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Inhibition of microRNA-346 inhibits myocardial inflammation and apoptosis after myocardial infarction *via* targeting NFIB

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Abstract. – OBJECTIVE: Acute myocardial infarction (AMI) is a sudden cardiovascular event that endangers human life. MicroRNA is considered to be an important participant in the pathophysiology of myocardial infarction (MI). This article aim was to study the function and mechanisms of microRNA-346 (miR-346) on myocardium after MI.

MATERIALS AND METHODS: To study the role of miR-346 in MI, we established H_2O_2-induced H9c2 cell injury model and rat MI model. Real-time polymerase chain reaction (RT-PCR) was used to detect miR-346 expression. Western blot was utilized to measure the expression of Bcl-2, Bax, TNF-α, IL-6 and NFIB. Apoptosis of H9c2 cells was detected by TUNEL staining and flow cytometry. Enzyme-linked immunosorbent assay (ELISA) assay was utilized to measure the levels of TNF-α and IL-6 in supernatant. Assessment of left ventricular function in rats was performed using echocardiography.

RESULTS: MiR-346 was significantly upregulated in H_2O_2-treated H9c2 cells and ischemic myocardium. In the H9c2 cell injury model, the expressions of Bax, TNF-α, and IL-6 were greatly increased while Bcl-2 expression was decreased, and the number of TUNEL-positive cells and apoptosis rate were also significantly increased. At the same time, the levels of TNF-α and IL-6 in the cell supernatant were markedly increased. However, after miR-346 expression was suppressed, these results were reversed. The expression of Bcl-2 increased, while the expression of Bax, TNF-α, and IL-6 decreased. The contents of TNF-α and IL-6 in the cell supernatant also decreased significantly. Both the number of TUNEL-positive cells and the apoptosis rate were markedly reduced. After injecting antagomir-346 into the myocardium of rats to silence miR-346, the cardiac function of MI rats was remarkably improved, and the LDH content in the serum of rats also decreased significantly. Using computational predictions tools, Western blotting and Luciferase activity assay, we found that nuclear factor I/B (NFIB) was targeted by miR-346.

CONCLUSIONS: The expression of miR-346 was increased in H9c2 cells and ischemic myocardium of MI rats. Silencing miR-346 can significantly inhibit the inflammatory response and the apoptosis of cardiomyocytes by targeting NFIB.

Key Words:
Myocardial infarction, MicroRNA-346, Inflammation, Apoptosis, NFIB.

Introduction

Sudden cardiac death (SCD) refers to the rapid, natural death, which is mainly characterized by sudden loss of consciousness caused by organic causes of the heart[1]. AMI is one of the main causes of SCD. MI is a serious type of coronary ischemic disease. It is mainly manifested by the continuous ischemia and hypoxia of myocardial cells caused by reduced or blocked blood flow in the coronary arteries in some areas of the heart, and then changes in the structure of cardiomyocytes manifested as necrosis of cardiomyocytes[2]. The further decline of cardiac function was mainly manifested as weakened myocardial contractility, significantly decreased myocardial compliance, and uncoordinated myocardial contractility in the infarcted and non-infarcted areas, etc[3]. The common clinical symptoms were pain in the anterior cardiac area accompanied by radiating pain in the shoulder. According to previous research results, about 12.2% of deaths worldwide are due to coronary ischemia, which ranks first among deaths in middle-and high-income countries[4]. About 700 million people worldwide suffer from MI each year[5]. Although global medical standards have gradually improved in recent years, cardiovascular disease is still one of the three leading causes of death in the United States, and MI is still a major factor in cardiovascular disease[6].

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MicroRNA is a type of non-coding protein RNA involved in the occurrence and development of various diseases and plays a very important role in the progress of disease[7,8]. MiRNAs have a significant effect on cell proliferation and apoptosis, thereby regulating the re-growth and development of cells, organs and tissues[9]. Previous researchers applied bioinformatics and microarray technology to analyze the differential expression profile of miRNA in diseased and normal tissues, and the results showed that the expression of the same miRNA could be upregulated or downregulated in different diseases. In the same disease, the expression level of different miRNAs also tends to change at different levels compared with normal people[10].

MicroRNA-346, as a member of the miRNA family, plays a key role in the occurrence and development of many diseases. MiR-346 can inhibit the proliferation of neural stem cells and promote their apoptosis[11] and can promote the progression of enteritis by down-regulating intestinal mucosal vitamin D receptors[12]. However, little is known about the role of miR-346 in the cardiovascular system. Therefore, this article aims to study the function of miR-346 in the development of MI and its mechanism of action.

Materials and Methods

Rat MI Model

A total of 30 Sprague-Dawley (SD) rats of 10 weeks of age, weighing 250 ± 20g, were purchased from Shanghai Experimental Animal Research Center. The living environment of rats was a standard clean-grade independent ventilating cage system. All rats were fed clean water and standard feed required by the rats. Thirty mice were divided into 3 groups: sham group, MI + negative control (NC) group, MI + antagomir-346 group. The rats were anesthetized with 2% sodium pentobarbital and connected to a small animal ventilator and an electrocardiograph. Anterior descending coronary artery was ligated at the lower edge of the left atrial appendage. When the myocardium turned white and the Electrocardiogram (ECG) showed significant elevation of the ST segment, it proved that the modeling was successful. At the same time, antagomir-346 or NC (RiboBio, Guangzhou, China) was injected into the ischemic heart muscle. Rats in the sham group were only opened the thorax, but the coronary artery was not ligated. Antagomir-346 is a specially chemically modified miR-346 antagonist. It strongly competes with mature miR-346 in the body to prevent the complementary pairing of miR-346 and their target genes, thereby inhibiting miR-346 from functioning. MiR-346 expression in myocardial cells was detected on day 1, 3, and 7 after antagomir-346 injection into myocardium of MI rats. This investigation was approved by the Animal Ethics Committee of Chinese PLA General Hospital Animal Center.

Echocardiographic Measurement

On day 7 of MI in rats, we measured left ventricular cardiac function, and calculated the left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS).

LDH Levels

On the seventh day of MI in rats, the LDH content in rat serum was measured by LDH enzyme linked immunosorbent assay (ELISA) kit (Dojindo Molecular Technologies, Shanghai, China).

Cell Culture and Transfection

H9c2 cells (Jining Shiye, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Procell, Wuhan, China), which contained 10% fetal bovine serum (FBS) (Procell, Wuhan, China) and 1% penicillin/streptomycin (Procell, Wuhan, China). To study the role of miR-346 in H9c2 cells, miR-346 inhibitor (INT) or negative control (NC) (KeyGEN, Shanghai, China) was transfected into H9c2 cells in accordance with the protocols. 24 hours later, H9c2 cells were then treated with 100 μM of H_2O_2 for 4 hours to establish H9c2 cell injury model. So, H9c2 cells were divided into 4 groups: control group, H_2O_2 group, H_2O_2 + NC group, H_2O_2 + INT group.

RNA Extraction and Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA from H9c2 cells and myocardial tissue was extracted using TRIzol reagent (MCE, Nanjing, China). 1 μl of total RNA was taken and the optical density value at 260 and 280 nm wavelengths was measured with a spectrophotometer respectively, and then the RNA concentration was calculated automatically. For miRNA cDNA synthesis, miScript RT II kit (Qiagen, Shanghai, China) was used to synthesize a miRNA cDNA library in a 20 μl reaction system using 1 μg of

total RNA as a template. The reaction system composition was 2 μl of 10 × miScript Nucleics Mix, 4 μl of 5 × miScript Hispec/Hiflex buffer, 2 μl of miScript reverse transcriptase, 10 μl of ribozyme-free water, and 2 μl of total RNA template. Immediately after the reverse transcription was completed, it was cooled on ice and stored at -20°C.

After reverse transcription was completed, the miScript SYBR Green PCR kit (Qiagen, Shanghai, China) was used for the amplification reaction using 10 ng miRNA cDNA as a template. The composition of 20 μl reaction system was 2 μl of 10 × miScript Universal Primer, 10 μl of 2 × QuantiTect SYBR Green PCR Master Mix, 2 μl of 10 × miScript miR-346 or U6 primer (Qiagen, Shanghai, China), 5 μl of ribonuclease-free water, 1 μl of miRNA cDNA template. U6 was the internal control of miR-346. All the primers were listed in Table I.

Western Blot

Total protein extraction kit (KeyGen, Shanghai, China) was used to extract the total protein of 4 groups of H9c2 cells. The protein concentration of the samples was then measured using a bicinchoninic acid (BCA) detection kit (KeyGen, Shanghai, China). The concentration of the protein standard is 1 mg/ml. In a 96-well plate, 10 μl, 8 μl, 6 μl, 4 μl, 2 μl, and 0 μl protein standards were added to the duplicate wells, and ddH_2O was added to make up to 20 μl. 2 μl of sample protein was added to each well and ddH_2O was then added to make up to 20 μl. Finally, the A and B solutions were mixed thoroughly at a ratio of 50: 1, and 200 μl of the A and B mixed solutions were added to each well. Then, the 96-well plate was covered with a transparent cover film and incubated at 37°C for 30 min. Microplate reader was used to read the OD value of each well at 570 nm. After calculating the standard curve, the protein concentration of the sample was calculated. After that, we took an appropriate amount of sodium dodecyl sulphate (SDS) loading buffer (KeyGen, Shanghai, China) and mixed it with the protein sample, heated at 100°C for 5 to 10 minutes, made a protein specimen, and stored it at 20°C.

30 μg of the above protein-sodium dodecyl sulfate (SDS) specimen was added to a 10% SDS-polyacrylamide gel and electrophoresed at 120 V. After the electrophoresis was completed, the protein was electro-transferred to a nitrocellulose membrane (EpiZyme, Shanghai, China) in a transfer buffer. The membrane was then immersed in a tris buffered saline (TBS) equilibration solution containing 2% bovine serum albumin (BSA; Jining Shiye, Shanghai, China) and incubated for 60 min at room temperature. Then, the primary antibodies (Bcl-2, Abcam, Cambridge, MA, USA, Mouse, 1:1000; Bax, Abcam, Cambridge, MA, USA, Mouse, 1:1000; TNF-α, Abcam, Cambridge, MA, USA, Mouse, 1:1000; IL-6, Abcam, Cambridge, MA, USA, Mouse, 1:1000; NFIB, Abcam, Cambridge, MA, USA, Mouse, 1:1000; GAPDH, Abcam, Cambridge, MA, USA, Mouse, 1:1000) were added and incubated at 4°C overnight. After that, the membrane was washed with TBS equilibration solution three times. Then, the secondary antibody containing horseradish peroxidase (HRP) label was used to incubate the specimen membrane at room temperature for 1 h. Finally, a chemiluminescence system was used to detect the signal of the membrane. The signal intensity was quantitatively analyzed using the NIH gel image analysis program.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) Assay

H9c2 cells were seeded in 96-well plate and transfected with miR-346 inhibitor or NC, and then treated with H_2O_2. Then, 20 μl of MTT solution (YEASEN, Shanghai, China) was added and incubated with the cells for 4 hours at 37°C, and then 150 μl of dimethyl sulfoxide (DMSO) solution were added to stop the cell's enzyme reaction. Finally, the absorption light intensity at 490 nm was measured with a spectrophotometer.

Table I. Real Time-PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
miR-346	TGTCTGCCTGAGTGCCT	GTTGTGGTTGGTTGGTTTGT
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

TUNEL Staining

After H9c2 cells were treated as above, they were fixed with paraformaldehyde, and then the cell membrane was destroyed with triton X-100. The cells were then incubated with the prepared TUNEL working solution (Roche, Basel, Switzerland) at 37°C for 1 hour in the dark. An appropriate amount of 4',6-diamidino-2-phenylindole (DAPI) (Roche, Basel, Switzerland) was then added for staining the nucleus. Finally, a fluorescent inverted microscope was used to observe the staining.

Flow Cytometry

H9c2 cells were seeded in a 6-well plate. After being treated as described above, the cells in the cell supernatant and adherent cells were collected by centrifugation. The cells were washed with phosphate-buffered saline (PBS) and collected by centrifugation for a total of 3 times, and then resuspend by adding 200 μl of Binding buffer. 5 μl of Annexin V-FITc (KeyGen, Shanghai, China) and PI (KeyGen, Shanghai, China) were added to each tube. Finally, the apoptosis rate of cells was detected by flow cytometry.

TNF-α and IL-6 Contents

The supernatants of the 4 groups of cells were taken and the contents of TNF-α and IL-6 were detected by TNF-α and IL-6 ELISA kits (Dojindo Molecular Technologies, Shanghai, China), respectively.

LDH Activity

The content of LDH in rat serum was detected by LDH ELISA kit (Dojindo Molecular Technologies, Shanghai, China).

Luciferase Activity Assay

We constructed luciferase reporters (RiboBio, Guangzhou, China) containing wild-type and mutant 3'UTR of NFIB. According to the instructions, the Luciferase reporters and miR-346 mimic or NC (KeyGEN, Shanghai, China) were co-transfected into HEK293T cells (Jining Shiye, Shanghai, China) to investigate whether miR-346 directly binds to 3'UTR of NFIB.

Statistical Analysis

Data were expressed as $\chi \pm s$ and were plotted using GraphPad Prism 5 software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Test level $\alpha=0.05$.

Results

MiR-346 Was Upregulated In Myocardial Ischemia and Hypoxia Injury

After treating H9c2 cells with H_2O_2 (100 μM, 4 h), we detected the expression level of miR-346 by PCR. After the treatment of H_2O_2, miR-346 expression in H9c2 cells was markedly up-regulated (Figure 1A). We also examined the expression of miR-346 in the infarcted zone, border zone and remote zone of the heart of MI rats. It was found that the expression of miR-346 in the infarcted zone and border zone increased remarkably, while the expression in remote zone did not change significantly (Figure 1B-D). Afterwards, we transfected miR-346 inhibitor or NC into H9c2 cells, and PCR detection revealed that miR-346 expression was successfully inhibited (Figure 1E). We also injected antagomir-346 or NC into the infarcted myocardium of rats and tested the expression of miR-346 in the myocardium 1 day, 3 days and 7 days after injection. It can be found from Figure 1F that antagomir-346 greatly inhibited miR-346 expression in the myocardium.

Downregulation of MiR-346 Inhibited Apoptosis and Inflammation of Cardiomyocytes

First, we used Western blot to detect the expression of apoptosis-related proteins and inflammatory factors in H9c2 cells in each group (Figure 2A). We can find that when H9c2 cells were treated with H_2O_2, the expression of Bax, TNF-α and IL-6 was greatly increased, and the expression of Bcl-2 was greatly reduced. However, the downregulation of miR-346 markedly reversed the expression of these proteins (Figure 2B). MTT assay found that inhibition of miR-346 expression can reduce the damage of H9c2 cell viability caused by H_2O_2 (Figure 2C). We also used TUNEL staining to study the effect of miR-346 on cardiomyocytes. The results showed that silencing of miR-346 significantly reduced the number of TUNEL-positive cells (Figure 2D). At the same time, the results of flow cytometry proved that the inhibition of miR-346 can inhibit the apoptosis of cardiomyocytes caused by H_2O_2 (Figure 2E). We also tested the levels of inflammatory factors in the cell supernatants of each group and found that H_2O_2 greatly increased the content of TNF-α and IL-6. However, the content of TNF-α and IL-6 decreased markedly after miR-346 was down-regulated (Figure 2F, 2G). In summary, the downregulation of miR-346 can

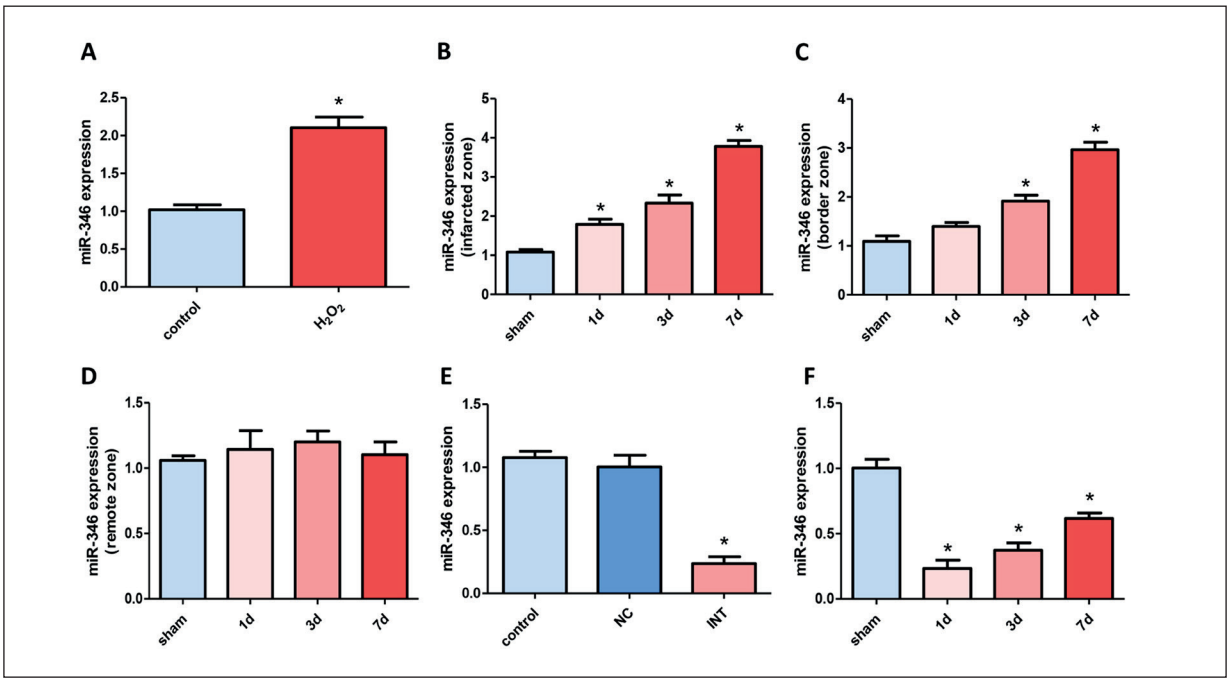

Figure 1. MiR-346 was upregulated in myocardial ischemia and hypoxia injury. **A,** Real-time PCR analysis showed the upregulation of miR-346 expression in H9c2 cells treated with H_2O_2 ("*" $p<0.05$ *vs.* control, n = 3). **B,** Real Time-PCR analysis indicated that miR-346 was increased in the infarcted zones of MI rat ("*" $p<0.05$ *vs.* sham, n = 3). **C,** Real-time PCR analysis indicated that miR-346 was increased in the border zones of MI rat ("*" $p<0.05$ *vs.* sham, n = 3). **D,** MiR-346 level in remote zones of rat ventricles. **E,** MiR-346 expression level was decreased in H9c2 cells after miR-346 inhibitor transfection ("*" $p<0.05$ *vs.* NC, n = 3). **F,** MiR-346 expression in heart tissue was decreased in MI+antagomir-346 group ("*" $p<0.05$ *vs.* sham, n = 3).

markedly inhibit the apoptosis and inflammation of cardiomyocytes caused by H_2O_2.

Downregulation of MiR-346 Improved Cardiac Function of MI Rats

We established a rat MI model to study the effects of miR-346. 7 days after myocardial injection of antagomir-346 or NC, we examined cardiac function of rats using cardiac ultrasonography (Figure 3A). The EF and FS values of MI rats decreased greatly, but the EF and FS values of MI rats injected with antigomir-346 intramyocardially increased remarkably (Figure 3B, 3C). On the 7th day after myocardial infarction in rats, we also tested the serum LDH content. The level of LDH in the serum of rats in the MI + NC group was markedly increased, while the level of LDH in the serum of rats in the MI + antagomir-346 group was markedly decreased (Figure 3D).

Downregulation of MiR-346 Inhibited Apoptosis and Inflammation of H9c2 Cells Via Regulation of NFIB

Using the TargetScan database for analysis, NFIB was predicted to be the target of miR-346 and Figure 4A showed the predicted binding sites of miR-346 and NFIB. We further demonstrated the regulatory relationship between miR-346 and NFIB by Western blot. The results showed that NFIB expression was inhibited in the H_2O_2 group, while NFIB expression was greatly increased in the H_2O_2 + INT group (Figure 4B, 4C). Most importantly, the results of Luciferase reporter gene detection showed that overexpression of miR-346 can remarkably inhibit the Luciferase activity in wild-type group but not the mutant group (Figure 4D).

Discussion

MI is an ischemic heart disease that causes damage and death of myocardial cells due to the interruption of local blood supply to the heart[13]. The resulting lack of oxygen and myocardial ischemia can cause severe myocardial damage and even death. Its high morbidity and mortality are major health issues facing the world[14]. The length of MI and the severity of MI greatly de-

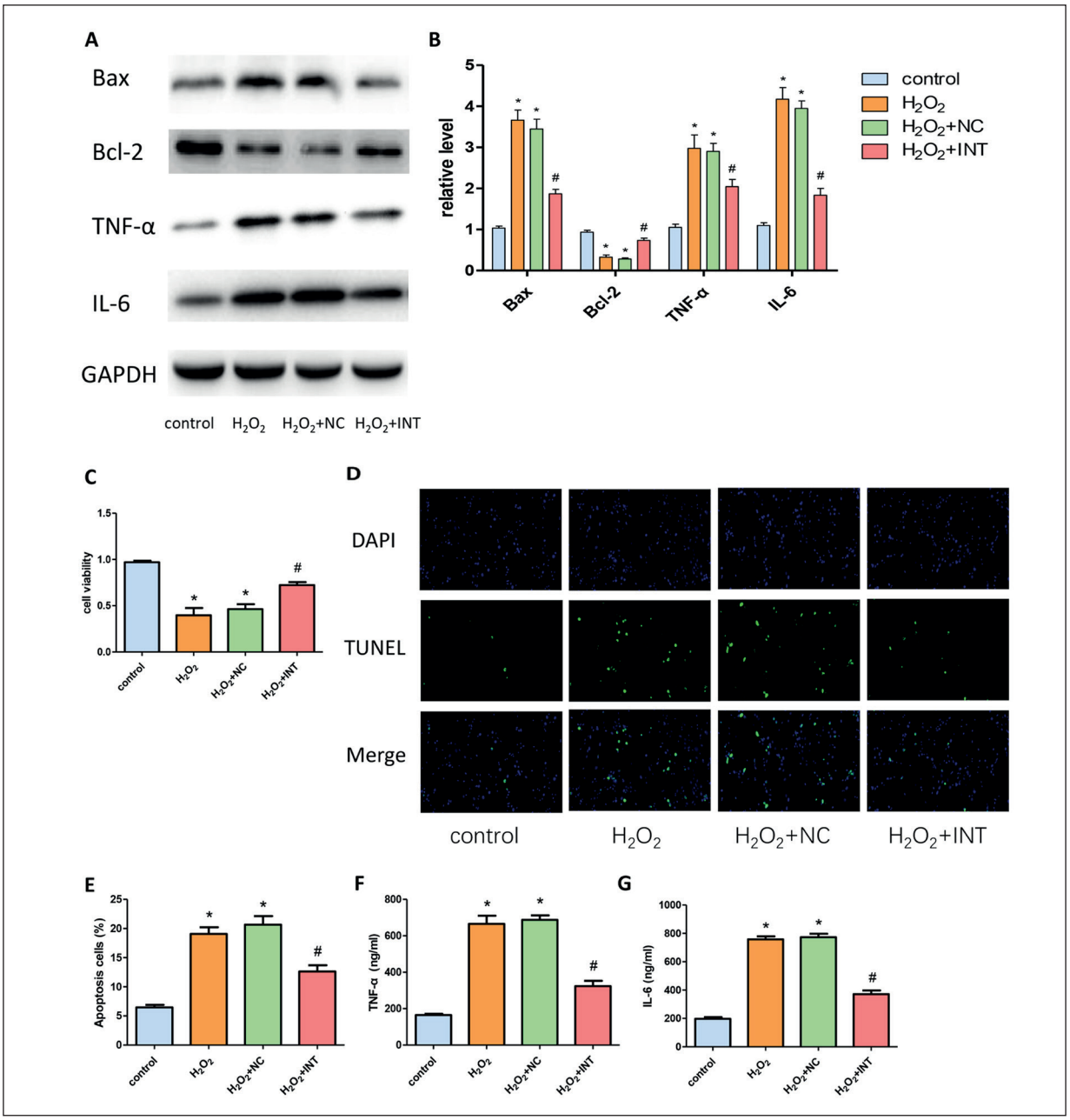

Figure 2. Downregulation of miR-346 inhibited apoptosis and inflammation of cardiomyocytes. **A,** The expression of Bax, Bcl-2, TNF-α and IL-6 was determined by Western blot analysis. **B,** Statistical results of protein levels ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **C,** MTT assay suggested that miR-346 inhibitor restored cell viability after H_2O_2 treatment ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D,** Representative images of TUNEL staining (magnification: 400×). **E,** The apoptotic rate of H_2O_2 group increased, and decreased in H_2O_2+INT group ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **F,** ELISA assay was used to detect the protein expression of TNF-α in the four groups ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3). **G,** ELISA assay was used to detect the protein expression of IL-6 in the four groups ("$_{*}$" $p<0.05$ *vs.* control, "$_{\#}$" $p<0.05$ *vs.* H_2O_2+NC, n=3).

termine the degree of damage and prognosis of cardiac tissue.

Hypoxia after MI can lead to the death of myocardial cells and cause a series of inflammatory reactions[15]. Damaged tissues can remove wound fragments through inflammatory cells. At the same time, fibroblasts and endothelial cells infiltrate and proliferate, and the extracel-

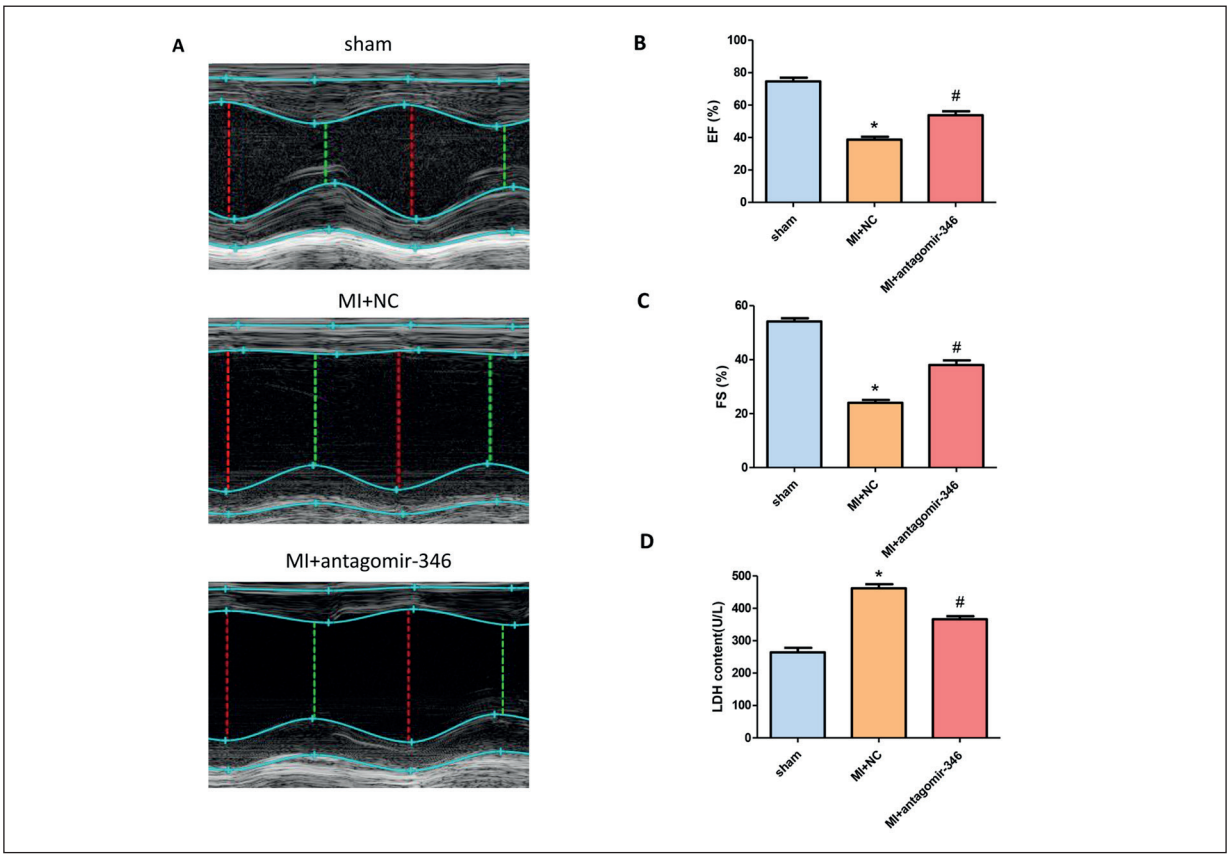

Figure 3. Downregulation of miR-346 improved cardiac function of MI rats. **A,** Representative photographs of rat echocardiography. **B, C,** (**B**) Ejection fractions (EF) and (**C**) Fractional shortening (FS) ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ vs MI+NC, n=6). **D,** Serum LDH activity was measured in MI and antagomir-346 injection rat ("*" $p<0.05$ *vs.* sham, "#" $p<0.05$ *vs.* MI+NC, n=6).

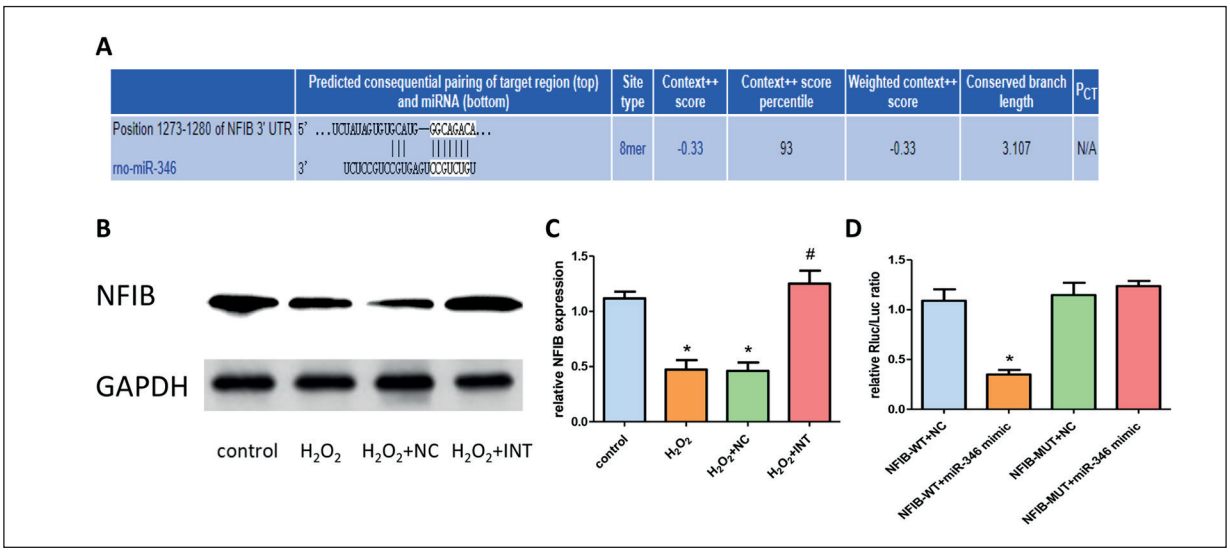

Figure 4. Downregulation of miR-346 inhibited apoptosis and inflammation of H9c2 cells via regulation of NFIB. **A,** Binding site predicted by the TargetScan database. **B,** Western blot showed the expression of NFIB. **C,** Statistical results of protein level of NFIB ("*" $p<0.05$ *vs.* control, "#" $p<0.05$ *vs.* H_2O_2+NC, n=3). **D,** MiR-346 overexpression significantly decreased the relative luciferase activity in WT group but did not decrease the relative luciferase activity in MUT group ("*" $p<0.05$ *vs.* WT+NC, n = 3).

lular matrix remodels, eventually leading to scar formation[16]. Apoptosis is different from cell necrosis. It is an active death process that requires energy support under the high regulation of genes. Its biggest feature is the controlled degradation of chromatin[17]. Anversa et al[18] explicitly proposed the relationship between myocardial cell apoptosis and AMI, that is, apoptosis is the main form of myocardial damage caused by coronary artery occlusion, and cell necrosis occurs after the cell activates the apoptosis cascade[18]. Roy et al[19] used chip technology to analyze gene expression in rat heart tissues 2 and 7 days after the occurrence of MI and found that the changed gene function is mainly divided into 3 categories, namely inflammation, apoptosis and extracellular matrix. Some genes were verified, and the roles of IL-6 and CCR2 in the early stage of MI were clarified.

The most basic biological function of miRNA is to bind the target gene that it regulates, so that the mRNA of the target gene is cut by RNase to achieve the purpose of inhibiting gene expression. Or it can inhibit the translation of mRNA by binding to the mRNA of the target gene[20]. In recent years, research has also found that miRNA can increase the expression level of the target gene by promoting the translation process. Generally speaking, miRNAs achieve target gene regulation by binding to the 3'UTR of the target gene. In this article, we found that miR-346 was highly expressed in ischemic myocardium. By inhibiting the expression of miR-346, the apoptosis and inflammation of the cells were remarkably suppressed, and the cardiac function of myocardial infarction rats was also remarkably improved.

NFIB is a member of the NFI family. This gene has high transcriptional activity in spinal organisms. NFIB gene can regulate the expression of more than 100 genes and participate in the regulation of cell proliferation, differentiation, apoptosis and other physiological and pathological processes[21]. Through prediction tools, we found that miR-346 has a binding site to NFIB. Through Western blot and Luciferase reporter gene experiments, we have demonstrated that miR-346 can directly target NFIB and inhibit NFIB expression.

Conclusions

The results of this study showed that miR-346 expression was elevated in ischemic myocardium. The downregulation of miR-346 significantly inhibited myocardial apoptosis and inflammation by targeting NFIB.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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