

Overexpression of microRNA-146 protects against oxygen-glucose deprivation/recovery-induced cardiomyocyte apoptosis by inhibiting the NF- κ B/TNF- α signaling pathway

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Abstract. MicroRNA (miR) has been reported to be associated with ischemia and reperfusion (I/R) and cell apoptosis. Suppression of cell apoptosis may reduce the irreversible damage induced by reperfusion. The aims of the current study were to explore the cytoprotective effects of miR-146 against oxygen-glucose deprivation/recovery (OGD/R)-induced injury in H9c2 rat myocardial cells, as well as the underlying mechanisms. Following stimulation with OGD/R, the cells were transfected with miR-146 mimics or negative controls. The levels of miR-146 were analyzed by reverse transcription-quantitative polymerase chain reaction. Thereafter, cell viability and cell apoptosis were analyzed by MTT assay and terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling assay, respectively. In addition, the levels of tumor necrosis factor (TNF)- α were determined by ELISA and the levels of B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2 and phosphorylated (p)-nuclear factor (NF)- κ B were measured by western blotting. The results demonstrated that overexpression of miR-146 significantly increased cell viability and decreased apoptosis ($P < 0.05$). It was observed that overexpression of miR-146 statistically reduced the levels of Bax, TNF- α and p-NF- κ B but markedly upregulated the levels of Bcl-2 ($P < 0.05$). These results indicate that overexpression of miR-146 may protect against OGD/R-induced cardiomyocyte apoptosis. Overexpression of miR-146 may alleviate the irreversible injury associated with reperfusion and

the effects may be achieved by inhibiting the NF- κ B/TNF- α signaling pathway.

Introduction

Myocardial infarction is a common cardiovascular event, which occurs following prolonged ischemia of the coronary arteries. It is responsible for heart failure and sudden death, and is associated with longer hospital stays (1). Timely and effective blood flow restoration to the ischemic myocardium helps cardiomyocyte survival, and reduces cardiac morbidity and mortality. However, reperfusion leads to ischemia and reperfusion (I/R) injury, which can cause irreversible damage and subsequent tissue remodeling (2). It has been well demonstrated that myocardial apoptosis is observed in human acute myocardial infarcts and is primarily triggered during reperfusion through various mechanisms (3-5). Increasing evidence suggests that suppression of myocardial apoptosis decreases infarct size and improves regional contractile dysfunction during reperfusion (3). Therefore, it is important to study how to inhibit myocardial apoptosis associated with reperfusion.

MicroRNAs (miRNAs/miRs) are a class of short (19-25 nucleotides), single-stranded, non-coding RNA molecules, which modulate gene expression at the post-transcriptional level by base pairing with the 3'untranslated region (6). It has been acknowledged that miRNAs are involved in diverse physiological and pathological processes, including cell proliferation and apoptosis (7,8). Emerging evidence has suggested that various miRNAs serve a critical role in I/R (9-12). Among miRNAs, miR-146 has been reported to protect against liver (13,14) small intestine (15) and myocardial I/R injury (16). In addition, miR-146 has been reported to negatively regulate the production of proinflammatory cytokines, including interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)- α , by the nuclear factor (NF)- κ B signaling pathway (17). Activation of NF- κ B has been reported to be involved in myocardial I/R (18). However, little information is available regarding the protective effects of miR-146 against I/R injury in the myocardium via inhibiting NF- κ B pathway and inflammatory cytokine production.

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The current study aimed to investigate whether miR-146 overexpression offered protection against myocardial I/R injury by inhibiting the NF- κ B pathway and production of the inflammatory cytokine, TNF- α . A cellular oxygen-glucose deprivation/recovery (OGD/R) model of I/R was induced in H9c2 rat myocardial cells. Following overexpression of miR-146 in the cells, cell viability and cell apoptosis, as well as the underlying mechanism, were analyzed.

Materials and methods

Cell culture and treatment. H9c2 rat myocardial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin G (Gibco; Thermo Fisher Scientific Inc., Waltham MA, USA), 100 μ g/ml streptomycin (Gibco; Thermo Fisher Scientific Inc.) and 2 mM glutamine (Gibco; Thermo Fisher Scientific Inc.). The cells were allowed to grow to 80% confluence and were then used for further experiments. The cells in the control group were untreated, and the cells in the miR-Ctrl and miR-146 mimics groups were first stimulated by OGD/R and then transfected with miR-146 mimic or mimic control. The cells were stimulated by OGD/R as previously described (19). Briefly, cells were washed twice with PBS and incubated in glucose-free DMEM. Thereafter, the cells were placed in an anaerobic chamber containing a mixture of 95% N₂ and 5% CO₂ at 37°C for 6 h, after which 4.5 mg/ml glucose was added. Subsequently, the cells were incubated in an atmosphere containing 95% air and 5% CO₂ for another 18 h.

Transfection. Mature miR-146 mimics and mimic control (miR-Ctrl) were designed and synthesized by Shanghai GenePharma Co., Ltd., (Shanghai, China). For stable transfection, the cells (5x10⁵ cells/well) were seeded on 6-well plates and were transiently transfected with 100 nM miR-146 mimic or miR-Ctrl for 48 h using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The sequences were as follows: miR-146 mimic, sense 5'-UGAGAACUGAAUCCAUGGGUU-3' and antisense 5'-CCCAUGGAAUUCAGUUCUCAUU-3'; and miR-Ctrl, sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'. The cell suspension was collected for further analyses. Untreated cells were considered the control group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Transfection efficiency was confirmed by RT-qPCR. Total RNA, including miRNA, was extracted from the cells with TRIzol (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. RT-PCR was performed using the Multiscribe RT kit (Applied Biosystems; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The contents of the kit included 1x100 μ l MultiScribe TM Reverse Transcriptase (50 U/ μ l), 1x100 μ l RNase Inhibitor (20 U/ μ l), 1x1 ml Dntp Mixture (2.5 mM each dNTP), 1x100 μ l Oligo d(T)16 (50 μ M), 1x100 μ l Random Hexamers (50 μ M), 1x1.5 ml 10X RT Buffer, and 1x1.5 ml MgCl₂ Solution. The

RNA was denatured at 70°C for 5 min and then put on ice for 2 min. Thereafter, the solution was added followed by incubation for 5 min at 25°C then at 42°C for 60 min and finally at 70°C for 5 min. The levels of miRNA were determined using SYBR Advantage qPCR Premix (Takara Bio Inc., Otsu, Japan), and the reactions were carried out with an ABI PRISM 7900HT Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific Inc.). The thermocycling conditions were as follows: 1 min at 95°C, 34 cycles of 94°C for 30 sec, 62°C for 30 sec and 72°C for 2 min, and a final extension at 72°C for 5 min. MiR expression was quantified by the comparative 2^{- $\Delta\Delta$ C_q} method (20) and U6 small nuclear RNA was used as a loading control. The primers used were as follows: miR-146, forward, 5'-CCGATGTGTATCCTCAGC TTTG-3' and reverse, 5'-GCTGAAGAACTGAATTTTCAGA GGTC-3'; and U6, forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'.

Cell viability. Following stimulation with OGD/R and transfection with miR-146 mimics or miR-Ctrl, cell viability was analyzed by an MTT colorimetric assay based on a standardized method (21). Briefly, the cells were seeded in 96-well plates and treated with OGD/R and transfected with miR-146 mimic or miR-Ctrl. After treatment for 0 and 72 h, and 7 days, the cells were treated with 5 mg/ml MTT (20 μ l; Invitrogen; Thermo Fisher Scientific Inc.) and were incubated at 37°C for 4 h. Subsequently, dimethyl sulfoxide (100 μ l; Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. Absorbance at 590 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay. A TUNEL assay was performed to analyze apoptosis using the *In Situ* Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland) according to a standard protocol (22). Briefly, H9c2 cells were treated with OGD/R and transfected with miR-146 mim or miR-Ctrl. Thereafter, the cells were incubated with terminal deoxynucleotidyl transferase and biotinylated dUTP. Flow cytometric analysis (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) was performed to analyze the apoptotic cells.

Western blotting. Following treatment with OGD/R and transfection with miR-146 mimics or miR-Ctrl, the cell suspension was harvested, centrifuged at 6,400 x g for 10 min at 4°C, and lysed in radioimmunoprecipitation assay lysis buffer with protease inhibitor (Merck & Co., Whitehorse Station, NJ, USA). The proteins were quantified using a Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific Inc.). Equal amounts of protein (20 μ g/lane) were separated by 10-12% SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.). Thereafter, the membranes were blocked with 5% skim milk in Tris-buffered saline with 1% Tween (TBST) for 1 h and probed with the following primary antibodies overnight at 4°C: Anti-B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. ab32503; 1:1,000; Abcam, Cambridge, UK), anti-Bcl-2 (cat. no. ab32124; 1:1,000; Abcam) and anti-phosphorylated NF- κ B (cat. no. ab16502; 1:1,000; Abcam). GAPDH (cat. no. ab9485; 1:2,000; Abcam) was used as an internal control. After

washing with TBST, the membranes were exposed to goat anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (cat. no. ab6721; dilution 1:5,000; Abcam) for 2 h at room temperature. Protein bands were visualized with WEST-ZOL-plus Western Blot Detection system (Intron Biotechnology, Inc., Seongnam, South Korea) using enhanced chemiluminescent reagents (ECL-Plus; Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Optical band densities were quantified using ImageJ software v1.41 (National Institutes of Health, Bethesda, MD, USA).

ELISA. Following treatment with OGD/R and transfection with miR-146 mimic or miR-Ctrl, the cell suspension was collected and centrifuged at 600 x g for 5 min. The supernatant was removed and stored at -80°C until further use. The levels of TNF- α were analyzed by sandwich ELISA (cat. no. RTA00; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analysis. Each experiment was run in triplicate. The data are presented as the mean \pm standard deviation. Statistic Package for Social Science (SPSS, version 16.0; SPSS, Inc., Chicago, IL, USA) statistical software was used to analyze statistical differences. One-way analysis of variance with a Bonferroni's post hoc test were performed for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression levels of miR-146. To explore the potential functional role of miR-146 in myocardial I/R, a cellular OGD/R model of I/R was generated using H9c2 cells. The cells were first stimulated by OGD/R and were then transfected with miR-146 mimics or miR-Ctrl. The levels of miR-146 were analyzed by RT-qPCR. Transfection with miR-146 mimics significantly increased the levels of miR-146 compared with in the miR-Ctrl group ($P < 0.05$; Fig. 1), indicating a high transfection efficiency.

Effects of miR-146 overexpression on cell viability. Following miR-146 overexpression, cell viability was analyzed by MTT. As shown in Fig. 2, the results revealed that compared to the untreated cells, cell viability was significantly reduced by OGD/R stimulation and transfection with miR-Ctrl ($P < 0.05$). However, cell viability significantly increased following transfection with miR-146 mimics compared with in the miR-Ctrl group ($P < 0.05$). These results demonstrated that overexpression of miR-146 promoted cell viability following I/R.

Effects of miR-146 overexpression on cell apoptosis, and on TNF- α and p-NF- κ B levels. A TUNEL assay was performed to determine the effects of miR-146 overexpression on cell apoptosis. As demonstrated in Fig. 3A, it was observed that the percentage of apoptotic cells was significantly greater following OGD/R stimulation and transfection with miR-Ctrl. However, apoptosis was significantly decreased by overexpression of miR-146 compared with the miR-Ctrl group

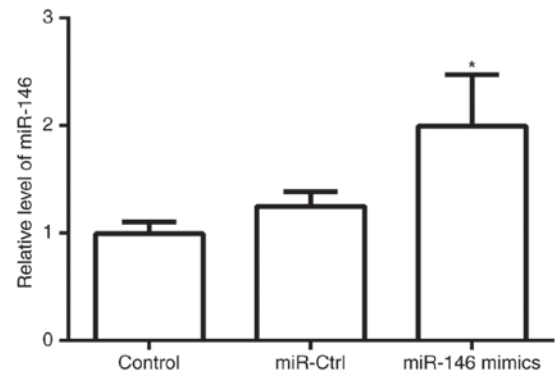


Figure 1. Expression levels of miR-146 in H9c2 cells that were first stimulated by OGD/R and were then transfected with miR-146 mimics or miR-Ctrl. The levels of miR-146 were analyzed by reverse transcription-quantitative polymerase chain reaction. The expression levels of miR-146 were increased by OGD/R stimulation and transfection with miR-146 mimics. * $P < 0.05$ vs. the miR-Ctrl group. miR, microRNA; OGD/R, oxygen-glucose deprivation/recovery.

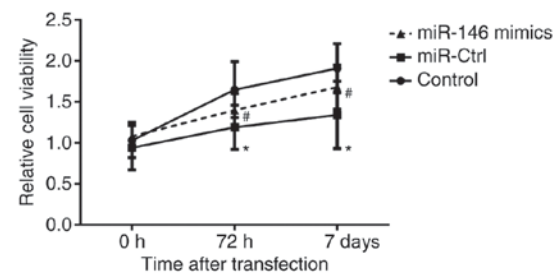


Figure 2. Effects of miR-146 overexpression on cell viability. Following stimulation with oxygen-glucose deprivation/recovery and transfection with of miR-146 mimics or miR-Ctrl, cell viability was analyzed by MTT. Cell viability was significantly increased by overexpressing miR-146 compared with the miR-Ctrl group. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. miR-Ctrl group. miR, microRNA.

($P < 0.05$). The underlying mechanisms of cell apoptosis were further explored through the expression of Bax and Bcl-2. The results revealed that miR-146 overexpression significantly decreased the protein expression levels of Bax but significantly increased the levels of Bcl-2 (Fig. 3B and C). This indicated that miR-146 overexpression inhibited apoptosis of ischemic cells by decreasing Bax and increasing Bcl-2. The underlying signaling pathway associated with the effects of miR-146 overexpression on ischemia cells was then investigated. TNF- α and p-NF- κ B levels were analyzed following miR-146 overexpression by ELISA and western blotting, respectively. Stimulation with OGD/R markedly upregulated the expression of TNF- α and p-NF- κ B ($P < 0.05$) in comparison to the untreated cells. p-NF- κ B levels were normalized to total NF- κ B levels (data not shown). Total NF- κ B protein was measured, including phosphorylated and non-phosphorylated forms. The ratio of phosphorylated form and non-phosphorylated form indicates the activity of NF- κ B. There effects were reversed by overexpressing miR-146 when compared with the miR-Ctrl group ($P < 0.05$; Fig. 3D and E). Representative images of the western blot are shown in Fig. 3F. Analysis of the data indicated that the overexpression of miR-146 inactivated the NF- κ B/TNF- α signaling pathway.

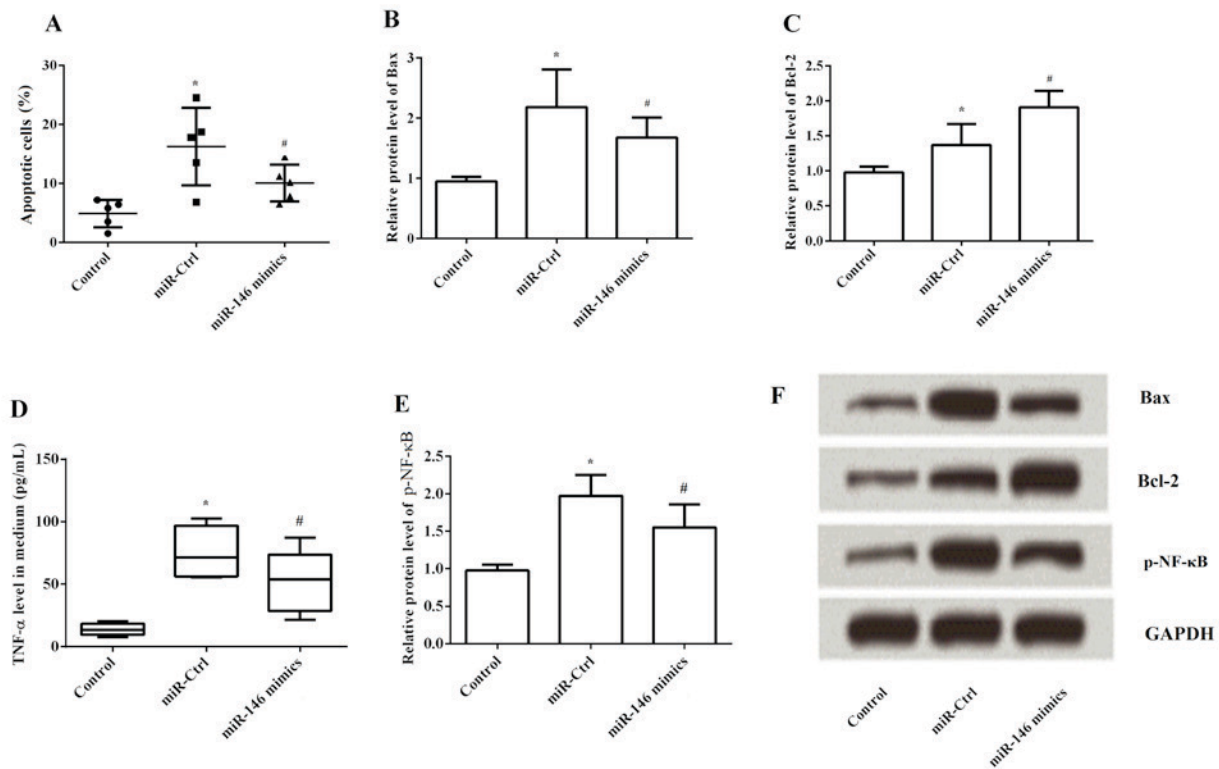


Figure 3. Effects of miR-146 overexpression on cell apoptosis, TNF- α and p-NF- κ B levels. A terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling assay, ELISA and western blotting were used to determine the effects of miR-146 overexpression on cell apoptosis, TNF- α and p-NF- κ B levels, respectively. (A) Percentage of apoptotic cells significantly decreased by overexpression of miR-146 compared with the miR-Ctrl group. (B) Semi-quantitative analysis of Bax protein expression, which was significantly reduced by overexpression of miR-146 compared with the miR-Ctrl group. (C) Semi-quantitative analysis of Bcl-2 protein expression, which was significantly increased by overexpression of miR-146 compared with the miR-Ctrl group. (D) Overexpression of miR-146 markedly decreased the levels of TNF- α . (E) Overexpression of miR-146 significantly reduced the levels of p-NF- κ B. (F) Representative western blot images. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. miR-Ctrl group. Bax, B-cell lymphoma 2-associated X protein; Bcl-2, B-cell lymphoma 2; p-NF- κ B, phosphorylated nuclear factor- κ B; TNF- α , tumor necrosis factor- α .

Discussion

In the present study, the potential cytoprotective effects of miR-146 against OGD/R-induced myocardial cell injury, as well as the underlying mechanisms, were investigated. The results demonstrated that miR-146 overexpression significantly increased cell viability and decreased apoptosis of H9c2 rat myocardial cells. In addition to this, it was observed that overexpression of miR-146 could significantly reduce the protein levels of Bax, TNF- α and p-NF- κ B, and increase the levels of Bcl-2. The results of the current study suggested that miR-146 overexpression may protect against OGD/R-induced cardiomyocyte apoptosis, which may be achieved by inhibiting the NF- κ B/TNF- α signaling pathway.

miRNAs are critically involved in the pathological alterations of the heart, including cardiac hypertrophy (23), arrhythmogenesis (24), heart failure (25) and angiogenesis (26), as well as myocardial I/R injury (27). It has been reported that specific protectomiRs may serve as potential therapeutic tools for treating I/R injury (28). For example, locked nucleic acid-modified antisense miR-92a exerts cell-protective, proangiogenic and anti-inflammatory effects on a porcine model of myocardial I/R. Inhibition of miR-92a may be a novel therapeutic tool to preserve cardiac function following ischemia (11). miR-146 was first identified as an immune system modulator that affects the mammalian response to

microbial infection (29). It has been reported that miR-146 serves a critical role in regulating numerous cell functions, including cell proliferation and apoptosis, and is involved in various human diseases, such as cancers, rheumatoid arthritis and Alzheimer's disease (30-32). In addition, increasing evidence has indicated that miR-146 is also involved in I/R. Jiang *et al* (13) proposed that miR-146a ameliorated liver I/R injury *in vivo* and hypoxia/reoxygenation injury *in vitro* by directly suppressing IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6). In addition, Wang *et al* (16) observed that the increased expression of miR-146a protected against myocardial I/R injury by significantly reducing myocardial infarct size and preventing I/R-induced cardiac dysfunction. The underlying mechanisms may be associated with the weakening of NF- κ B activation and inflammatory cytokine production through the inhibition of IRAK1 and TRAF6. The protective role of miR-146 in myocardial I/R injury in the current study was partly similar. miR-146 exerted its cytoprotective effects against OGD/R-induced cardiomyocyte apoptosis by inhibiting the NF- κ B/TNF- α signaling pathway.

In addition to necrosis, several lines of evidence suggest that myocardial apoptosis is also initiated and enhanced during reperfusion through numerous mechanisms (3,33,34). Therefore, an improved understanding of the cellular mechanisms underlying apoptosis may aid in the prevention

of reperfusion-associated irreversible injury. Apoptosis is a highly regulated process and the balance between apoptosis-promotion and apoptosis-inhibition decides the cell fate. Bcl-2 and Bax are two such regulatory proteins that serve pivotal roles in apoptosis, and have been investigated in myocardial I/R injury (35,36). Bcl-2 is an apoptosis inhibitor, whereas Bax is a proapoptotic protein. Overexpression of Bcl-2 and down-regulation of Bax attenuate apoptosis. The results the present study demonstrated that overexpression of miR-146 increases cell viability and decreases cell apoptosis. The mechanism underlying cell apoptosis may be associated with increasing and decreasing levels of Bcl-2 and Bax, respectively.

It has been demonstrated that reperfusion injury initiated by inflammatory cascades results in perpetual damage to cardiac tissue following ischemia (37). Therefore, regulation of the inflammatory response may be a potential pharmacological target to protect the heart from I/R injury (38). Among the inflammatory cascades, the transcription factor NF- κ B is one of the central players in this cascade and is known for modulating the transduction of inflammatory signals and cytokine production. NF- κ B is activated early on in reperfusion. Activation of NF- κ B results in translocation to the nucleus, where numerous effector genes are stimulated, many of which are important regulators of cell apoptosis and inflammation (39). For example, some cytokines, including TNF- α and IL-6, are motivated by NF- κ B (40), which then enhance the production of proinflammatory cytokines. Previous studies have confirmed higher levels of TNF- α following I/R (41,42) and upregulation of TNF- α subsequently results in amplified cytokine effects. TNF- α is responsible for myocardial dysfunction by direct inhibition of contractility and stimulation of myocyte apoptosis (43). Furthermore, it has been reported that a neutralizing antibody against TNF- α exerts protective anti-apoptotic effects in cardiomyocytes (44,45). Because of the important role of NF- κ B/TNF- α in I/R, the effects of overexpression of miR-146 on the expression of TNF- α and p-NF- κ B were analyzed. The expression levels of TNF- α and p-NF- κ B were significantly decreased through the overexpression of miR-146, which suggested an inactivating effect of miR-146 on NF- κ B/TNF- α .

In conclusion, the results from the current study suggested that miR-146 protects against OGD/R-induced cardiomyocyte apoptosis, and the effects may be achieved by inhibiting the NF- κ B/TNF- α signaling pathway. Overexpression of miR-146 may provide a novel therapeutic and preventive target for the treatment of myocardial I/R injury.

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