Biotechnology, China), following the manufacturer's instructions. The absorbance was read at 532 nm on a microplate reader, and MDA content was presented as nmol/mg protein.

The glutathione peroxidase (GSH-Px) activity was determined using a GSH-Px assay kit (Beyotime Institute of Biotechnology, China). The absorbance at 340 nm (A340) was recorded every minute for 5 min at 25°C using a microplate reader. The GSH-Px activity was reported as U/mg protein.

The superoxide dismutase (SOD) activity levels were assessed using the SOD assay kit (Beyotime Institute of Biotechnology, China), following the manufacturer's protocols. The absorbance was measured spectrophotometrically at 560 nm, and SOD activity was reported as U/mg protein.

Analysis of caspase-3 activity

Caspase-3 activity was measured using a caspase-3 activity assay kit (Jiamei Technology, Beijing, China). Cell pellets from each group were collected, washed with PBS, and centrifuged (600 *g* at 4°C for 10 min). Cell lysis and centrifugation (18,000 *g* at 4°C for 15 min) were performed to obtain the supernatant. Protein concentration was adjusted to 1 μg/μl using the Bradford method. 50 μL of each sample was combined with 40 μL of reaction buffer and 10 μL of Ac-DEVD-pNA (2 mM) substrate in a 96-well plate. The plate was incubated at 37°C for 1 h, after which the reaction was halted. The absorbance of pNA was measured at 405 nm using a microplate reader. Three independent experiments were performed. Caspase-3 activity was reported in terms of U/μg cytoplasmic protein, defined as the quantity of enzyme that can cleave 1 nmol Ac-DEVD-pNA to produce 1 nmol pNA, caspase-3/h at 37°C under saturated substrate conditions.

Quantitative real-time PCR

Cells from each group were rinsed twice with PBS before total RNA was extracted using Trizol reagent and subsequently reverse transcribed into cDNA. The utilized primers were as follows: for HO-1, forward primer CACAGACAGAGTTTCTTCGC and reverse primer ATGAGACAGAGTTCACAGCCT; for β-Actin, forward primer TGTTGTCCCTGTATGCCTC, and reverse primer ATGTCACGCACGATTTCC. A reaction mixture was then created comprising 2 μL of cDNA template, 10 μL of supermix, 0.4 μL of passive dye, 0.4 μL of upstream primers, 0.4 μL of downstream primers, and 6.8 μL of sterile distilled water. The mixture was subjected to 40 cycles of amplification: 95°C for 3 min, 95°C for 30 s, and 55°C for

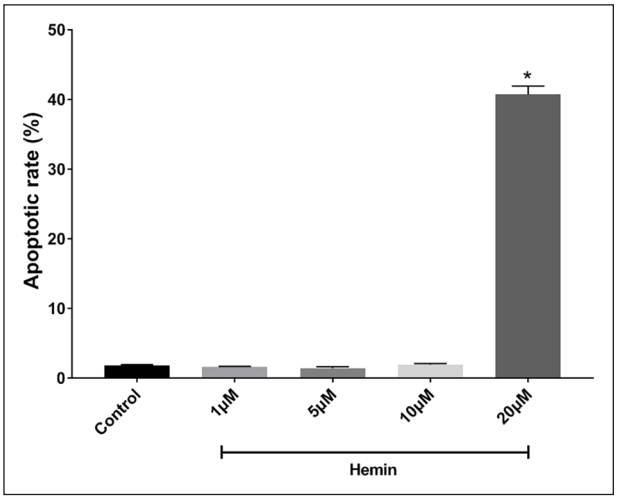

Figure 1. H9c2 cells were treated with various hemin concentrations (1, 5, 10, and 20 μM); cell viability was detected by flow cytometry. The results were reported as the mean ± standard error of the mean ($n = 5$ per group).
*$p < 0.001$ compared with the other groups.

20 s. RT-qPCR products were quantified using the $2^{-\Delta\Delta Cq}$ method with ACTB serving as the endogenous control for RNA expression. A melting curve analysis was performed by gradually increasing the temperature from 60°C to 95°C over 20 min. Each sample was run in triplicate.

Western blotting

H9c2 cells from each group were harvested, washed thrice with PBS, and lysed using a cell lysis buffer containing phenylmethylsulfonyl fluoride (PMSF) (Amresco, OH, USA). Protein concentrations were determined using a BCA kit (Beyotime Institute of Biotechnology, China). After mixing 30 μg of cell lysate with 5× sample buffer, samples were loaded onto a 10% polyacrylamide gel. Proteins were subsequently separated by SDS-PAGE electrophoresis and transferred to a PVDF membrane (Bio-Rad, no. 162-0177). After blocking with 3% BSA for 2 h at room temperature, membranes were incubated overnight at 4°C with secondary antibodies against β-actin, HO-1, Bax, Bcl2, and cleaved caspase-3. Membranes were washed thrice with tris-buffered saline (TBST) to remove unbound primary antibodies, followed by a 2-h incubation with HRP secondary antibodies (Solarbio, Beijing, China) at room temperature. Protein bands were visualized using ECL reagent under the GelDoc imaging system (Bio-Rad no. 170-5060). β-actin served as the internal reference protein for normalizing protein expression levels.

Statistical analysis

Prior to applying parametric tests (e.g., *t*-tests, one-way ANOVA), we evaluated data normality using the Shapiro–Wilk (or Kolmogorov–Smirnov) test and assessed homogeneity of variances with Levene's test. Data meeting these assumptions were analyzed using *t*-tests or one-way ANOVA, whereas data violating normality or homogeneity assumptions were analyzed using nonparametric methods (e.g., Mann–Whitney *U* test). For comparisons among more than two groups, one-way ANOVA was followed by a post hoc test (Tukey or Bonferroni) to adjust for multiple comparisons.When conducting multiple outcome assessments, *p*-values were further corrected either by Bonferroni adjustment or by controlling the false discovery rate. In all such cases, a corrected p-value < 0.05 was considered statistically significant. Enumeration data were expressed as counts (n) and percentages, and analyzed using the Chi-square test. The sample size (n) in each experimental group was determined based on preliminary data and literature guidelines. Each experiment included at least three biological replicates, and each replicate was measured in duplicate or triplicate. All statistical analyses were performed using SPSS 27.0 (IBM Corp., Armonk, NY, USA). All tests were two-sided. Where applicable, effect sizes (e.g., Cohen's d, partial η^2) were calculated to provide additional context for the magnitude of the observed effects. We regarded $p < 0.05$ (two-tailed) as statistically significant.

Results

Dose-dependent effect of hemin on H9c2 cell viability

To determine an optimal concentration for hemin pretreatment, cell apoptosis rates were assessed after exposure to varying concentrations of hemin (1, 5, 10, and 20 μM) for 6 h under standard culture conditions using flow cytometry. Relative to the control group, treatments with 1, 5, and 10 μM hemin had no discernable effect on the viability of H9c2 cells, whereas 20 μM hemin treatment significantly reduced H9c2 cell viability (Figure 1). Consequently, a concentration of 5 μM was selected for subsequent hemin pretreatment experiments.

Hemin attenuates H/R-induced apoptosis in H9c2 cells

The effect of hemin on H/R-induced myocyte apoptosis was investigated by pretreating H9c2 cells with 5 μM hemin for 1 h prior to H/R, followed by an analysis of cell viability. H/R treatment significantly increased apoptosis in H9c2 cells compared to control. However, this H/R-induced apoptosis was substantially mitigated with 5 μM hemin pretreatment for 1 h, indicating a protective effect of hemin against H/R-mediated injury in H9c2 cells (Figure 2).

Hemin reduces cleaved caspase-3 and caspase-3 activity while elevating Bcl-2/Bax ratio

Given that Bcl-2, Bax, and caspase-3 have well-established roles in governing cell survival or death upon exposure to

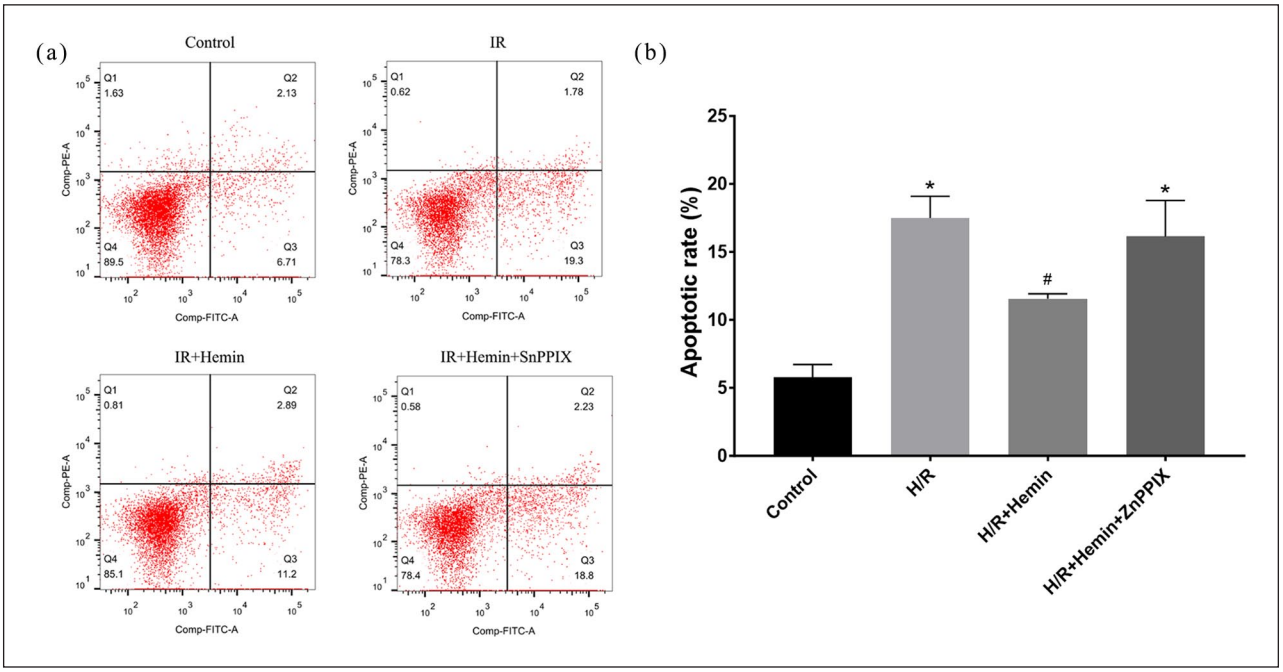

Figure 2. Hemin attenuates H/R-induced apoptosis of H9c2 cells. (a) Representative flow cytometric pictures of cell apoptosis for each of the four groups. (b) Flow cytometry assay analysis of H9c2 cell apoptosis. H/R: hypoxia-reoxygenation; H/R + Hemin: H9c2 cells pretreated with 5 μM hemin for 1 h before H/R; H/R + Hemin + ZnPPIX: H9c2 cells pretreated with 5 μM hemin for 1 h before H/R, then ZnPPIX (10 μM) was added throughout H/R.
*$p < 0.001$ versus control, #$p < 0.001$ versus H/R and H/R + Hemin + ZnPPIX groups.

apoptotic stimuli, we explored their expression levels in H9c2 cells to understand how hemin attenuates apoptosis during H/R. The Bcl-2/Bax ratio in the H/R group was notably lower than that of the control group. However, this ratio was significantly restored by hemin pretreatment (Figures 3(a) and 4). Concurrently, we observed an upsurge in cleaved caspase-3 and caspase-3 activity in H9c2 cells post-H/R (Figures 3(b) and 4), a rise that was effectively countered by hemin pretreatment.

Hemin mitigates H/R-induced oxidative damage

Next, we investigated the effects of hemin on oxidative stress in H9c2 cells subjected to H/R. H/R treatment escalated both ROS production and MDA content in comparison to the control group (Figure 5), while markers of antioxidant activity, namely SOD and GSH-Px, were notably diminished. Interestingly, hemin pretreatment substantially curtailed H/R-induced oxidative stress as indicated by decreased intracellular ROS and MDA levels, bolstered SOD activity, and GSH-Px expression (Figure 5).

Hemin augments HO-1 expression post-H/R

To further unravel the molecular mechanisms underpinning the cardioprotective effects of hemin, we assessed HO-1 expression levels. Both RT-PCR and Western blotting were used to detect endogenous HO-1 expression. Remarkably, HO-1 expression was significantly amplified post-H/R and was further boosted by hemin pretreatment (Figures 3(a) and 4).

HO-1 inhibition diminishes the protective effect of hemin pretreatment

To shed additional light on the mechanism underlying hemin's cardioprotective properties, we introduced an HO-1 inhibitor, tin-protoporphyrin IX (ZnPPIX), at a dose of 10 μM to H9c2 cells during simulated H/R. Intriguingly, the antiapoptotic effects of hemin on H9c2 cells were effectively nullified in the presence of ZnPPIX (Figure 2). Additionally, we examined if ZnPPIX treatment could influence the effects of hemin on the expression levels of oxidative stress markers and apoptotic proteins. The findings revealed that ZnPPIX supplementation significantly augmented ROS and MDA production while depleting SOD activity and GSH expression, effectively reverting these parameters to levels akin to the H/R group (Figure 5). Similarly, the effect of hemin in augmenting Bcl-2/Bax ratio and curtailing caspase-3 activity was also negated following ZnPPIX addition (Figures 3 and 4).

Discussion

Hemin, a critical prosthetic group in various cytochromes,[14] enzymes,[15] and globulins,[16] plays a pivotal role in oxygen storage and transport, regulation of oxidative damage, and energy production.[17] Hemin has also been recognized as an inducer of HO-1, a vital antioxidant enzyme involved in antioxidative stress and antiapoptotic processes during ischemia-reperfusion (H/R) injury.[18,19]

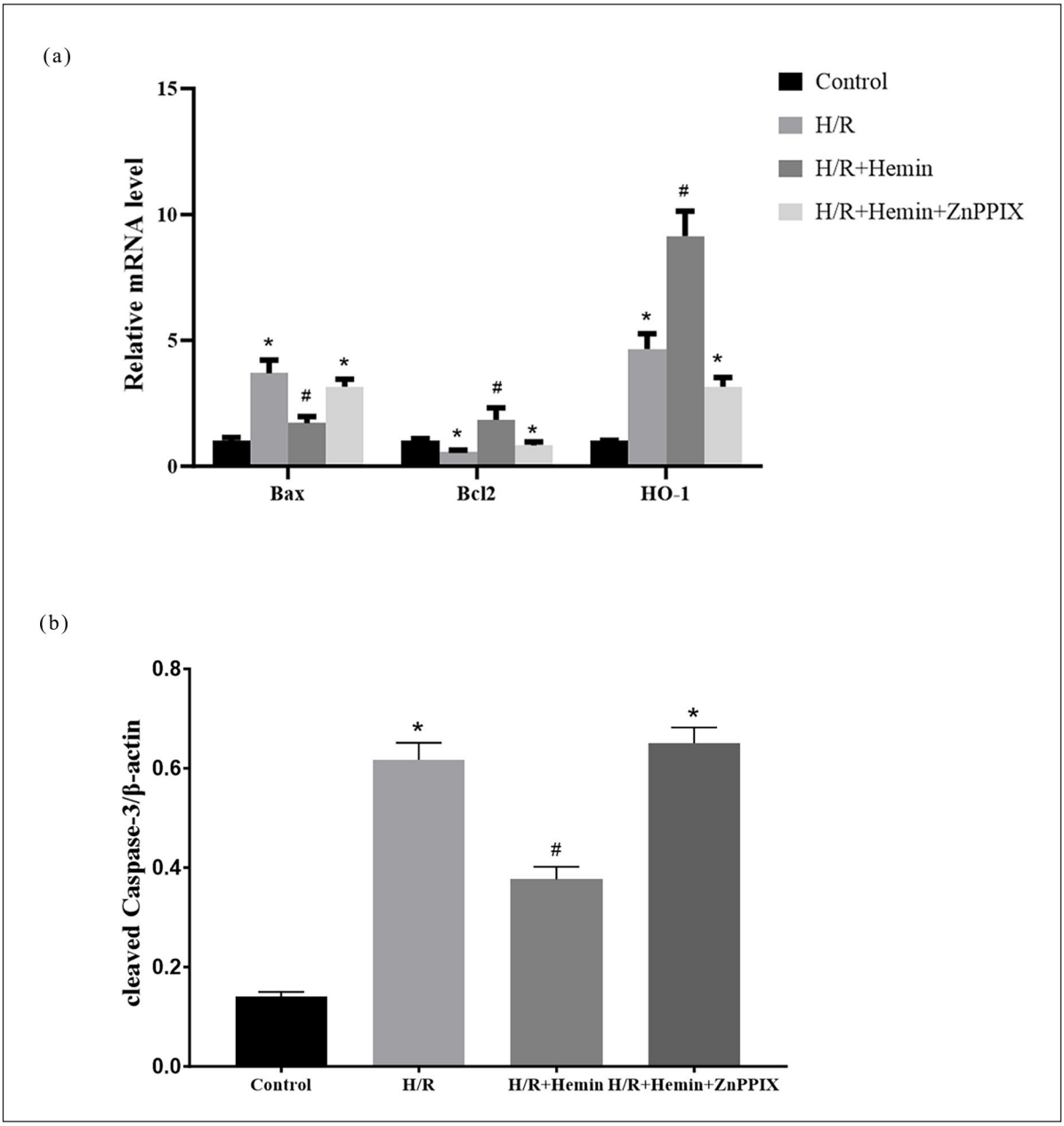

Figure 3. (a) The mRNA expression of Bax, Bcl2, and HO-1. (b) The caspase-3 activity in H9c2 cells. H/R, hypoxia-reoxygenation; H/R + Hemin, before H/R, H9c2 cells were pretreated for 1 h with 5 μmol/L hemin. H/R + Hemin + ZnPPIX: H9c2 cells were pretreated with 5 μmol/L hemin for 1 h before H/R, then ZnPPIX (10 μM) was added throughout H/R.
*$p < 0.001$ versus control, #$p < 0.001$ versus H/R group. The values indicate the mean ± standard error of the mean of five separate experiments.

Our in vitro investigations focused on hemin's role in modulating oxidative stress within H9c2 cardiomyocytes under H/R conditions, a key factor in myocardial cell death. Although this study employed H9c2 cells to simulate ischemia-reperfusion through a H/R protocol, it should be noted that in vitro conditions cannot fully capture the complexity of clinical ischemia-reperfusion scenarios. Consequently, using the term "H/R" in reference to our experimental setup more accurately reflects the limited, simulated nature of the cardiomyocyte injury model. It is well documented that IR injury precipitates oxidative stress responses in cardiomyocytes, leading to myocardial damage. Hemin, interestingly, exhibits a bidirectional influence on oxidative stress. While high-dose hemin administration postinjury can exacerbate IR-induced acute kidney injury, pretreatment with hemin has been observed to alleviate renal damage and oxidative stress in other models.[20] In our study, we specifically examined the effects of various concentrations of hemin on H9c2 cell viability. Our observations revealed that hemin concentrations of 1, 5, and 10 μmol/L did not significantly increase apoptosis in these cells. However, at

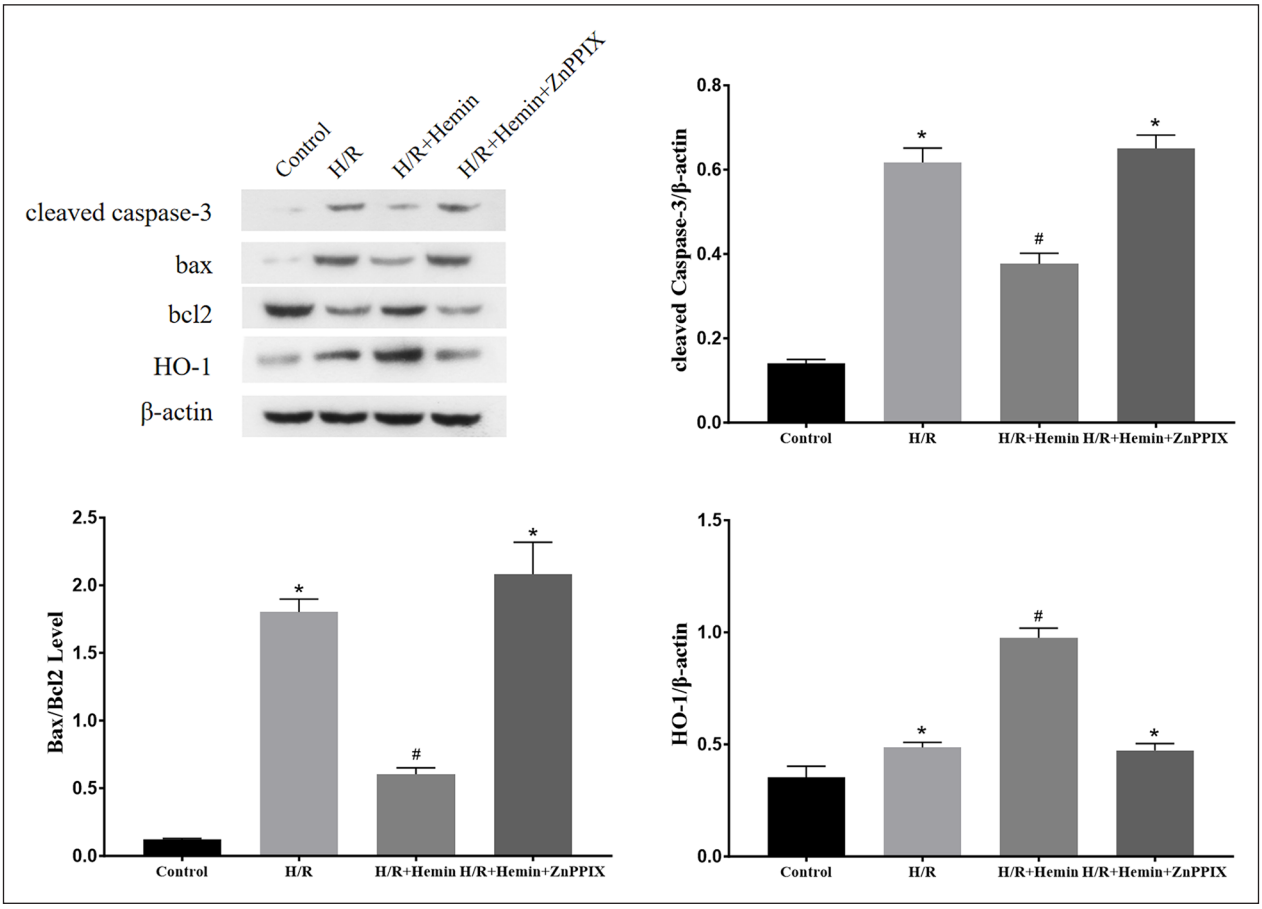

Figure 4. Protein expression levels of HO-1, cleaved caspase-3, Bax, and Bcl2 were examined by western blotting. H/R, hypoxia-reoxygenation; H/R + Hemin, H9c2 cells pretreated with 5 μmol/L hemin for 1 h before H/R. H/R + Hemin + ZnPPIX: H9c2 cells were pretreated with 5 μmol/L hemin for 1 h before H/R, then ZnPPIX (10 μM) was added throughout H/R.
*$p < 0.001$ versus control, #$p < 0.001$ versus H/R. Values represent mean ± standard error of the mean of three independent experiments performed.

a higher concentration of 20 μmol/L, hemin induced notable apoptosis, indicating a dose-dependent biological effect. Crucially, our results showed that low-dose (5 μmol/L) hemin pretreatment effectively mitigated the oxidative stress and apoptosis induced by H/R in H9c2 cells, suggesting a potential protective role of hemin in myocardial H/R injury.

Importantly, these findings provide new insights into the dose-dependent effects of hemin in an H9c2 model of H/R injury. Previous studies have rarely highlighted the protective window of low-dose hemin, particularly prior to the onset of H/R injury. By pinpointing a safe and efficacious concentration (5 μmol/L) for hemin, our current work adds to the understanding of its nuanced role in cardio protection and establishes a framework for future investigations on optimal dosing regimens.

The precise mechanism by which hemin impacts cardiomyocytes during oxidative stress remains elusive.[21] Numerous studies have suggested that hemin may safeguard various organs from ischemia-reperfusion injury by inducing HO-1 upregulation. For instance, Kumar et al.[22] demonstrated that hemin significantly promoted wound healing in diabetic rats by mitigating inflammation and oxidative stress via HO-1 activation. Hong et al.[23] found that hemin pretreatment upregulated HO-1 expression and lessened ischemia-reperfusion injury in the mouse liver. Furthermore, Attuwaybi et al.[24] showed that hemin upregulated HO-1 protein expression to diminish mucosal damage during intestinal ischemia-reperfusion injury in rats. In a rat model of ischemia-reperfusion injury, Hangaishi et al.[25] found that sequential intraperitoneal injections of hemin 2 days prior to ischemia-reperfusion could decrease the area of myocardial infarction, an effect linked to increased HO-1 expression in mononuclear macrophages and myofibroblasts.

In our experiments, we demonstrated that short-term, low-dose hemin pretreatment enhanced HO-1 expression in H9c2 cells. Using ZnPPIX inhibition experiments, we further confirmed that the protective effect of hemin pretreatment was mediated through HO-1 induction.

In addition, although our current study demonstrates that low-dose hemin pretreatment confers protective effects against hypoxia/reoxygenation (H/R) injury in cardiomyocytes via the HO-1 pathway, we acknowledge that the NRF2

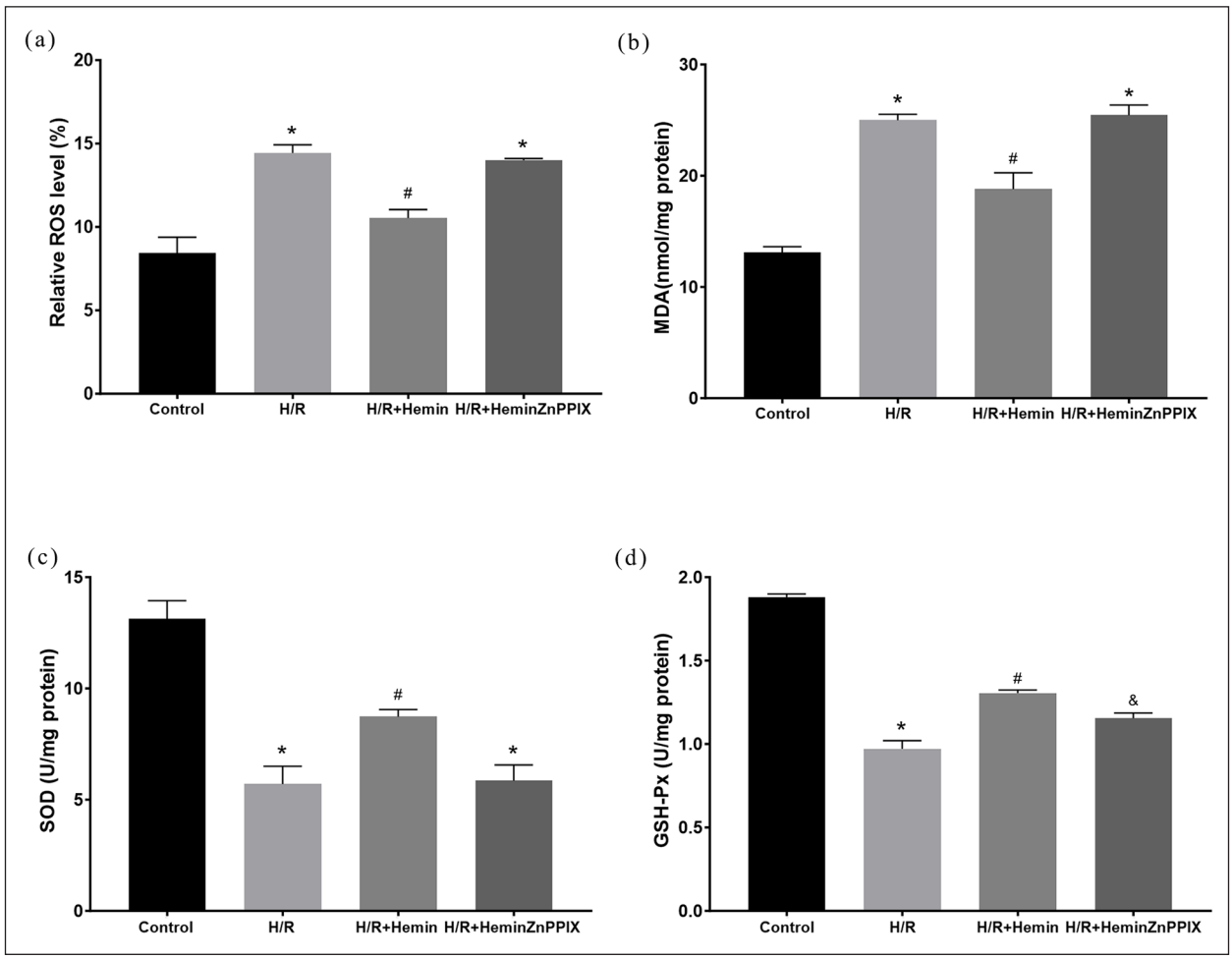

Figure 5. The effect of 5 μM hemin pretreatment on the H/R-induced oxidative stress in H9c2 cells. (a) ROS production, (b) MDA content, (c) SOD activity levels, and (d) GSH-Px expression were assayed using appropriate kits. H/R, hypoxia-reoxygenation; H/R + Hemin, H9c2 cells pretreated with 5 μM hemin for 1 h before H/R. H/R + Hemin + ZnPPIX: H9c2 cells were pretreated with 5 μM hemin for 1 h before H/R, then ZnPPIX (10 μM) was added throughout H/R.
*$p < 0.001$ versus control, #$p < 0.001$ versus H/R, &$p < 0.01$ versus H/R and H/R + Hemin groups. The values indicate the mean ± standard error of the mean of five separate experiments.

pathway likely plays a significant role in this process as well. Existing evidence suggests that NRF2 not only orchestrates antioxidant responses but may also interact with HO-1 to reinforce cytoprotective mechanisms.[26,27] To more thoroughly delineate how NRF2 might mediate or enhance HO-1–related cardio protection, further experiments involving NRF2 modulation and comprehensive molecular analyses are warranted. Due to constraints in project funding and timeline, we were not able to perform these experiments in the present study; however, we intend to explore the NRF2–HO-1 axis in follow-up investigations to broaden our understanding of the cardioprotective signaling network.

However, while our data emphasize the potential benefits of low-dose hemin, it is essential to address its limitations and possible adverse effects. Hemin can act on multiple cell types and organs beyond cardiomyocytes, leading to unwarranted outcomes. For instance, high concentrations of hemin may induce ferroptosis in neuronal cells and fibroblasts.[28,29] and can also modulate immune responses in ways that exacerbate inflammation.[30] Indeed, potential nephrotoxicity has been observed in some I/R models, further underscoring the importance of delineating a detailed safety profile across tissues prior to clinical application. In line with these observations, our own results revealed that at 20 μM, hemin substantially increased H9c2 apoptosis, hinting at a narrow therapeutic window. Thus, while low-dose hemin pretreatment appears effective for myocardial protection in this model, its dose-sensitive nature highlights the necessity of carefully optimized regimens in any future translational or clinical context.

Conclusion

Our findings suggest that short-term, low-dose hemin pretreatment confers protection against hypoxia/reoxygenation

injury in cardiomyocytes by attenuating oxidative stress and apoptosis through HO-1 upregulation. This study highlights the therapeutic potential of hemin in mitigating myocardial ischemia-reperfusion injury. However, the narrow therapeutic window of hemin, evidenced by its toxicity at higher doses, underscores the necessity for precise dosing regimens in future applications. Further research is warranted to elucidate the detailed molecular mechanisms of hemin's protective effects and to explore its clinical translation in myocardial injury management.

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Statements and Declarations

Ethics approval

Ethical approval was not sought for this study because it was conducted entirely in vitro using cell lines and did not involve human or animal participants.

Author contributions / CRediT

Zuoyan Wang: Conceptualization, Methodology, Supervision, Writing – Original Draft, Writing – Review & Editing. Wei Liu: Investigation, Formal Analysis, Data Curation, Visualization, Writing – Original Draft.

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conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Informed consent

Not applicable, as the study did not involve human subjects.

Trial registration

Not applicable, as this study was not a clinical trial.

Data availability

All data generated or analyzed during this study are included in this published article. Any additional information related to the data may be provided by the corresponding author upon reasonable request.

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