

Protective Effect of MiR-223-3p against Hypoxia/Reoxygenation Injury in H9c2 Cardiomyocytes via Targeting the TLR4/NF- κ B Pathway

Fuqiang Kan^{1,a,*}, Lingyu Li^{2,b}

¹Department of Cardiology, Yiwu Central Hospital, The Affiliated Yiwu Hospital of Wenzhou Medical University, Yiwu, Zhejiang, 322000, China

²Department of Encephalopathy, Yiwu Traditional Chinese Medicine Hospital, Yiwu, Zhejiang, 322000, China

^a kfq_2006@163.com, ^b 1453585815@qq.com

*Corresponding author

Abstract: This study aimed to investigate the role and mechanism of miR-223-3p in hypoxia/reoxygenation (H/R)-induced injury in H9c2 cardiomyocytes. An in vitro H/R injury model was established, and cells were divided into Control, H/R, H/R + Negative Control (NC), and H/R + miR-223-3p inhibitor groups. Cell viability was assessed by CCK-8 assay, miR-223-3p expression was measured via RT-qPCR, protein levels of TLR4 and phosphorylated NF- κ B, p-p65 were analyzed by Western blot, and the release of inflammatory factors and lactate dehydrogenase (LDH) was detected using ELISA. The results showed that compared with the Control group, H/R treatment significantly decreased cell viability, upregulated miR-223-3p expression, and increased the levels of TLR4, p-p65, TNF- α , IL-6, and LDH. No significant difference was observed between the H/R+NC and H/R groups. However, inhibition of miR-223-3p further reduced cell viability and markedly exacerbated the expression of TLR4 and p-p65, as well as the release of inflammatory factors. These findings suggest that miR-223-3p may alleviate H/R-induced cardiomyocyte injury by negatively regulating the TLR4/NF- κ B signaling pathway, whereas its inhibition aggravates inflammatory response and cellular damage, indicating its potential protective role in myocardial ischemia/reperfusion injury.

Keywords: miR-223-3p, TLR4/NF- κ B signaling pathway, hypoxia/reoxygenation, cardiomyocyte injury

1. Introduction

Myocardial ischemia-reperfusion (I/R) injury is a common clinical complication following acute myocardial infarction, cardiac surgery, and percutaneous coronary intervention. It is characterized by secondary cardiomyocyte damage triggered by the restoration of blood flow after a transient ischemic episode, which significantly impacts patient prognosis [1]. Although reperfusion therapy is essential for salvaging ischemic myocardium, the process of reperfusion itself exacerbates cellular injury through mechanisms such as oxidative stress, calcium overload, mitochondrial dysfunction, and inflammatory responses, potentially leading to malignant arrhythmias or heart failure [2, 3]. While current clinical interventions can partially mitigate this damage, limitations remain, including suboptimal efficacy and lack of targeted therapies [4]. Therefore, a deeper understanding of the molecular mechanisms underlying I/R injury and the identification of novel therapeutic targets are of paramount clinical importance.

MicroRNAs (miRNAs), as key regulators of gene expression, play a significant role in myocardial I/R injury [5, 6]. Studies have shown that miRNAs can influence the progression of injury by regulating processes such as apoptosis, autophagy, and inflammatory pathways [5]. For instance, miR-21 reduces apoptosis and promotes cardiomyocyte survival by activating the PI3K/Akt signaling pathway [7]. These findings suggest that miRNAs may exert bidirectional regulatory effects at different pathological stages, with their functions being highly dependent on the specificity of their target genes and signaling pathways.

Recent studies have revealed a complex role for miR-223-3p in cardiovascular diseases [8, 9]. In an atherosclerosis model, miR-223-3p alleviates plaque inflammation by inhibiting the NLRP3 inflammasome [10]; whereas in mouse hearts upon I/R, miR-223-3p decreased I/R-triggered cardiac

necroptosis, infarction size, and dysfunction [11]. Notably, miR-223-3p has been demonstrated to directly target the 3'UTR of TLR4 mRNA, thereby inhibiting the TLR4/NF- κ B signaling pathway [12]. However, its specific role in myocardial I/R injury remains unclear. The TLR4/NF- κ B signaling pathway, a central regulatory axis of inflammation, drives cardiomyocyte death in I/R injury by releasing pro-inflammatory factors such as TNF- α and IL-6 [13, 14]. Nevertheless, direct inhibitors of this pathway face challenges in clinical translation due to systemic toxicity. If miR-223-3p can specifically modulate TLR4/NF- κ B activity, it may offer a novel direction for developing precise therapeutic strategies. Based on this rationale, the present study established a H/R model in H9c2 cardiomyocytes to investigate the effects of miR-223-3p on cell viability, the release of inflammatory factors, and TLR4/NF- κ B pathway activity. This study aims to elucidate the regulatory mechanism of miR-223-3p in I/R injury, providing an experimental basis for miRNA-targeted cardioprotective strategies.

2. Materials and Methods

2.1 Cells and Reagents

H9c2 cells, a rat cardiac myoblast line, were acquired from iCell Bioscience. The miR-223-3p inhibitor and its negative control (NC) were sourced from RiboBio (Guangzhou, China). The Cell Counting Kit-8 (CCK-8) for assessing cell viability was obtained from Beyotime (Shanghai, China). Antibodies against TLR4, NF- κ B p-p65 were purchased from Bioss (Beijing, China). ELISA kits for detecting TNF- α , IL-6, and LDH were acquired from Mlbio (Shanghai, China).

2.2 Cell Culture

Cryopreserved H9c2 cells were rapidly thawed in a 37°C water bath, centrifuged at 1,000 rpm for 5 min, and resuspended in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were seeded in 60 mm dishes and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Upon reaching 80% confluence, cells were detached with 0.25% trypsin–EDTA and subcultured at a 1:2 ratio. Cells from passages 3 to 5 were used for all experiments.

2.3 H/R Model

Upon reaching adherence, cells were washed and incubated in glucose-free DMEM under hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) at 37°C for 6 h in a hypoxia workstation. The glucose-free medium was then replaced with normal DMEM containing 10% FBS, and cells were returned to normoxic conditions (5% CO₂, 95% air) at 37°C for 16 h for reoxygenation.

2.4 Cell Transfection and Experimental Grouping

Four groups were established: (1) Control group — cells cultured under normoxic conditions without H/R; (2) H/R group — cells subjected to H/R treatment only; (3) H/R + NC group — cells transfected with negative control before H/R; and (4) H/R + miR-223-3p inhibitor group — cells transfected with miR-223-3p inhibitor before H/R. For transfection, miR-223-3p inhibitor or NC was mixed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a 1:2 ratio and incubated at room temperature for 15 min to form transfection complexes. The complexes were added to serum-free DMEM and incubated with cells at 37°C for 6 h, after which the medium was replaced with complete DMEM. After 48 h of further incubation, cells were subjected to H/R treatment.

2.5 Cell Viability Assay

H9c2 cells were seeded in 96-well plates at a density of 1×10^4 cells/well with six replicate wells per group. After H/R treatment, 10 μ L of CCK-8 reagent was added to each well, and the plates were incubated at 37°C in the dark for 2 h. Absorbance was measured at 450 nm using a microplate reader.

2.6 RNA Extraction and RT-qPCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). RNA concentration and purity were determined using a NanoDrop spectrophotometer (A260/A280 > 1.8). Complementary DNA (cDNA) was synthesized using a miRNA-specific stem-loop reverse transcription primer and a

PrimeScript RT reagent kit (TaKaRa, RR047A, Shiga, Japan). Quantitative PCR was performed using SYBR Green Master Mix (TaKaRa, RR820A) with specific primers. The amplification protocol was as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s. The miR-223-3p forward primer sequence was 5'-GCTCGCCTGTCAGTTTGTCAAATACC-3'; the reverse primer and U6 snRNA primers were provided with the tailing-based RT-qPCR kit (Sangon Biotech, B532451, Shanghai, China). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method with U6 as the internal reference.

2.7 Western Blot Analysis

Cells were lysed in RIPA buffer containing 1% phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were determined using the bicinchoninic acid (BCA) method. Equal amounts of protein (30 µg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against TLR4 (1:1,000) and p-p65 (1:800). After washing three times with Tris-buffered saline containing Tween-20 (TBST), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) substrate, and band intensities were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). GAPDH served as the loading control.

2.8 ELISA

Cell culture supernatants were collected and centrifuged at 3,000 rpm for 10 min at 4°C to remove debris. The concentrations of TNF- α , IL-6, and LDH were measured using commercial ELISA kits according to the manufacturers' instructions. Briefly, standards and samples were added to pre-coated wells, followed by sequential incubation with biotinylated detection antibody and HRP-conjugated streptavidin at 37°C. After tetramethylbenzidine (TMB) substrate development, the absorbance was measured at 450 nm, and cytokine concentrations were calculated against standard curves.

2.9 Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software. Quantitative data are expressed as mean $\pm$ standard deviation. Differences among multiple groups were compared using one-way analysis of variance (ANOVA). A P-value of less than 0.05 was considered statistically significant. Data are expressed at least three independent experiments.

3. Results

3.1 MiR-223-3p Expression

The PCR results demonstrated that the expression of miR-223-3p was up-regulated after cell modeling and was significantly down-regulated following the addition of the miR-223-3p inhibitor (Figure 1).

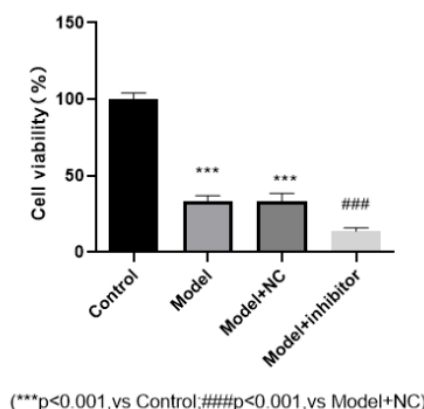


Figure 1: The cell viability was detected by CCK8.

3.2 MiR-223-3p Regulates H9c2 Cell Viability

According to the CCK8 assay, cell viability was reduced following modeling, and this reduction was exacerbated by the miR-223-3p inhibitor(Figure 2).

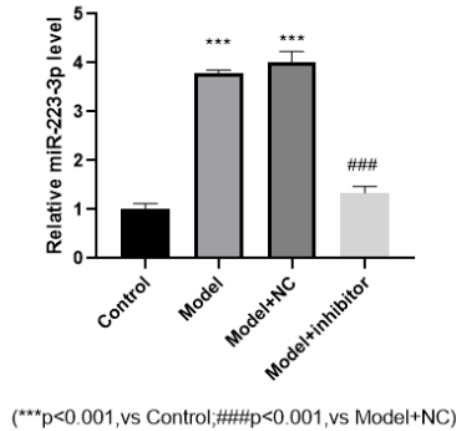


Figure 2: RT-qPCR detection of miR-223-3p expression in different cell groups.

3.3 Western Blot

Western blot analysis revealed that the expression of p-p65 and TLR4 was up-regulated after establishing the cardiomyocyte H/R model and was further up-regulated following treatment with the inhibitor (Figure 3).

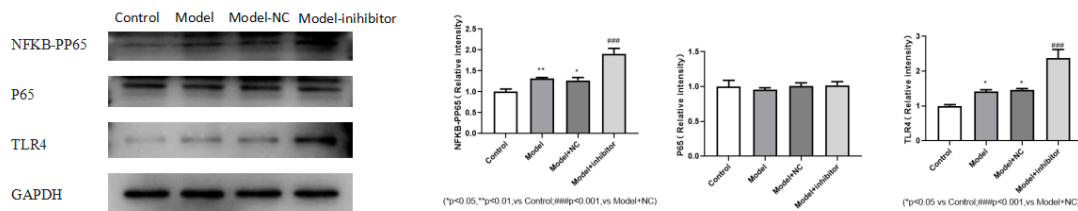


Figure 3: The protein expression levels of TLR4, p-p65 were detected by Western Blot.

3.4 ELISA

The ELISA results demonstrated that the expression levels of TNF- α , IL-6, and LDH were up-regulated after cell modeling and were further up-regulated following intervention with the inhibitor (Figure 4).

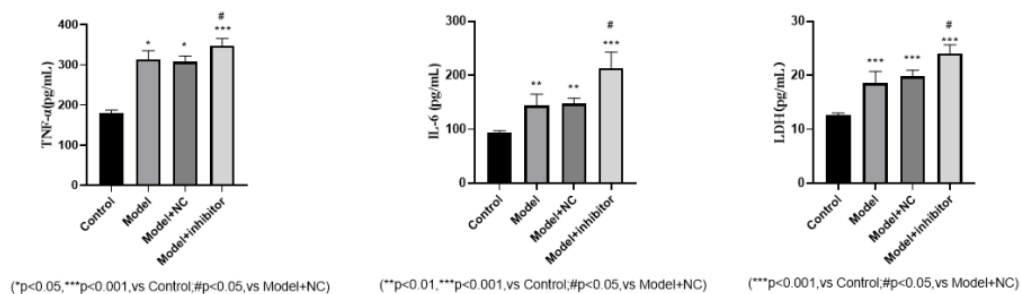


Figure 4: The protein expression levels of TNF- α , IL-6 and LDH were detected by Western Blot.

4. Discussion

This study demonstrates that miR-223-3p plays a critical role in H/R-induced cardiomyocyte injury by regulating the TLR4/NF- κ B signaling pathway. The expression of miR-223-3p was up-regulated in the H/R group. However, following the inhibition of miR-223-3p, the protein levels of TLR4 and p-p65, as well as the levels of inflammatory factors (TNF- α , IL-6, LDH), were further significantly elevated, suggesting that miR-223-3p may alleviate inflammatory injury by negatively regulating the TLR4/NF- κ B pathway. The TLR4/NF- κ B pathway is a central mechanism in myocardial I/R injury, and its activation induces the release of pro-inflammatory factors such as TNF- α and IL-6 [15, 16]. This mechanism aligns with the findings of Wang et al. In the atherosclerotic ApoE^{-/-} mice models, where miR-223 negatively regulated the activation of the TLR4/NF- κ B pathway [12]. Although this study did not directly validate the targeting relationship, the observed trends in protein expression support a potential regulatory interaction between them. No significant difference was observed between the H/R+NC group and the H/R group, which ruled out non-specific effects and strengthened the reliability of the conclusion. Notably, the up-regulation of miR-223-3p under H/R stress may represent an endogenous protective response of the cells. However, this protective effect appears to have a ceiling; its excessive inhibition leads to over-activation of the TLR4/NF- κ B pathway and exacerbates inflammatory injury. Furthermore, the regulatory network of miR-223-3p may not be limited to TLR4. Previous studies have suggested that it can also target the NLRP3 inflammasome [10] or the STAT3 pathway [17] to co-regulate apoptosis and inflammatory responses. Future studies should combine dual-luciferase reporter assays to identify its direct targets and employ animal models to verify its *in vivo* efficacy.

The limitations of this study include the lack of causal validation between miR-223-3p and TLR4, as well as insufficient investigation into other relevant pathways such as apoptosis and autophagy. Future work could involve constructing TLR4-knockout cell lines and establishing a mouse I/R model to evaluate the therapeutic potential of miR-223-3p and facilitate its clinical translation.

5. Conclusions

Our study demonstrates that miR-223-3p may alleviate H/R-induced cardiomyocyte injury by inhibiting the TLR4/NF- κ B signaling pathway, whereas inhibiting its expression significantly exacerbates inflammatory responses and cell death. These findings provide a novel perspective on miRNA-mediated regulation in myocardial I/R injury, yet further *in vivo* experiments are required to validate its clinical potential.

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