

## Original Article

# Protective effect of targeted inhibition of p38MAPK/NOX4 signaling pathway with microRNA-182 on the inflammatory injury of endothelial cells in coronary artery disease

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**Abstract:** Objective: To study the protective effect of miR-182/p38MAPK/NOX4 signaling pathway on inflammatory injury of endothelial cells in coronary artery disease (CAD). Methods: Seventy healthy male SD rats were selected and sixty of them were given a daily high-fat diet to establish the CAD model and the rats were divided into 7 groups (10 in each group). The ECG of each group was tested. Dual luciferase reporter gene experiment was used to validate the targeted relationship between miR-182 and NOX4, and the contents of TNF- $\alpha$ , IL-6 and ICAM-1 in serum were detected by ELISA. NO secretion activity was also detected. MTT method, Hoechst staining, qRT-PCR and Western blot were used to detect the cell proliferation activity, cell apoptosis, mRNA and relative expression levels of in each group, respectively. Results: Compared with the normal group, changes of ST segment of ECG in model group were significantly higher ( $P < 0.05$ ). The content of TNF- $\alpha$ , IL-6, and ICAM-1 in rat serum of the other groups was significantly increased, the secretion of NO was small, the proliferation slowed down, the apoptosis rate was higher, and expression of NOX4, p-p38MAPK, NF- $\kappa$ Bp65, miR-182 and Bcl-2 was downregulated (all  $P < 0.05$ ). Compared with the blank group and NC group, the miR-182 in miR-182 mimic group was up-regulated, and the contents of inflammatory factors in rat kidney tissues of miR-182 mimic group and si-NOX4 group were significantly decreased, the NO secretion of cells was higher, the proliferation was faster, the cell apoptosis rate was lower, and expression of NOX4, p-p38MAPK, and NF- $\kappa$ Bp65 were down-regulated, while Bcl-2 was upregulated, which were converse to the results in miR-182 inhibitor group (all  $P < 0.05$ ). Conclusion: Highly-expressed miR-182 can downregulate NOX4 and inhibit the p38MAPK NOX4 signaling pathway, which can promote a protective effect on inflammatory injury of myocardial microvascular endothelial cells of rats with CAD.

**Keywords:** miR-182, NADPH oxidase 4, p38MAPK/NOX4 signaling pathway, coronary artery disease, endothelial cells

## Introduction

Coronary artery disease (CAD), also known as the ischemic heart disease, is mainly caused by myocardial ischemia, hypoxia or necrosis by coronary artery steosis [1]. In recent years, a large number of studies have found that inflammation response plays an important role in the pathogenesis of CAD [2]. The cause of plaques is also related to the activation of inflammatory response [3]. Vascular endothelial injury and cell dysfunction have now been shown to be important factors in triggering cardiovascular disease [4]. Therefore, exploring the pathological process of endothelial cell injury is very important for the treatment of CAD and other

cardiovascular diseases. Some studies have found that inflammatory response can accelerate the endothelial cell injury in patients with CAD, and the severity of the inflammatory response is closely related to the severity of the disease [5]. NADPH oxidase 4 (NOX4) is an active NADPH oxidase, composed of 578 amino acids and expressed in osteoblasts, kidneys, nerve, and hematopoietic stem cells. Bartoloni et al. explored the relationship between vascular endothelial cell injury and NOX4, and found that NADPH oxidase 4 was activated in human umbilical vein endothelial cell line during injury process [6]. This further explains the close relationship between NOX4 and endothelial cell injury. The protective effect of calcitonin gene

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related peptide on vascular endothelial cell injury may be related to the down-regulation of p38MAPK phosphorylation and the inhibition of NOX4 expression [7]. At present, clinical research has mainly focused on the relationship between NOX4 and oxidative stress of vascular endothelial cells in patients with CAD, but the mechanism remains unclear. From the perspective of targeted therapy of CAD, this study explored the relationship between the p38MAPK/NOX4 signaling pathway inhibited by targeted-downregulation of NOX4 in miR-182 and the endothelial cell injury in CAD, with the hope to provide a new basis for the development of drugs.

### Materials and methods

#### *Research subject*

Seventy healthy male SD rats of clean grade (provided by Animal Experimental Center of Hebei Medical University with Certificate No. 810054) were selected with the age of eight to nine weeks and the body weight from 160 to 180 g. They were supplied with sufficient food and water, and subjected to 12 hours of light and dark conditions. The room temperature of laboratory was controlled between 18-22°C with a humidity of 40%-70% and the noise was lower than 50 dB. The rats were randomly divided into the model group (60 rats to establish CAD model) and the normal control group (10 rats). Rats in CAD group were given a daily high-fat diet (87.3% basal diet, 10.0% lard, 2.0% cholesterol, 0.5% sodium cholate, 0.2% propylthiouracil) [2]. The last intragastric administration started 72 h before taking medicine, the pituitrin was injected into the peritoneal cavity of the rats 30 min after the administration and the injection dose was 30 U/kg. Such injection was performed once every 24 h for 3 times to construct the myocardium ischemia rate model of CAD. Rats in normal group were injected with the same amount of normal saline. Twelve days after the establishment of myocardial ischemia rat model of CAD, the rat ECG was measured to judge the result. During the experiment, the rats which failed in the model construction were eliminated. In this study, for all the animals, *Declaration of Helsinki* was strictly abided by the researchers, and the protocol and animal usage scheme have been approved by the Experimental Animal Ethics Committee of Hebei Medical University.

#### *ECG test*

The ECG of each group was monitored. The ECG was found to be positive for myocardial ischemia if any of the following conditions was satisfied: the ST segment was shifted downward or upward horizontal by more than 0.1 mV; the T wave was high with ST segment shift; the T wave was higher than 1/2 of the same lead R wave. Negative: The horizontal offset or oblique offset of ST segment was below 0.1 mV; the T wave was flat or bi-directionally inverted.

#### *Dual luciferase reporter gene experiment*

The target gene analysis of miR-182 was carried out on the biological prediction site microRNA.org, and the dual luciferase reporter gene experiment was used to determine whether NOX4 is a direct target of miR-182. Cells containing the reporter plasmid pMIR-reporter (Promega, Madison, WI, USA) were inoculated in 24-well plates and cultured in DMEM with 10% fetal bovine serum in an incubator at 37°C with 5% CO<sub>2</sub> for 24 h. The pMIR-reporter (Promega, Madison, WI, USA) was introduced into the synthetic NOX4 3'UTR gene fragment using the endonuclease sites SpeI and Hind III. The mutant site sequence was designed on the wild type NOX4. After restriction enzyme digestion, the target fragment was inserted into the pMIR-reporter reporter plasmid using T4 DNA ligase, thus producing the pNOX4-Wt. According to bioinformatics prediction, the site of miR-182 binding to the target gene was mutated to construct pNOX4-Mut carrier. The pRL-TK carrier (E2241, Promega, USA) with Renilla luciferase was used as an internal control to adjust the difference in transfection efficiency and the number of cells. miR-182 mimics, negative control and luciferase reporter carrier were co-transfected into the cells respectively. Forty-eight hours after transfection, the DMEM medium was removed and cells were washed twice with PBS. The cells were harvested and lysed with the dual luciferase reporter assay system (Dual-Luciferase® Reporter Assay System, E1910, Promega) for luciferase activity. Firefly luciferase activity was inspected by adding 50 µL firefly luciferase working solution to each 10 µL cell sample. Then, 50 µL Renilla luciferase working solution was added to detect Renilla luciferase activity. Relative luciferase activity was calculated according to the firefly luciferase activity/Renilla luciferase activity. The experiment was repeated three times [3].

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### *Plasmid construction and grouping*

According to the sequence of NOX4 (NC 000086.7) on Genbank website, following the design principles of RNA sequence, unrelated sequence, overexpression sequence, interference fragment and NOX4 interference fragment targeting miR-182 were designed and synthesized by Shanghai Jikai Genetic Chemical Co., Ltd. All fragments went through the Blast gene homologous analysis to ensure specificity. The amplified target fragment and p-AAV-CMV plasmid were double-digested (restriction endonuclease, Thermo) and ligated overnight at 16°C under the action of T4 DNA ligase (Thermo). The ligated product was transformed into DH5 $\alpha$  competent cells (CB101, Tiangen Biochemical Science (Beijing) Co., Ltd.) and the positive clones were picked and sequenced (Changsha Engine Technology Co., Ltd.). The constructed carrier, the pRC2-mi342 and pHelper carrier were co-transfected to the AAV2 virus which are packed and contain the exogenous fragments in mammalian cells. After purification, the virus titer was determined [4].

Twelve days after modeling, the rats were divided into 6 groups: blank group (model rats), negative control group (NC group, model rats injected with blank plasmids), miR-182 mimic group (injected with miR-182 mimic plasmids), miR-182 inhibitor group (injected with miR-182 inhibitor plasmids), siRNA-NOX4 group (si-NOX4 group, injected with small interfering si-NOX4 plasmids) and miR-182 inhibitor + si-NOX4 group (injected with miR-182 inhibitor + si-NOX4). The animals were anesthetized with 2% sodium pentobarbital, the rats were placed on the operating table with their limbs in the supine position, and the plasmids were injected into the caudal vein of rats, and then the feeding was continued.

### *ELISA method*

The contents of TNF- $\alpha$ , IL-6, and ICAM-1 in rat serum were measured by ELISA method. Venous blood was collected, set aside for 1 h and centrifuged at 3,000 r/min for 10 min. Then the serum samples were stored in -20°C freezer. ELISA kit (numbers of TNF- $\alpha$ , IL-6, and ICAM-1 detection kit are: 69-25328, 69-40133, 69-30497, Wuhan MSK, China) and the test specimens were operated according to kit instructions. The experiment was repeated for three times.

### *Separation and culture of myocardial micro-vascular endothelial cells in rats of each group*

Adult male SD rats were initially injected with 0.3% pentobarbital sodium intraperitoneally and anesthetized by intraperitoneal injection of 40 mg/kg to ensure that the left ventricle of the rats was removed under aseptic conditions. The removed rat heart was immersed in a vessel containing heparin and PBS solution for 5 min, washed for 3 times with PBS solution, and then soaked in 75% alcohol for about 15 s. The ophthalmic scissors were used to cut the rat ventricular adventitia, and the myocardial tissue was cut into cuts with a size of 1 mm<sup>3</sup>. Then, 3 mL of 0.2% collagenase type II (Sigma, USA) was added, followed by water bathing at 37°C for 6 mins. Then, 3 mL trypsin with a concentration of 0.25% (Sigma, USA) was added, and it was bathed and digested for 5 min. Then, 3 mL DMEM culture medium containing 10% fetal bovine serum was added, and the culture medium was filtered using a 100  $\mu$ m filter, and the filtrate was centrifuged at 2,000 r/min for 10 min. The supernatant was discarded and washed twice with D-hanks. The cells were placed in a previously prepared complete medium (DMEM 4 mL, fetal bovine serum 1 mL, penicillin/streptomycin double resistant 500  $\mu$ L), and then incubated in 5% CO<sub>2</sub> at 37°C. After 6 h continuous culture, the liquid was replaced, and the cells not adherent to the walls were removed. After 24 h, the liquid was replaced again, and then replaced every 3 days. After the cells grew to 80%, 0.25% trypsin-0.02% EDTA was used for digestion and passage, and the 2-3 passages of digested cells were used for the next experiment [5].

### *Detection of NO secretion activity*

The cells in the logarithmic growth phase were digested with 0.25% trypsin-0.02% EDTA medium and the complete medium was resuspended after centrifuge processing (DMEM 4 mL, fetal bovine serum 1 mL, penicillin/streptomycin double resistant 500  $\mu$ L). The cells were seeded in a 24-well culture plate at 1\*10<sup>4</sup> cells/well and cultured in an incubator at 37°C in 5% CO<sub>2</sub>. After each group grew and adhered to the wall, four parallel wells were established in each group, and after 24 h, the cell supernatant was collected. The NO<sup>3-</sup> and NO<sup>2-</sup> concentrations in the cell culture supernatants of each group were measured according to the procedure of NO kit (K262-200, AmyJet Scientific,

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**Table 1.** qRT-PCR primer sequence

Gene	Sequences
miR-182	Upstream: 5'-ACGGGCTTTGGCAATGGTAGAA-3' Downstream: 5'-CAGTGCAGGGTCCGAGGTAT-3'
NOX4	Upstream: 5'-TATCAGCATTAGAAAACACCCA-3' Downstream: 5'-GCCTCCAGCCACACACAGACT-3'
p38MAPK	Upstream: 5'-TCCAAGGGCTACACCAAATC-3' Downstream: 5'-TGTRCCAGGTAAGGGTGAGC-3'
NF-κBp65	Upstream: 5'-AAGATCAATGGcTACACAGG-3' Downstream: 5'-CCTCAAGTCTTCTTCTGC-3'
GAPDH	Upstream: 5'-CACGGCAAGTTCAACGGCACAGTCA-3' Downstream: 5'-GTGAAGACGCCAGTAGACTCCACGAC-3'
U6	Upstream: 5'-GCTTCGGCAGCACATACTAAAT-3' Downstream: 5'-CGCTTCACGAATTTGCGTGCAT-3'

**Table 2.** ECG test result

Group	Case	Negative pole	Positive pole	$\chi^2$	P
Normal group	10	10	0	57.367	<0.0001
Model group	60	1	59	58.349	<0.0001

China) and the amount of secreted NO was calculated [6].

### MTT method

The cells in logarithmic growth phase were digested with 0.25% trypsin-0.02% EDTA medium, and then resuspended after centrifuge processing. The final concentration was 1 M and the cells were seeded in 96-well culture plate at  $1 \times 10^4$  cells/well in an incubator of 5% CO<sub>2</sub> at 37°C. After the cells grew to a monolayer of 60%, serum-free culture medium was adopted, 8 parallel wells each group, and then 20 μL of MTT solution (Thermo Fisher Scientific, a concentration of 5 g/L) was added for 4 h culture. After the culture was completed, the culture solution was sucked out and 150 μL of dimethylsulfoxide (DMSO) was added to each well, and the solution was thoroughly shaken for 10 min till the crystals were dissolved. The absorbance A value of each well was read by using an ELISA detector (DG5031, Nanjing Huadong Electronics Group Medical Equipment Co., Ltd., China) at a wavelength of 490 nm. The experiment was repeated for three times [7].

### Hoechst staining

The sterile coverslips were placed in 24-well plate and the cells in logarithmic growth phase

were digested with 0.25% trypsin-0.02% EDTA medium and resuspended after centrifuge processing. The final concentration was 1 M and the cells were seeded in a 24-well plate at a density of 500 μL ( $6 \times 10^5$ /holes), and cultured in an incubator of 5% CO<sub>2</sub> and 37°C [8]. Each group was set up with 4 parallel wells. After the cells were fully integrated with the wall, the coverslips were removed, rinsed for 3 times with PBS, each time for 5 min. Hoechst 33258 working solution (C1011, Beyotime) was adopted for 5 min staining at room temperature. The solution was washed with distilled water and dried. Five high magnification fields of vision were randomly selected for observation [9]. Apoptotic nuclei showed pyknosis with high concentration and bright color. The number of positive (apoptotic) cells

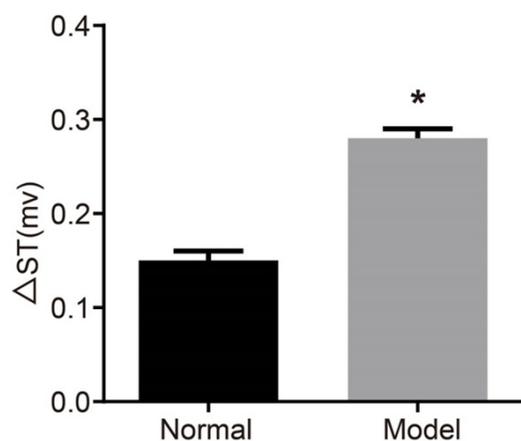
was calculated, and the apoptosis rate—the number of positive cells/the total number of cells. The experiment was repeated for three times.

### qRT-PCR

Total RNA was extracted according to Trizol instruction manual and the RNA concentration and purity were tested with ultraviolet spectrophotometry (UV1901, Shanghai Aucy Scientific Instruments Co., Ltd., Shanghai, China). The concentration of all samples with a purity of A260/A280=1.8-2.0 was adjusted to 50 ng/μL, and the RNA was reversely transcribed into cDNA (50 ng/μL) using PrimeScript™ RT reagent Kit (Takara, RR047A, Beijing Zhijie Fangyuan Technology Co., Ltd., Beijing, China), and frozen under -80°C for future use. The primers were designed by Genetool software. Primer synthesis was completed by Beijing Tsingke Biological Technology Co., Ltd. (**Table 1**).

According to the ABI 7900HT real-time PCR two-step method, U6 and GAPDH were set as internal reference. The reaction conditions: Pre-denaturation at 95°C for 30 s; denaturation at 95°C for 5 s, annealing at 58°C for 30 s, extension at 72°C for 15 s, 40 cycles in total. The relative expression levels of miR-182, NOX4, p38MAPK, and NF-κB mRNA in the cells

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**Figure 1.** Histogram of ECG ST changes. Normal, the normal group, Model, the model group. Compared with the normal group, \* $P < 0.0001$ ,  $t = 38.4500$ .

were calculated by  $2^{-\Delta\Delta Ct}$  method. Three replicate wells were established for each gene in each sample. The experiment was repeated for three times.

### Western blot

The cells in the logarithmic growth phase were centrifuged at 3,000 r/min and 4°C for 20 min, and the supernatant was discarded. The cyto-compound PCV (volume of centrifuged compact cells) was estimated and every 100  $\mu\text{L}$  cell lysate (P0013B, Beyotime Biotechnology Institute and 1  $\mu\text{L}$  phosphatase inhibitors (Roche, Switzerland) were added per 20  $\mu\text{L}$  of cells) for 30 min lysis on ice. It was centrifuged under low temperature at 12,000 r/min for 10 min. The supernatant protein was taken, and the microplate protein content was detected. 50  $\mu\text{g}$  protein was taken and dissolved in 2x SDS loading buffer and the sample was boiled for 5 min at 100°C. Then, gel electrophoresis was performed by 10% SDS-PAGE. After the electrophoresis, the sample was transferred to a PVDF membrane (TE77XP semi-dry membrane system, TE77XP, Shanghai Genetimes Technology Co., Ltd.). The skim milk powder with a concentration of 5% was blocked at room temperature for 1 h and rinsed by PBS for 2 min. The PVDF membrane and diluted mouse antibody were incubated, including anti-NOX4 (1:1,000, ab-216654), anti-p38MAPK (1:1,000, ab31828) and anti-NF- $\kappa\text{B}$  (0.5  $\mu\text{g}/\text{mL}$ , ab16502), anti-Bax (1:5,000, ab32503), anti-Bcl-2 (1:500, ab692) antibodies. The above antibodies are from Abcam, Cambridge, UK, and kept overnight under

4°C, and washed by TBST for three times, each time for 5 min. 1:100 diluted HRP-labeled secondary anti-goat and anti-mouse IgG antibody (HA1003, Shanghai Yanhui Biotechnology Co., Ltd.) was incubated for 1 h. Finally, ECL chemiluminescence kit (O164, Shanghai Surej Biotechnology Co., Ltd., Shanghai, China) was used for development, and Image-Proplus image analysis software (Media Cybernetics, USA) was used for analysis. Using GAPDH as internal reference, the ratio of the gray values of target and internal reference bands was taken as the relative protein expression [9]. Each experiment was repeated for 3 times.

### Statistics analysis

All the data were processed using SPSS 21.0 statistical software (SPSS Inc, Chicago, USA). The measurement data are expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm \text{sd}$ ). The normal distribution variables were compared as independent samples t-test while the non-normal distribution variables were compared using rank sum tests. One-way ANOVA should be used for comparison among groups. Counting data was represented by the percentage, and chi-square test was adopted for analysis.  $P < 0.05$  indicates that the difference is statistically significant.

## Results

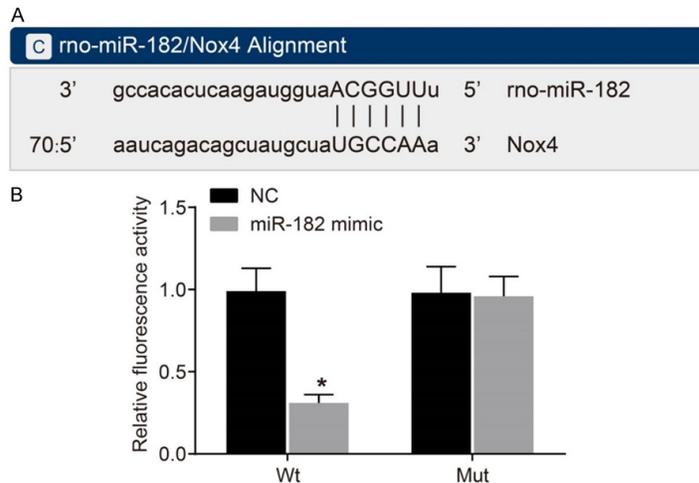
### Changes of ECG ST

No animal died during the modeling process. The ST changes of all animals are shown in **Table 2**. As shown in **Figure 1**, the changes of ECG ST segment in model group were significantly higher than those in normal group ( $P < 0.05$ ).

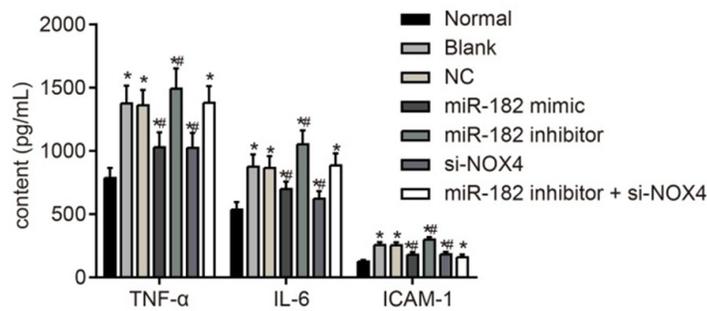
### Verification result of target relationship

Online analysis software was adopted for analysis, and there were binding sites between miR-182 and NOX4 3'UTR (**Figure 2A**). Therefore, NOX4 is the target gene of miR-182. Dual fluorescent reporter system results showed that compared with NC group, the luciferase signal of wild-type miR-182/NOX4 co-transfection group was lower in miR-182 mimics transfection group ( $t = 7.9230$ ,  $P = 0.0014$ , **Figure 2B**). However, there was no significant difference in the luciferase signal between mutant-type miR-182/NOX4 plasmid group ( $t = 0.1732$ ,  $P =$

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**Figure 2.** Verification of target relationship between miR-182 and NOX4. A: The predicted binding site of miR-182 on NOX4 3'UTR; B: The result of luciferase activity test, compared to NC group, \*P<0.05. NC group, negative control group; NOX4, NADPH oxidase 4.



**Figure 3.** Contents histogram of inflammatory factors, including TNF- $\alpha$ , IL-6, and ICAM-1 in rat serum in each group. Compared with normal group, \*P<0.05; compared with blank group and NC group, #P<0.05. NC group, negative control group.

0.8709). Therefore, NOX4 is the target gene of miR-182 [5, 9].

### Contents inspection results of inflammatory factors, including TNF- $\alpha$ , IL-6, and ICAM-1 in the rat serum of each group

The ELISA results are shown in **Figure 3**. Compared with the normal group, the contents of inflammatory factors, including TNF- $\alpha$ , IL-6 and ICAM-1 in the other groups were significantly increased (all P<0.05). There was no significant difference between blank group and NC group (P>0.05). Compared with blank group and NC group, the contents of TNF- $\alpha$ , IL-6 and ICAM-1 in serum of miR-182 mimic group and si-NOX4 group were significantly decreased (all P<0.05) while the contents of TNF- $\alpha$ , IL-6, and

ICAM-1 were significantly increased in miR-182 inhibitor group (all P<0.05), but there was no obvious change in miR-182 inhibitor + si-NOX4 group (all P>0.05) [10-12].

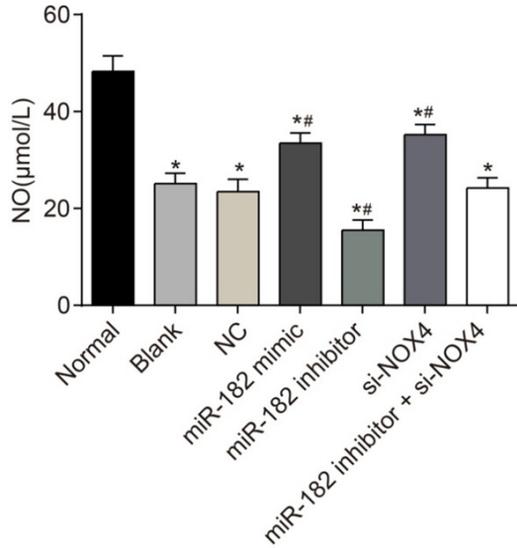
### Inspection results of NO secretion activity in various groups

**Figure 4** shows the NO secretion activity in various groups. Compared with normal group, the NO secretion in Blank group, NC group, miR-182 mimic group, miR-182 inhibitor group, si-NOX4 group, miR-182 inhibitor + si-NOX4 group was lower (t=10.3900, P=0.0005; t=10.4300, P=0.0005; t=6.6590, P=0.0026; t=14.7200, P=0.0001; t=5.8580, P=0.0042; t=10.7700, P=0.0004); there was no significant difference between Blank group and NC group (t=0.8667, P=0.4350). Compared with blank group and NC group, NO secretion in miR-182 mimic group and si-NOX4 group was higher while NO secretion in miR-182 inhibitor group was lower (t=5.5780, P=0.0051; t=4.1510, P=0.0143), and there was no significant difference in miR-182 inhibitor + si-NOX4 group.

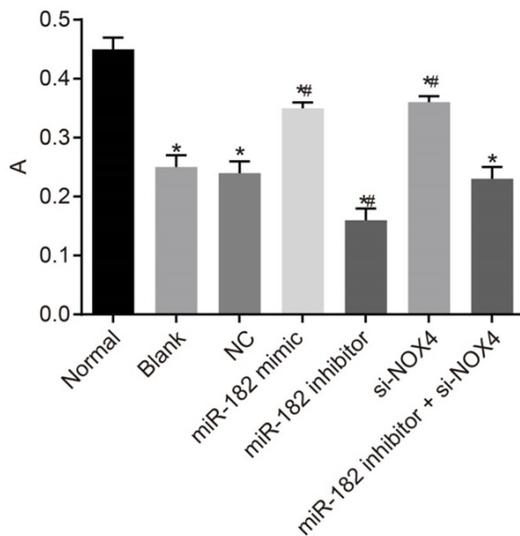
### Proliferation activity of various groups

As shown in **Figure 5**, compared with normal group, the proliferation of myocardial microvascular endothelial cells in blank group, NC group, miR-182 mimic group, miR-182 inhibitor group, si-NOX4 group and miR-182 inhibitor + si-NOX4 group slowed down (t=12.2500, P=0.0003; t=12.8600, P=0.0002; t=7.7460, P=0.0015; t=17.7600, P<0.0001; t=6.9710, P=0.0022; t=13.4700, P=0.0002); there was no significant difference between the blank group and the NC group (t=0.6124, P=0.5734). Compared with the blank group and the NC group, the proliferation of myocardial microvascular endothelial cells in miR-182 mimic group and si-NOX4 group accelerated, and there was no significant difference in miR-182 inhibitor + si-NOX4 group.

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**Figure 4.** NO secretion contents histogram in various groups. Compared with normal group, \* $P < 0.05$ ; compared with blank group and NC group, # $P < 0.05$ . NC group, negative control group.



**Figure 5.** Cell proliferation of various groups. Compared with normal group, \* $P < 0.05$ ; compared with blank group and NC group, # $P < 0.05$ . NC group, negative control group.

### Cell apoptosis of various groups

Hoechst staining results of each group of cells are shown in **Figure 6**, and the cells were uniformly light blue and at the normal nuclear state. The uniform light blue indicates normal cell nucleus nuclear pyknosis, those with high concentrated chromatin and pyknosis are apoptotic nuclei. Compared with the normal group,

the apoptosis rate of myocardial microvascular endothelial cells in the blank group, the NC group, the miR-182 mimic group, the miR-182 inhibitor group, the si-NOX4 group and the miR-182 inhibitor + si-NOX4 was higher ( $t=12.2500$ ,  $P=0.0003$ ;  $t=12.8600$ ,  $P=0.0002$ ;  $t=7.7460$ ,  $P=0.0015$ ;  $t=17.7600$ ,  $P<0.0001$ ;  $t=6.9710$ ,  $P=0.0022$ ;  $t=13.4700$ ,  $P=0.0002$ ); there was no significant difference between blank group and NC group ( $t=0.3744$ ,  $P=0.7271$ ). Compared with the blank group and the NC group, the apoptosis rate of myocardial microvascular endothelial cells in the miR-182 mimic group and the si-NOX4 group was lower, the apoptosis rate of miR-182 inhibitor was higher and there was no significant difference in the miR-182 inhibitor + si-NOX4 group ( $t=4.8780$ ,  $P=0.0082$ ,  $t=5.0130$ ,  $P=0.0074$ ).

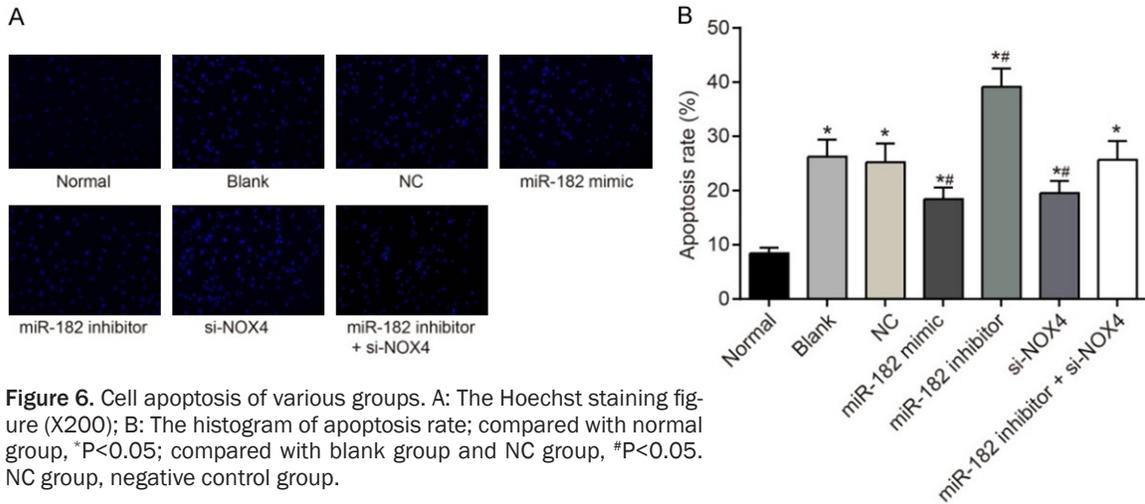
### Expression levels of miR-182, NOX4, p38MAPK, and NF-κBp65 mRNA in various groups

The qRT-PCR results in each group are shown in **Figure 7**. Compared with normal group, the expression of NOX4, p38MAPK, and NF-κBp65 mRNA was up-regulated and the miR-182 mRNA was down-regulated in all other groups (all  $P < 0.05$ ). There was no significant difference between the NC group and the blank group. Compared with the blank group and the NC group, the expression of miR-182 in the miR-182 mimic group was upregulated, the expression of NOX4, p38MAPK, NF-κBp65 mRNA in the miR-182 inhibitor group and the si-NOX4 group was downregulated, the miR-182 in miR-182 inhibitor + si-NOX4 group was downregulated (all  $P < 0.05$ ), and there was no other significant change [5-20].

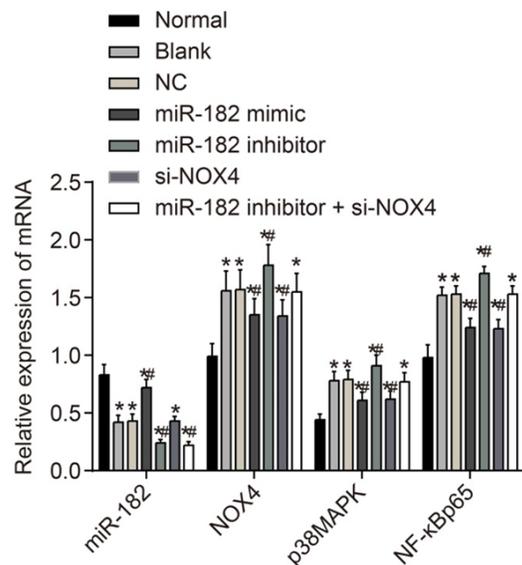
### Protein expression levels of NOX4, p38MAPK, NF-κBp65, Bax, and Bcl-2 in each group

**Figure 8** shows the inspection results of Western blot. Compared with normal group, protein expression of NOX4, p38MAPK, NF-κBp65 and Bax was upregulated and the protein expression of Bcl-2 was downregulated in all the other groups (all  $P < 0.05$ ); there was no significant difference between the blank group and the NC group. Compared with the blank group and the NC group, protein expression of NOX4, p38MAPK, NF-κBp65, and Bax in the miR-182 mimic group and the si-NOX4 group was downregulated and the protein expression of Bcl-

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**Figure 6.** Cell apoptosis of various groups. A: The Hoechst staining figure (X200); B: The histogram of apoptosis rate; compared with normal group, \* $P < 0.05$ ; compared with blank group and NC group, # $P < 0.05$ . NC group, negative control group.



**Figure 7.** Related mRNA expression histogram of various groups. Compared with normal group, \* $P < 0.05$ ; compared with blank group and NC group, # $P < 0.05$ . NC group, negative control group.

κBp65, Bax, and Bcl-2 was downregulated (all  $P < 0.05$ ), and there was no obvious difference in protein expression in the miR-182 inhibitor + si-NOX4 group.

### Discussion

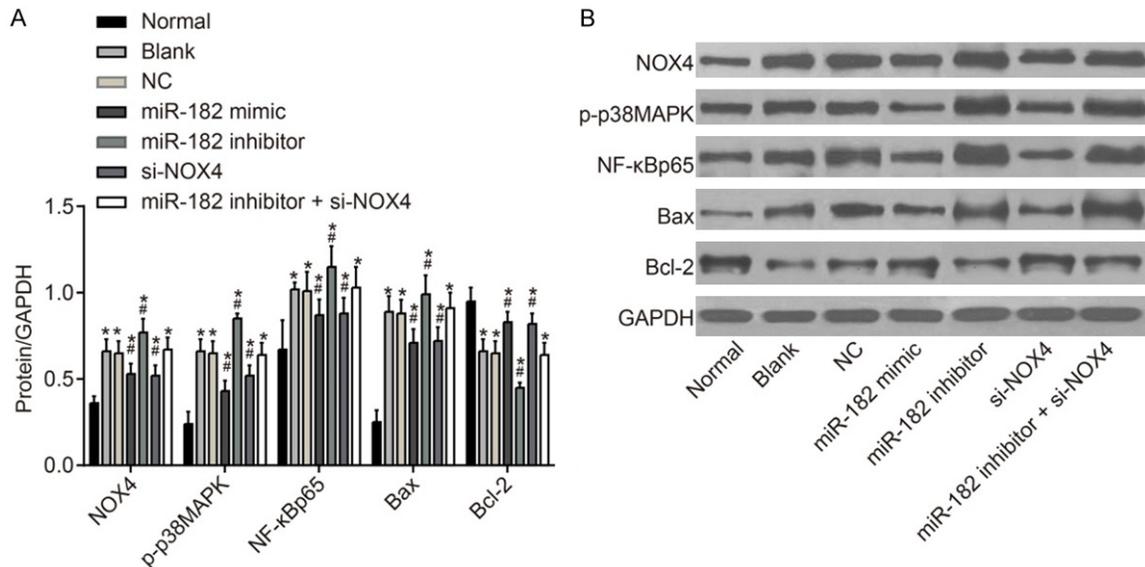
Cardiovascular disease is one of the most common diseases that pose serious threats to human health worldwide. Endothelial cell injury is the leading cause of cardiovascular injury [8]. As a barrier between vascular smooth muscle and circulating blood, vascular endothelium has the function of receiving and transmitting

information, and regulating the permeability and tension of blood vessels as well as coagulation and fibrinolysis states [9]. Once vascular endothelial cells are injured, cell-synthesis ability and the release of vasoactive peptides are easily decreased. Therefore, understanding the regulatory mechanism of endothelial cell molecules is of great significance for the treatment of cardiovascular diseases caused by endothelial injury [11, 13].

As a non-coding RNA, miRNA plays an important role in human physiological functions and has been shown to play an important role in the transformation of tumor epithelial stroma and the invasion and metastasis of tumor cells [11]. Clinical studies have found that miR-182 is closely related with a variety of cancer cells [12]. For example, miR-182 and prostate cancer studies have found that miR-182 can promote proliferation of prostate cancer cells, but in lung squamous cell carcinoma, miR-182 has the effect of inhibiting cancer cell proliferation [14, 15]. The current incidence of CAD cannot be completely explained through the hyperlipemia, hypertension, diabetes and other reasons. More and more studies confirm that inflammatory reaction and oxidative stress play an important role in the pathogenesis process of the disease [16, 17].

In order to further explore the protective effect of p38MAPK/NOX4 signaling pathway induced by NOX4 gene on inflammatory injury of endothelial cells in CAD, we constructed a SD rat model. The change of ECG ST segment in the model group was significantly higher, which suggests that the modeling was successful.

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**Figure 8.** Protein expression levels of NOX4, p38MAPK, NF-kBp65, Bax, and Bcl-2 in various groups. A: The protein expression histogram; B: The protein electrophoresis map; compared with normal group, \* $P < 0.05$ ; compared with blank group and NC group, # $P < 0.05$ . NC group, negative control group; NOX4, NADPH oxidase 4.

Through online analysis software, there were binding sites between miR-182 and NOX4 3'UTR, which indicate that NOX4 is the target gene of miR-182. The content of inflammatory factors, including TNF- $\alpha$ , IL-6, and ICAM-1 in rat serum in each group were detected by ELISA, which shows that the contents of inflammatory factors, including TNF- $\alpha$ , IL-6, and ICAM-1 in rat serum in miR-182 mimic group and si-NOX4 group were significantly decreased while the contents of TNF- $\alpha$ , IL-6, and ICAM-1 in miR-182 inhibitor group were significantly increased (all  $P < 0.05$ ). The results showed that inflammatory factors in miR-182 group and NOX4 plasmids were significantly increased while there was no obvious change in miR-182 inhibitor + si-NOX4 group. NO has an important active molecule regulating vascular function, and can not only dilate blood vessels, but also maintain the integrity and elasticity of blood vessels. Once the NO secretion is imbalanced, it is easy to cause vascular lesions [18]. Compared with the blank group and the NC group, NO secretion in miR-182 mimic group and si-NOX4 group were much higher ( $P < 0.05$ ), which further suggests that after the inhibition of miR-182 expression, the NO secretion in the rat endothelial cells was decreased, and the risk of vascular disease was increased. Some studies explored the role of oxidative stress products and inflammatory factors in the pathogenesis of CAD and found that both in patients with CAD were significantly

increased and well correlated [19]. Some studies explored the p38MAPK signaling pathway and vascular endothelial cell oxidative stress injury and found that downregulation of p38MAPK phosphorylation and expression inhibition of NOX4 can effectively inhibit human umbilical vein endothelial cell injury [20]. We detected proliferation of myocardial microvascular endothelial cells by the MTT method and found that proliferation of myocardial microvascular endothelial cells in the miR-182 mimic group and the si-NOX4 group accelerated and proliferation of the miR-182 inhibitor group slowed down (all  $P < 0.05$ ), which indicates that miR-182 can regulate NOX4 down to inhibit p38MAPK/NOX4 signaling pathway and promote proliferation of rat cardiac microvascular endothelial cells. Hoechst staining showed that the apoptosis rate of myocardial microvascular endothelial cells in other groups was higher than that in the normal group ( $P < 0.05$ ). Compared with the blank group and the NC group, the apoptosis rate of myocardial microvascular endothelial cells in the miR-182 mimic group and the si-NOX4 group was lower while the apoptosis rate of the miR-182 inhibitor group was higher (all  $P < 0.05$ ), which indicates that miR-182 overexpression can downregulate NOX4 to inhibit the p38MAPK/NOX4 signaling pathway and thus inhibit the apoptosis rate of rat cardiac microvascular endothelial cells. The expression levels of miR-182, NOX4, p38MAPK,

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and NF- $\kappa$ Bp65 mRNA in each group were observed. It was found that miR-182 was regulated up in miR-182 mimic group compared with the blank group and the NC group, expression of NOX4, p38MAPK, and NF- $\kappa$ Bp65 mRNA was regulated down in the miR-182 inhibitor group and the si-NOX4 group, suggesting that p38MAPK/NOX4 signaling pathway induced by NOX4 of miR-182 targeted inhibition has a good anti-inflammatory effect. Expression of NOX4, p38MAPK, NF- $\kappa$ Bp65, Bax, and Bcl-2 in each group also confirmed that high expression of miR-182 could regulate down the NOX4 to activate the p38MAPK/NOX4 signaling pathway and inhibit expression of apoptosis-related protein expression in rat cardiac microvascular endothelial cells. In this study, different rat groups were constructed by RNA interference, and the relationship between p38MAPK/NOX4 signaling pathway induced by NOX4 gene of miR-182 targeting and the inflammatory injury of endothelial cells in CAD was investigated. However, considering the limited sample size and other factors and that there are several different miRNA target genes, NOX4 may also be regulated by other target genes apart from miR-182, which we hope to further explore in future study.

In summary, the p38MAPK/NOX4 signaling pathway induced by NOX4 gene of miR-182 targeting has a certain protective effect on the endothelial injury recovery of CAD. The high expression of miR-182 can promote the repair of inflammatory injury of endothelial cells in CAD. The p38MAPK/NOX4 signaling pathway is blocked and plays a protective role in inflammatory injury of endothelial cells in CAD. This study provides a new basis for the targeted treatment of CAD and the research and development of targeted drugs.

### Disclosure of conflict of interest

None.

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