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Down-regulation of miR-320 exerts protective effects on myocardial I-R injury via facilitating Nrf2 expression

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Abstract. – OBJECTIVE: Nuclear factor NF-E2 related factor 2 (Nrf2) plays crucial roles in the regulation of oxidative stress (OS) or myocardial ischemia-reperfusion (I-R) injury. During the process of I-R injury, the miR-320 is down-regulated. Bioinformatics analysis showed complementary binding sites between miR-320 and 3'-UTR of Nrf2 mRNA. Therefore, this study aimed to investigate the role of miR-320 in mediating Nrf2 expression and myocardial I-R injury.

MATERIALS AND METHODS: Rat I-R model was established. Fluorescent quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot were used to measure the expression of miR-320, Nrf2, HO-1 in myocardial tissues. Contents of malondialdehyde (MDA), superoxide dismutase (SOD) and caspase-3 activity, serum assay of creatine kinase (CK) and lactate dehydrogenase (LDH) activity were evaluated. I-R rat models were transfected with antagomir-320 followed by measuring those proteins. Cultured H9C2 cells were transfected with antagomir-320 to measure miR-320, Nrf2 and heme oxygenase 1 (HO-1) expression, cell apoptosis and reactive oxygen species (ROS) content.

RESULTS: Compared to the sham group, I-R rats had significantly lower miR-320 or HO-1 expression in the myocardium, plus higher levels of Nrf2, MDA, CK and LDH, and decreased SOD activity ($p<0.05$). Antagomir-320 transfection suppressed miR-320 expression, elevated Nrf2 and HO-1 expression, decreased levels of MDA, CK or LDH, and increased SOD activity. In H9C2 cells, antagomir-320 transfection also elevated Nrf2 and HO-1 expression and suppressed myocardial cell apoptosis or ROS production under I-R treatment.

CONCLUSIONS: Down-regulation of miR-320 exerts protective effects on myocardial I-R injury. The inhibition of mir-320 expression can enhance OS potency of the myocardium, alleviate I-R injury or reduce cell apoptosis by facilitating Nrf2 expression.

Key Words
I-R injury, Nrf2, HO-1, Cell apoptosis, ROS, MiR-320.

Introduction

Reperfusion for coronary artery after acute myocardial infarction (AMI) is the most effective treatment for retrieving ischemic myocardium, protecting cardiac function and saving patients' life[1,2]. However, the re-opening of blood supply inevitably leads to ischemia-reperfusion (I-R) injury of infarcted myocardium. Myocardial I-R injury frequently occurs during treatment of AMI, and may cause severer injury to myocardial tissues[3,4].

Under the challenge of oxidative stress (OS), the body will initiate endogenous protective mechanism against OS to ameliorate the tissue injury which is caused by OS and reactive oxygen species (ROS) products[5,6]. Nuclear factor E2 related factor 2 (Nrf2)/antioxidant response element (ARE) is the most important endogenous anti-OS signal pathway, whose activation and nuclear translocation can alleviate OS damage or cell apoptosis, thus protecting pathological tissues or cells[7-9].

MicroRNA (miR) is a type of endogenous non-coding small molecule single-stranded RNA with 22-25 nucleotides length and can mediate target gene expression by complementary binding to 3'-untranslated region (3'-UTR) of target gene mRNA to degrade mRNA or inhibit its translation. The abnormal expression or function of miR has been indicated in tissue injuries of heart[10], spinal cord[11] or kidney[12]. Previous studies[13,14] showed significantly decreased miR-320 expression in the heart or spinal cord tissues after I-R injury, suggesting important roles of miR-320 in mediating tissue I-R injury. Further bioinformatics analysis showed the existence of complementary binding sites between miR-320 and 3'UTR of Nrf2 mRNA, suggesting a possible relationship. This work generated a rat I-R injury model after myocardial infarction, followed by measuring the expression of miR-320 and Nrf2. In addition, we interfered miR-320 expression in cultured rat

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myocardial cell line H9C2 to investigate the role of miR-320 in mediating Nrf2 expression and I-R injury of myocardial cells.

Materials and Methods

Major Reagent and Materials

Healthy adult male Sprague Dawley (SD) rats (6 weeks old, body weighting from 240 g to 30 g) were purchased from the Guizhou Medical University (Guizhou, China). H9C2 cell was purchased from Jiniou Biotech. (Shanghai, China). Type II collagenase and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA, USA). Rabbit anti-rat Nrf2, heme oxygenase 1 (HO-1) and β-actin antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Horseradish peroxide (HRP) conjugated secondary antibody was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). PrimeScript™ RT reagent Kit was purchased from TaKaRa (Otsu, Shiga, Japan). pGL3 plasmid and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, MI, USA). MiR-NC, miR-320 mimic, miR-320 inhibitor, antagomir-NC and antagomir-320 were purchased from RiboBio (Guangzhou, China). Lipid peroxidation product malondialdehyde (MDA), superoxide dismutase (SOD), fluorescein isothiocyanate (FITC)-Annexin V/Propidium Iodide (PI) apoptotic assay kit and bicinchoninic acid (BCA) protein quantification kit were purchased from Beyotime Biotech. (Shanghai, China). DCFH-DA fluorescent dye was purchased from Sigma-Aldrich (St. Louis, MO, USA). Creatine kinase (CK) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Creatine kinase (CK) and lactate dehydrogenase (LDH) assay kit were purchased from Solarbio (Beijing, China). Stat Fax 4200 microplate reader was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

Generation of Rat Myocardial I-R Model

Rats were anesthetized through intraperitoneal injection of 10% hydrate chloral (Sigma-Aldrich, St. Louis, MO, USA) and fixed in a supine position. The chest cavity was opened during the fourth intercostal site to expose the heart. Left anterior descending branch of coronary artery was identified and ligated. Electrocardiogram (ECG) monitor showed the elevation of ST segment convert by 0.1 mT on Q-lead, or T wave peak. The myocardial tissues presented whitening and weakened pulsation. After 60 min, the ligating suture was released to recover cardiac blood supply. The reddish of myocardial tissue indicates successful reperfusion, for generating a myocardial I-R model. The sham group was employed receiving open-chest surgery but without ligation of the left anterior descending branch of the coronary artery as the control. Rats from both groups were partially sacrificed at 6 h or 12 h after surgery for collecting myocardial tissues, on which gene and protein expression were examined. Myocardial tissues which were harvested from 12 h post-surgery rats were homogenized for measuring MDA and SOD content. Blood samples were also collected from abdominal aorta at 12 h after surgery for separating serum in further assays.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the Guizhou People's Hospital (Guizhou, China).

Antagomir-320 Treatment of I-R Model Rats

I-R model rats were randomly assigned into two groups (n=5 each): antagomir-NC group, in which rats received 200 nmol antagomir-NC at 24 h before surgery via intravenous injection; and antagomir-320 group with rats receiving 200 nmol antagomir-320 at 24 h before surgery. Blood samples were collected via abdominal aorta at 12 h after surgery to obtain serum. Myocardial tissues were collected after blood collection, and the protein expression was measured by Western blot. Caspase-3 activity was tested by spectrometry. The activity of MDA and SOD was measured by the test kit.

Caspase-3 Enzymatic Activity Assay

The assay for measuring caspase-3 activity was performed followed the manual instruction of the test kit. In brief, pNA standard samples were diluted in serial gradients to make samples with 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM and 0 μM. Absorbance (A) values at a wavelength of 405 nm (A405) were measured for plotting the standard curve. Myocardial tissues were homogenized to prepare the homogenate, which was mixed with Caspase Lysis Buffer for 20 min on ice. The tissue lysate was centrifuged at 12000 ×g for 10-15 min at 4°C. The supernatant was collected for protein quantification us-

ing bicinchoninic acid (BCA) test kit. Total of 65 µl of assay buffer was added into 96-well plate, along with 25 µl of lysate supernatant, and 10 µl of Ac-DEVD-pNA (2 mM) into 96-well plate. After 37°C incubation for 2 h, A405 values were measured immediately after the significant color change. The relative enzymatic unit was defined as A405 of experimental group/A405 of control group.

Assays for MDA, SOD, CK, and LDH

Following the manual instruction of test kits, the content of MDA and OSD in rat myocardial tissue lysate was quantified to evaluate OS condition and anti-oxidation potency. The rat serum was used to measure CK and LDH activity following the manual instruction of test kits.

Rat H9C2 Cell Transfection and I-R Treatment

I/R treatment: H9C2 cells were cultured in low-glucose, serum-free DMEM medium to mimic ischemia conditions, and kept in a chamber (Mode: HERAcell 240i, Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 and 95% N_2 for incubation. 12 h later, the normal serum-containing medium was switched for 12 h incubation in a second chamber with 5% CO_2 and 95% air. Cells were collected for further assays.

Rat H9C2 cells at log-growth phase were divided into miR-NC transfection group and miR-320 inhibitor transfection group. After 72 h transfection, I-R treatment was performed as described in the previous section. H9C2 cells under normal culture conditions were used as the control group. Cells were collected for measuring gene and protein expression of relevant molecules. Flow cytometry was used for measuring cell apoptosis and ROS level.

Flow Cytometry for Cell Apoptosis

Cells were rinsed in Phosphate-Buffered Saline and Tween 20 (PBST) twice and digested in 0.25% trypsin (Beyotime Biotechnology, Shanghai, China). Cells were collected and centrifuged at 300 ×g for 5 min, followed by Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) rinsing twice. 100 µlL of binding buffer was added followed by gentle re-suspension. Then, 5 µl of Annexin V-FITC and 5 µl of Propidium Iodide (PI) solutions were sequentially added and incubated in the dark for 15 min at room temperature. After that, 400 µl of binding buffer was added and cell apoptosis was measured on a FC500MCL flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Flow Cytometry for ROS

ROS was measured in cultured myocardial cells. In brief, cells were rinsed in PBS twice and digested in 0.25% trypsin for collection, followed by centrifugation at 300 g for 5 min. Cell precipitation was mixed with serum-free DMEM medium diluted 0.1% DCFH-DA probe, and incubated at 37°C for 30 min. Cells were gently mixed 3-5 times. After rinsing in PBS twice, cells were re-suspended in 500 µl of PBS followed by analysis of ROS level by an FC500MCL flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Luciferase Reporter Gene Assay

Total mRNA was extracted from HEK293T cells by TRIzol (Beyotime Biotech. Shanghai, China). Using mRNA of HEK293T cells as the template, full length or mutant fragment of 3'-untranslated region (3'-UTR) of Nrf2 gene was amplified and sub-cloned to the pGL3 plasmid. After transforming DH5α competent cells, sequencing was performed to screen out plasmids with correct sequence and was named as pGL3-Nrf2-WT or pGL3-Nrf2-MUT. Lipofectamine 2000 was used to co-transfect pGL3-Nrf2-WT (or pGL3-Nrf2-MUT) and miR-320 mimic (or miR-320 inhibitor, miR-NC) into HEK293T cells. After 48 h incubation, Dual-Glo Luciferase Assay System was used to measure the enzymatic activity of Luciferase.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) for Measuring Gene Expression

Total RNA was extracted by TRIzol approach and reversely transcripted into cDNA using PrimeScript™ RT reagent Kit (Invitrogen/Life Technologies, Carlsbad, CA, USA). Under TaqDNA polymerase, Polymerase Chain Reaction (PCR) amplification was performed using complementary DNA (cDNA) as the template. In a 10 µl reaction system, the followings were added: 5.0 µl 2× SYBR Green Mixture, 0.5 µl forward and reverse primer (5 µm/l), 1 µ cDNA template, and ddH_2O up to 10.0 µl. Reverse transcription conditions were: 50°C 15 min, followed by 85°C for 5 min. PCR was performed under the following conditions: 95°C for 5 min denature, followed by 40 cycles each containing 95°C for 15 sec, and 60°C for 1 min. 40 cycles were performed on a CFX96 Real Time-fluorescent quantitative PCR cycler for data storage. The primers were listed in Table I.

Table I. Primers for the RT-PCR assay.

Genes		Primers
For human		
miR320	Forwards	5'-ACACTCCAGCTGGGAAAAGCTGGGTTCAGA-3'
	Reverse	5'-GTCGGCAATTCAGTTGAG-3'
Nrf2	Forwards	5'-CCTCAACTATAGCGATGCTGAATCT-3'
	Reverse	5'-AGGAGTTGGGCATGAGTGAGTAG-3'
GAPDH	Forwards	5'-GCCTCAAGATCATCAGCAATGC-3'
	Reverse	5'-CCTTCCACGATAC CAAAGTTGTCAT-3
For rat		
miR320	Forwards	5'-AAAAGCTGGGTTGAGAGGG-3'
	Reverse	5'-TGCGTGTCGTGGAGTC-3'
Nrf2	Forwards	5'-GCAACTCCAGAAGGAACAGG-3'
	Reverse	5'-GGAATGTCTCTGCCAAAAGC-3'
HO-1	Forwards	5'-CTTTCAGAAGGGTCAGGTGTC-3'
	Reverse	5'-TGCTTGTTTCGCTCTATCTCC-3'
GAPDH	Forwards	5'-TATGACAACTCCCTCAAGAT-3'
	Reverse	5'-GGCATGGACTGTGGTCATGA-3'

Western Blot for Protein Expression

Total proteins were extracted from cells and tissue samples using radioimmunoprecipitation assay (RIPA) lysis buffer (Tiangen Biotech Co. Ltd., Beijing, China). BCA approach was used to measure protein quality and concentration. 40 μg of samples were separated in Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE; 10% separating gel and 4% stacking gel), and transferred to polyvinylidene difluoride (PVDF) membrane (300 mA, 90 min). The membrane was blocked in PBST containing 5% defatted milk powder for 60 min at room temperature. Primary antibody (Nrf2 at 1:1000, HO-1 at 1:2000, β-actin at 1:10000) was then added for 4°C overnight incubation. Unbounded primary antibody was eliminated by PBST rinsing, and HRP conjugated goat anti-rabbit IgG (H+L) secondary antibody was added for 60 min room temperature incubation. After PBST rinsing three times, BeyoECL Plus working solution A and B were mixed in equal volume and was added onto the membrane. After 2-3 min of dark incubation, the membrane was exposed and the film was scanned for data storage.

Statistical Analysis

SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student's *t*-test was used to compare measurement data between the two groups. The comparison of measurement data among multiple groups was performed by one-way analysis of variance (one-way ANOVA), followed by Bonferroni post-hoc test. A statistical significance was defined when $p<0.05$.

Results

Targeted Regulation Between MiR-320 and Nrf2

Online prediction using MicroRNA.org showed the existence of complementary binding sites between miR-320 and 3'-UTR of Nrf2 mRNA (Figure 1A). Dual-Luciferase gene reporter assay showed that transfection of miR-320 mimic significantly reduced the relative Luciferase activity in HEK293T cells after transfected with pGL3-Nrf2-WT, and transfection of miR-320 inhibitor remarkably increased the relative Luciferase activity of HEK293T cells after transfected with pGL3-Nrf2-WT. However, either miR-320 mimic or miR-320 inhibitor had no effects on relative Luciferase activity of HEK293T cells which were transfected with pGL3-Nrf2-MUT (Figure 1B). These results suggested a targeted regulation relationship between miR-320 and Nrf2.

I-R Rats Had Prominent Myocardial Injury and Elevated OS Level

We found significantly elevated MDA content in myocardial tissues of I-R rats compared to the sham group (Figure 2A), whilst SOD enzymatic activity was remarkably decreased (Figure 2B). Spectrometry results showed markedly increased caspase-3 activity in I-R model rat myocardial tis-

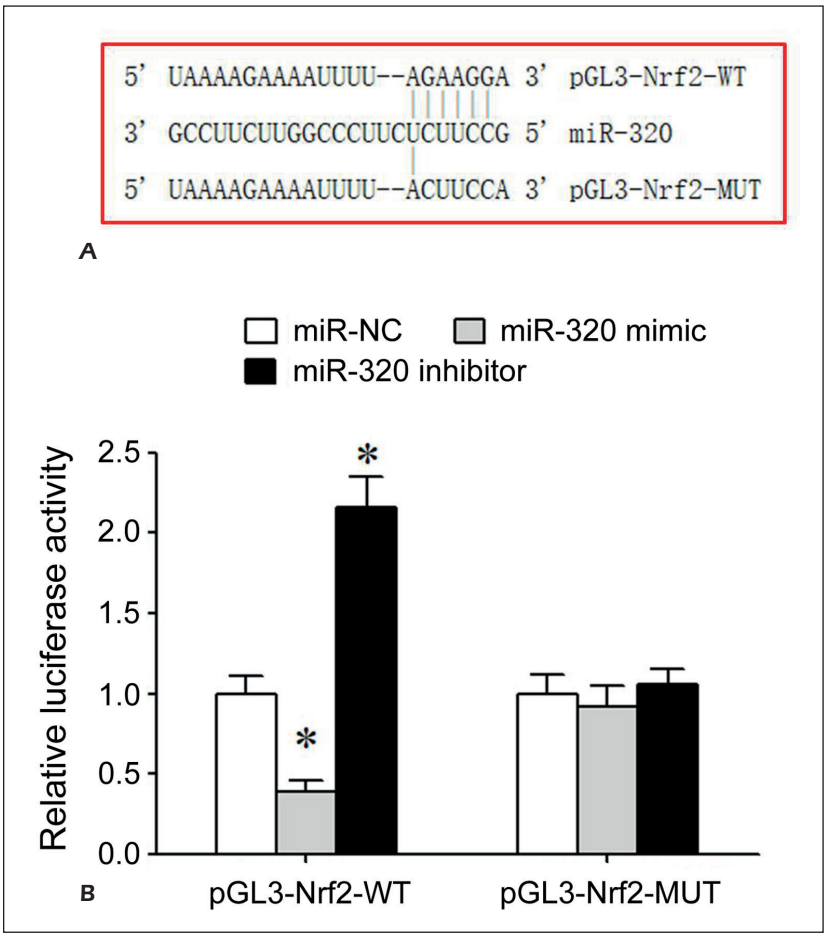

Figure 1. Targeted regulation between miR-320 and Nrf2. **A**, Illustration for the functioning site be-tween miR-320 and 3'-UTR of Nrf2 mRNA. *p<0.05 compared to the miR-NC group.

sues compared to the sham group (Figure 2C). Further assays showed remarkably elevated CK (Figure 2D) and HDL (Figure 2E) levels in I-R model rat myocardial tissues compared to the sham group.

Prominent Down-Regulation of MiR-320 and Up-Regulation of Nrf2 in I-R Rat Myocardial Tissues

The qRT-PCR results showed that, compared to rats in the sham group, I-R rats showed decreased miR-320 expression in myocardial tissues, which was further decreased with elongated I-R time (Figure 3A). Meanwhile, I-R rats had significantly higher Nrf2 mRNA expression in the myocardium (Figure 3B), together with decreased HO-1 mRNA expression in myocardial tissues 6 h post-surgery compared with rats in the sham group. Although HO-1 mRNA level was increased at 12 h post-surgery, it was still lower than the sham group (Figure 3C). Western blot analysis also showed enhanced Nrf2 protein expression in myocardial tissues at 6 h and 12 h post-surgery, plus decreased HO-1 protein expression (Figure 3D).

Injection of Antagomir-320 Decreased OS Level and Caspase-3 Activity in Rat Myocardial Tissues

Furthermore, we found significantly decreased MDA content in myocardial tissues in rats receiving antagomir-320 injection compared to antagomir-NC injection (Figure 4A), together with significantly elevated SOD activity (Figure 4B). Spectrometry results showed that injection of antagomir-320 remarkably suppressed caspase-3 activity in rat myocardial tissues (Figure 4C). Compared to rats in the antagomir-NC group, antagomir-320 injected rats presented decreased CK and LDH activity in the myocardium (Figure 4D). The qRT-PCR results showed that, compared to the antagomir-NC group, antagomir-320 injected mice showed significantly decreased miR-320 expression in myocardial tissues, plus markedly elevated Nrf2 and HO-1 mRNA expression (Figure 4E). Western blot showed that, compared to the antagomir-NC group, antagomir-320 injected rats had remarkably elevated expression of Nrf2 and HO-1 proteins in myocardial tissues (Figure 4F).

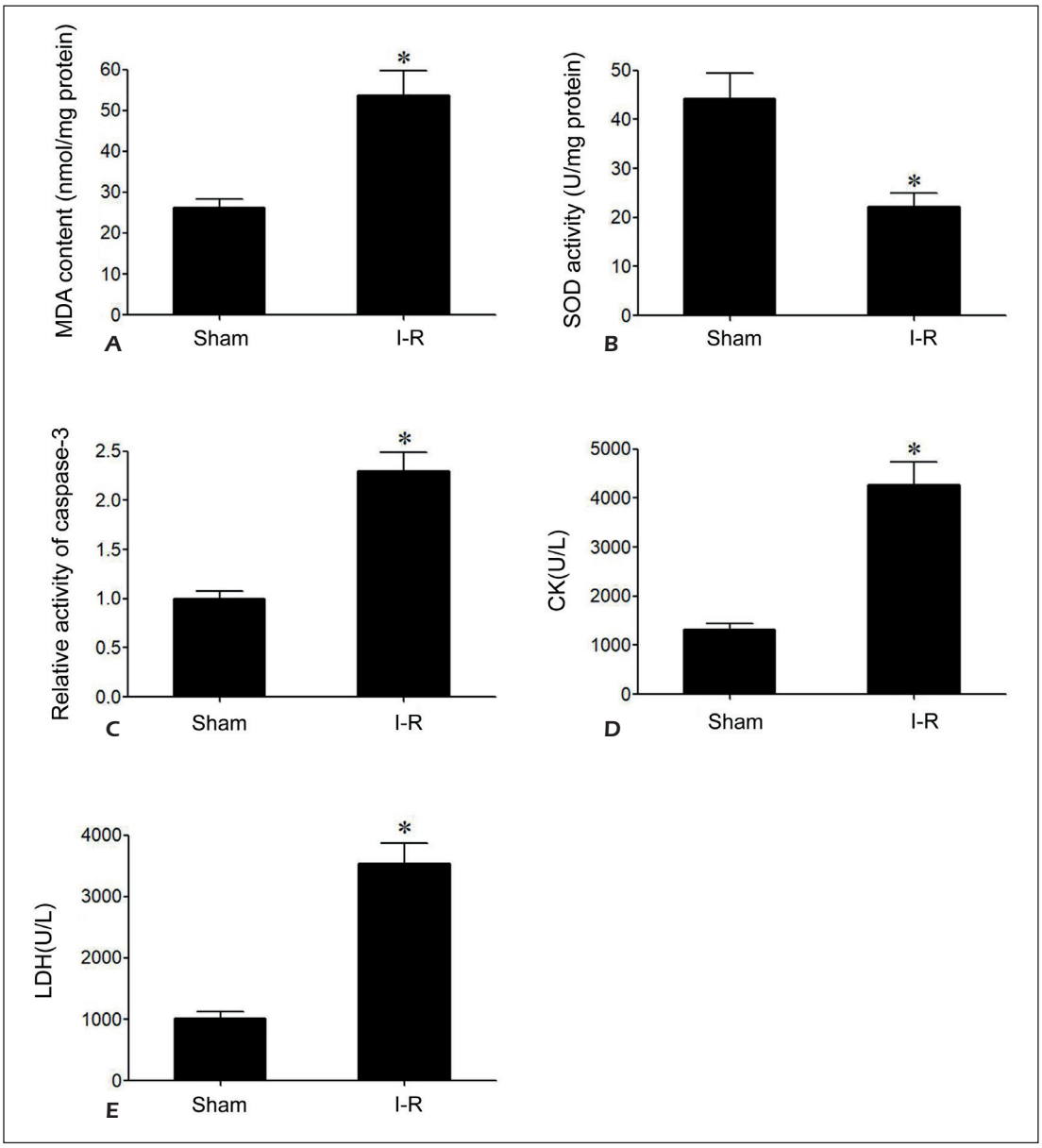

Figure 2. Significantly OS and higher caspase-3 activity in I-R model rat myocardial tissues. **A**, Test kit for measuring MDA content in rat myocardium. **B**, Test kit assay for SOD enzymatic activity in rat myocardial tissues. **C**, Spectrometry quantification of caspase-3 activity in rat cardiac tissues. **D**, CK activi-ty in rat myocardial tissues. E, LDH activity in rat myocardial tissues. *$p<0.05$ compared to the miR-NC group.

Down-Regulation of MiR-320 Elevated Nrf2 Expression and Weakened Myocardial Cell Oxidative Damage and Cell Apoptosis

The qRT-PCR showed that, compared to the control group, I-R treated H9C2 cells had decreased expression of miR-192 (Figure 5A) and HO-1 mRNA (Figure 5C), but increased Nrf2 mRNA expression (Figure 5B). Transfection of antagomir-320 significantly suppressed miR-320 expression in H9C2 cells under I-R treatment, enhanced Nrf2 mRNA expression and HO-1 mRNA expression. Western blot results showed that, compared to the control group, I-R treated H9C2 cells had elevated Nrf2 protein expression and lower HO-1 protein expression. The transfection of antagomir-320 further elevated Nrf2 protein expression, and increased HO-1 expression (Figure 5D). Flow cytometry assay showed that I-R treatment remarkably elevated ROS production in

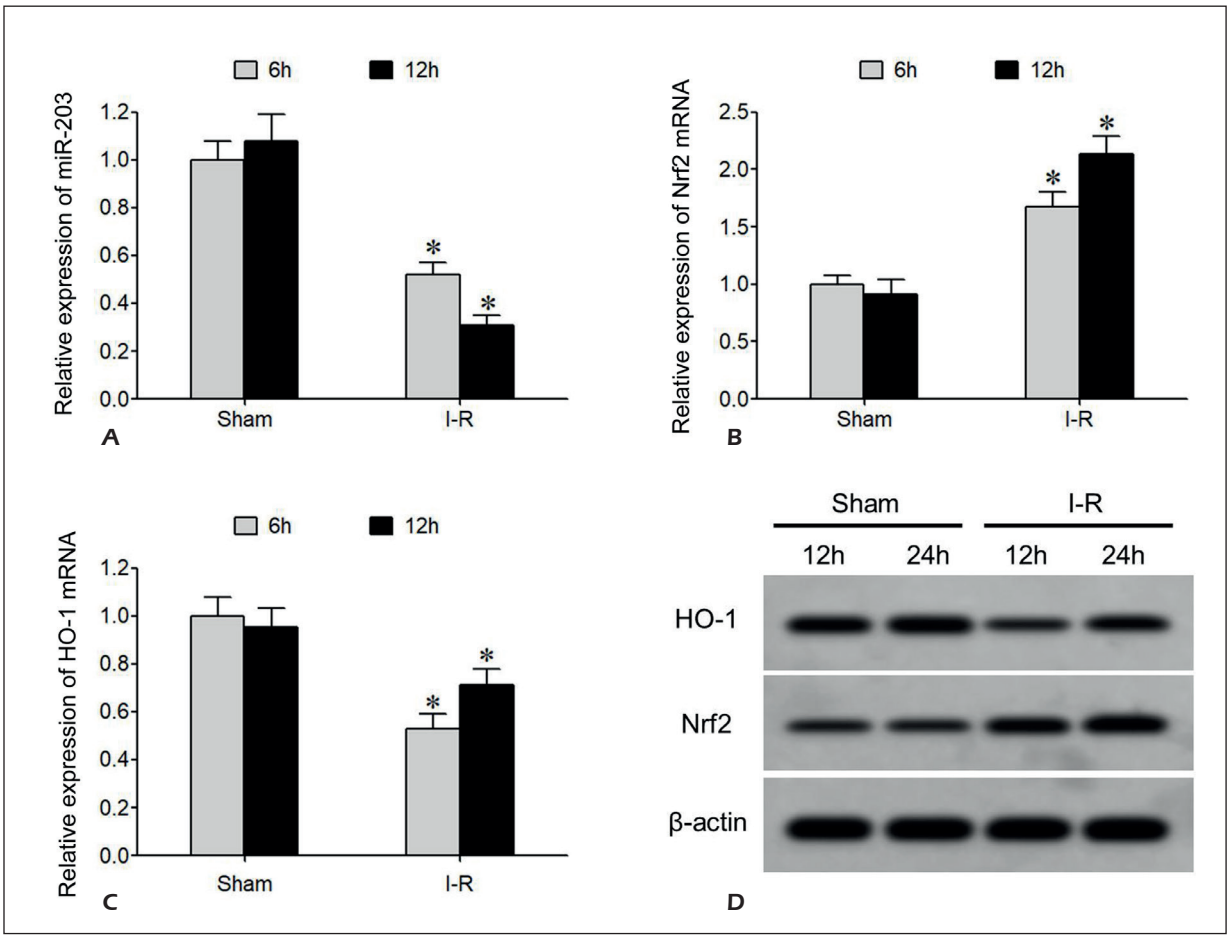

Figure 3. Significantly decreased miR-320 expression and elevated Nrf2 expression in I-R rat myocardial tissues. **A**, qRT-PCR for rat myocardial expression of miR-320. **B**, qRT-PCR assay for Nrf2 mRNA expression in rat myocardium. **C**, qRT-PCR analysis for HO-1 mRNA expression in rat myocardial tis-sues. **D**, Western blot for measuring protein expression in myocardial tissues. *$p<0.05$ compared to the miR-NC group.

H9C2 cells (Figure 5E) and enhanced cell apoptosis (Figure 5F). However, transfection of antagomir-320 significantly depressed ROS production under I-R condition (Figure 5E) and suppressed cell apoptotic rate (Figure 5F).

Discussion

Myocardial cell apoptosis and necrosis are involved in I-R injury after myocardial infarction. Apoptosis of myocardium occurs early and throughout the whole process of I-R injury post-myocardial infarction, thus playing roles in inducing I-R injury. Both OS and mitochondrial dysfunction are required for induction of myocardial cell apoptosis and I-R injury[15-17]. Therefore, investigation of signaling molecules with abnormal changes during myocardial I-R injury is of critical importance for revealing I-R injury mechanism and alleviating I-R damage.

Under OS condition, the body can initiate and activate endogenous Nrf2/ARE signal pathway, which is the most important endogenous anti-OS signal pathway ever been found. When OS level is within the normal physiological range, Nrf2 couples with Keap1 to bind cytoplasmic myosin for anchoring into the cytoplasm, and is under the minimal transcriptional activity with continuous degradation and low expression. With prominent OS, Keap undergoes structural alternation, leading to dissociation from Nrf2 which was subsequently released into the nucleus where it binds with ARE to up-regulate the expression of various anti-oxidative proteasome proteins including heme oxygenase-1 (HO-1), γ-glutamine cysteine synthase (γ-GCS), glutathione S-transferase (GST), and NAD(P)H, thus alleviating OS level and depressing OS injury[18-21].

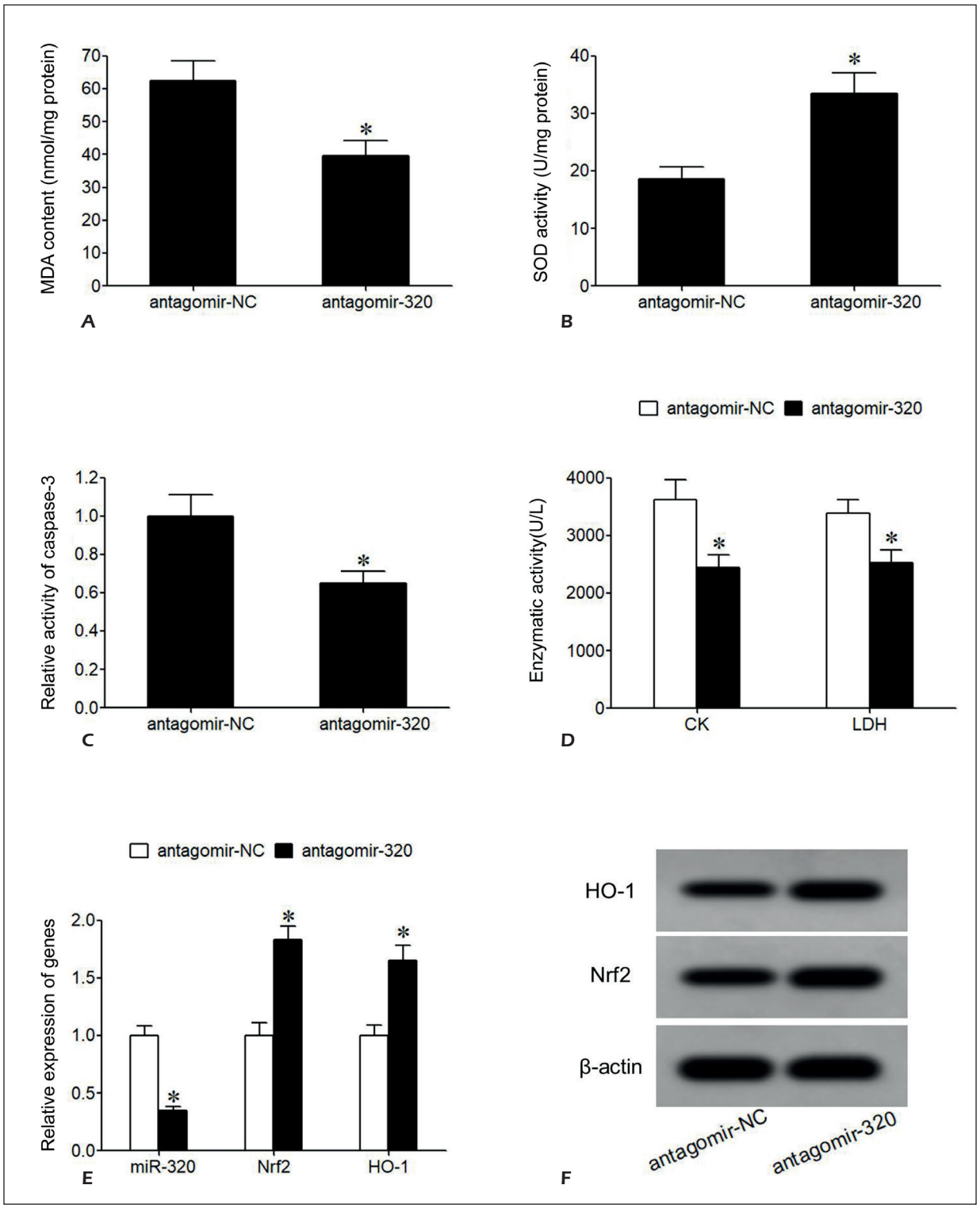

Figure 4. Antagomir-320 injection suppresses OS level and caspase-3 activity in rat myocardial tissues. **A**, Test kit for MDA content in rat myocardial tissues. **B**, SOD activity in rat myocardium by test kits. **C**, Spectrometry assay for caspase-3 activity in rat myocardial tissues. **D**, Test kit for CK and LDH enzyme activity in rat myocardial tissues. **E**, qRT-PCR for gene expression in rat myocardial tissues. **F**, Western blot for measuring myocardial protein expression. *p<0.05 compared to the miR-NC group.

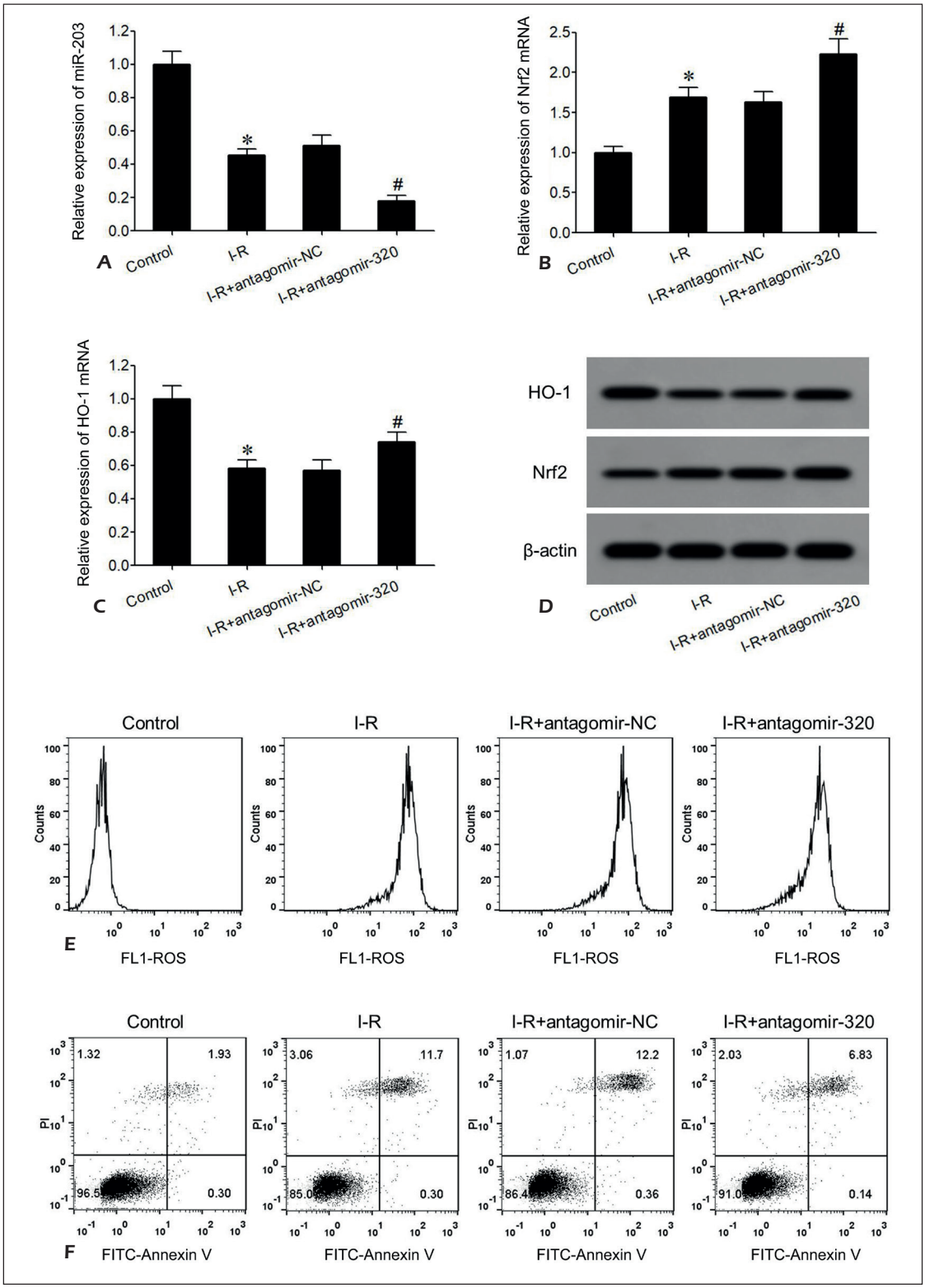

Figure 5. Down-regulation of miR-320 elevated Nrf2 expression and weakened myocardial cell oxidative stress or apoptosis. ***A***, qRT-PCR for measuring miR-320 expression. ***B***, qRT-PCR measuring Nrf2 mRNA expression. ***C***, qRT-PCR for quantification of HO-1 mRNA expression. ***D***, Western blot for protein expression assay. ***E***, Flow cytometry measuring intracellular ROS content. ***F***, Flow cytometry measuring cell apoptosis. *$p<0.05$ comparing I-R group and control group. $^{\#}p<0.05$ compared to I-R + antagomir-320 and I-R + antagomir-NC group.

Scholars[22-24] have demonstrated the important regulatory role of enhanced expression of Nrf2 and functional activation in anti-OS and alleviating I-R injury of the myocardium. After I-R injury in heart and spine cord tissues, miR-320 is found to be significantly decreased[13,14], suggesting the important role of miR-320 in tissue I-R injury. Bioinformatics analysis showed the existence of targeted complementary binding sites between miR-320 and 3'-UTR of Nrf2 mRNA, suggesting a possible regulation relationship. This study established a rat I-R model post-infarction to measure expressional profiles of miR-320 and Nrf2. In addition, we performed intervention of miR-320 in rat myocardial cell line H9C2 *in vitro* to investigate the role of miR-320 in mediating Nrf2 expression and myocardial I-R injury.

Dual-Luciferase gene reporter assay found that transfection of miR-320 mimic remarkably depressed relative Luciferase activity of HEK293T cells transfected with pGL3-Nrf2-WT, and transfection of miR-320 inhibitor remarkably elevated relative Luciferase activity in HEK293T cells transfected with pGL3-Nrf2-WT, showing the targeted regulation relationship between miR-320 and Nrf2. We also found that, compared to the sham group, I-R model rats had abnormally elevated serum CK and LDH enzymatic activity, indicating prominent myocardial injury. Compared to the sham group, I-R model rats had significantly elevated MDA content, higher caspase-3 enzymatic activity, plus significantly decreased SOD activity. These results revealed that I-R model preparation led to remarkably enhanced OS level in rat myocardial tissues, with prominent cell apoptosis. Both gene and protein assay showed that, compared to the sham group, I-R rats had decreased miR-320 expression in myocardial tissues at 6 h and 12 h post-surgery, plus higher Nrf2 expression and lower HO-1 expression. These results indicated the under I-R challenge, the body can initiate anti-OS mechanism by enhancing Nrf2 expression and function. However, due to the relatively lower anti-oxidative potency of the body, the expression of anti-oxidase HO-1 was still lower, suggesting a higher OS level than the sham group. Due to the targeted inhibition between miR-320 and Nrf2, plus our observation showing decreased miR-320 expression and higher Nrf2 expression at 6 h and 12 h after I-R, we proposed that the body may exert more potent anti-OS protective effect *via* suppressing miR-320 expression, leading to Nrf2 up-regulation. In a correlation study between miR-320 and I-R injury, He et al[13] observed that, compared to the sham group, I-R model of the spinal cord showed significantly decreased miR-320 expression, plus remarkably elevated expression of its target gene HSP20. Ren et al[14] further referred that compared to the sham group, I-R model rats had significantly depressed miR-320 expression in myocardial tissues. Song et al[25] found that compared to the sham group, I-R model rats had remarkably decreased miR-320 expression in myocardial tissues. This study observed a decreased miR-320 expression during I-R injury process of the myocardium, as similar with previous results.

Furthermore, this work injected antagomir-320 into I-R model rats and detected that, compared to the antagomir-NC injection group, antagomir-320 injected rats had decreased MDA content and caspase-3 activity in myocardial tissues, plus higher SOD activity, and lower serum CK or LDH activity. The protein assay showed that compared to the antagomir-NC group, antagomir-320 injected rat myocardial tissues had remarkably elevated Nrf2 and HO-1 protein expression. In addition, injection of antagomir-320 can enhance Nrf2 expression by further down-regulating miR-320, potentiating the expression of anti-oxidase HO-1. Our *in vitro* assays revealed that down-regulation of miR-320 elevated Nrf2 and HO-1 expression to potentiate anti-oxidative potency of rat myocardial cells, suppress ORS production or cell apoptosis. Both *in vivo* and *in vitro* assays demonstrated that under I-R conditions, down-regulation of miR-320 further enhanced Nrf2 expression, elevated the expression of anti-oxidase HO-1 expression, potentiated anti-oxidative potency of myocardial cells, and suppressed OS level or myocardial cell apoptosis, thus relieving I-R injury. He et al[13] showed that antagomir-320 treatment on rats can remarkably elevate the expression of target gene HSP20, improve hindlimb motor function and relieve spinal cord injury of I-R model rats. Ren et al[14] found that injection of AdmiR-320 into the myocardium of I-R model rats could elevate miR-320 expression, and aggravate I-R injury and myocardial infarction area; the injection of AdasmiR-320 for inhibition of miR-320 expression in myocardial tissues relieved cardiac infarction area and I-R injury condition. A further *in vitro* assay detected that transfection of AdmiR-320 could elevate miR-320 expression in cultured rat myocardial tissues and aggravated myocardial cell apoptosis or I-R injury; the transfection of AdasmiR-320 decreased miR-320 expression in cultured rat myocardial tissues, decreased cell apoptosis and suppressed I-R induced

injury sensitivity[14]. Song et al[25] found that compared to I-R model, I-R + antagomir-320 treated rats had significantly relieved myocardial tissues injury, weakened myocardial fibrosis, and less cell apoptosis, suggesting that miR-320 down-regulation can protect myocardium and relieve I-R injury. Song et al[26] observed that miR-320 could target and inhibit insulin growth factor-1 (IGF-1), and miR-320 down-regulation can suppress myocardial cell apoptosis, improve cardiac function and relieve myocardial I-R injury. All these results demonstrated the relationship between miR-320 up-regulation and aggravation of I-R injury, whilst miR-320 inhibition can relieve I-R injury. In contrast to those studies, we combined miR-320 and Nrf2 targeted relationship, and revealed that miR-320 could regulate Nrf2 expression, affect body anti-oxidative potency and related mechanism, thus modulating I-R injury. These results have not been reported before and are thus novelty of this study. However, whether such regulatory relationship between miR-320 and Nrf2 existed in human is still unclear and requires further studies to fulfill the weakness of this work.

Conclusions

We found that the down-regulation of miR-320 exerts a protective effect on rat myocardial I-R injury. The inhibition of miR-320 expression can enhance anti-OS potency of the myocardium, relieve I-R injury and reduce cell apoptosis by facilitating Nrf2 expression.

Conflict of Interests

The authors declare that they have no conflict of interest.

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