

# MicroRNA-146a/NAPDH oxidase4 decreases reactive oxygen species generation and inflammation in a diabetic nephropathy model

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**Abstract.** The present study investigated the role of microRNA (miR)-146a in a diabetic nephropathy (DN) model, and its molecular mechanism. DN mice were given intraperitoneal injections of streptozotocin (55 mg/kg/day) for 5 consecutive days as an *in vivo* DN model. The HK-2 human kidney cell line were exposed to 45% D-glucose as an *in vitro* DN model. Firstly, it was demonstrated that miR-146a expression was inhibited and NAPDH oxidase 4 (Nox4) was increased in DN mice. In HK-2 cells, overexpression of miR-146a inhibited Nox4 protein expression and decreased reactive oxygen species (ROS) generation, oxidative stress and inflammation, and suppressed vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) protein expression. N-acetylcysteine, a Nox4 inhibitor, was demonstrated to inhibit ROS generation, suppress VCAM-1 and ICAM-1 protein expression, and decrease oxidative stress and inflammation in HK-2 cells following overexpression of miR-146a. In conclusion, these results indicated that miR-146a/Nox4 decreases ROS generation and inflammation and prevents DN. Therefore, miR-146a may represent a novel anti-inflammatory and -oxidative modulator of DN.

## Introduction

Diabetic nephropathy (DN) is the most common complication of diabetes, as one of the main causes of renal failure. Its incidence has increased yearly, which has become a serious global public health problem (1). According to the estimation from the World Trade Organization, the global diabetes incidence will reach 370 million by 2025, of which 30% will develop into DN (2). Pathological features primarily include glomerular hypertrophy, hyperplasia, a thickened basement

membrane, increase of extracellular matrix components, and gradual progression to glomerular sclerosis, interstitial fibrosis and loss of function, which eventually leads to chronic renal failure, reducing the quality of life of the patient (3). The morbidity of DN has many causes, and the pathogenesis is complex, which remains to be fully elucidated.

The function of a large number of microRNAs (miRNAs) have been studied *in vivo*. One-third of human genes are subject to the regulation of miRNAs (4). They are involved in various physiological and biochemical processes, including cell growth, differentiation, apoptosis, sugar metabolism, fat metabolism, insulin secretion, brain formation, cardiogenesis and stem cell differentiation, and are closely associated with the occurrence of many diseases (including cancer) (5). Different from other genes, miRNAs have an independent transcription method, of which the product is not translated into a protein; they identify specific targets and bond with them, thereby regulating target expression after transcription (6). A large number of studies have indicated that miRNAs are involved in the development of DN, and form a very complex network (7). The identification of miRNAs provides a novel way to study the pathogenesis of DN (8).

At present, the pathogenesis of DN remains to be fully elucidated. However, it is understood that the significant pathological features at early stages are glomerular hypertrophy caused by glomerular mesangial cell proliferation, and the elevated glomerular filtration rate induced by mesangial cell (MC) systolic dysfunction (9). Oxidative stress is enhanced in the state of high glucose, and the generation of reactive oxygen species (ROS) is increased (10). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main source of ROS in MCs, and NAPDH oxidase 4 (Nox4) is the most important NADPH oxidase subunit in MCs (9). NADPH oxidase and Nox4-derived ROS is involved in the abnormal proliferation of MCs induced by high glucose (11).

Previous study have suggested that DN is a low-grade inflammatory disease, proposing an 'inflammatory theory' of diabetes. Inflammation is considered to promote the pathogenesis of diabetes, with significance in the development of diabetic complications including DN (12). Previous have shown that during the development of DN, inflammation is obvious, renal tissue is accompanied with the infiltration of various inflammatory cells, including mononuclear macrophages, and significant increase in the concentration of various

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pro-inflammatory cytokines can often be detected in the tissues and circulating blood (12,13). The control of diabetes kidney inflammation is helpful for delaying the development of DN. Therefore, the oxidative stress of DN and inflammation are considered as a whole in current research, in which a large amount of active oxygen generated by enhanced oxidative stress caused by a high glucose environment is regarded as a major cause of DN inflammation, and inflammation increases oxidative stress in turn, indicating a significant interaction between them (14,15). Therefore, the present study aimed to investigate whether miRNA-146a/Nox4 decreases ROS generation and inflammation in a DN model.

## Materials and methods

**Animal studies.** The animal experimental protocol was approved by the Institutional Ethics Committee on Animal Care and Experimentation at the Hospital of Tianjin Nankai (Tianjin, China). Male C57BL/6 mice (age, 8 weeks; weight, 20–25 g) were obtained from the Animal Laboratory of Tianjin Nankai and maintained in a specific pathogen free environment with a 12-h light/dark cycle at 22–24°C and 55–60% humidity and free access to food and water. All mice were randomly distributed into two groups: Control (n=6) and DN model (n=6). DN mice were given intraperitoneal injections of streptozotocin (STZ; 55 mg/kg/day; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 consecutive days as the DN model. Control mice were given intraperitoneal injections of saline for 5 consecutive days. All mice were sacrificed using decapitation under anesthesia (35 mg/kg/day of pentobarbital sodium) and renal tissue samples were collected, washed PBS and stored at -80°C.

**Cell culture and transfection.** HK-2 human kidney cells were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in keratinocyte serum-free media (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B, and incubated at 37°C in a 100% humidified incubator containing 5% CO<sub>2</sub>. The groups were as follows: DN model and D-glucose control, exposed to 45 D-glucose; control, exposed to D-glucose; negative, exposed to 5% D-glucose and transfected with a negative control plasmid; miR-146, exposed to 45% D-glucose and transfected with an miR-146 mimic and treated with N-acetylcysteine (NAC, 3 mM, Beyotime Institute of Biotechnology, Haimen, China). The miR-146 mimic (30 nM) and negative control duplex mimic (30 nM) plasmids were transfected into HK-2 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from HK-2 cells transfected with mimics using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then cDNA was synthesized using a Takara RNA PCR kit (Baoshengwu, Dalian, China). The gene expression of microRNA-146a and Nox4 were measured by qPCR using a SYBR Select Master mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers used were as follows: Forward, 5-CTCTGCTCCTCCTGTTCGAC-3 and reverse, 5-ACGACCAATCCGTTGACTC-3

for GAPDH; forward, 5-GCTGACGTTGCATGTTTCAG-3 and reverse, 5-CGGGAGGGTGGGTATCTAA-3 for Nox4. A CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.) was run at 50°C for 1 min and at 95°C for 5 min, followed by 40 cycles at 95°C for 30 sec and 60°C for 1 min. miRNA expression was measured using the quantitation cycle (Ct) according to the  $\Delta\Delta Cq$  method.  $\Delta\Delta Cq = \Delta Cq$  (gene of interest) -  $\Delta Cq$  (endogenous control) (16,17).

**Assessment of ROS concentration.** ROS levels were analyzed by an Oxiselect Intracellular ROS Assay kit (Cell Biolabs, Inc., San Diego, CA, USA). HK-2 cells were incubated with the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Cell Biolabs, Inc.) for 20 min at 37°C and 5% CO<sub>2</sub> in the dark. ROS levels were analyzed at wavelengths of 485 and 525 nm using a fluorescence microscope (Eclipse Ti-S, Nikon Corporation, Tokyo, Japan) and analyzed using NIS-Elements Basic Research software version 4.10 (Nikon Corporation). Cellular levels of ROS were measured by means of a previously described (18) semi-quantitative DCFH-DA fluorescence technique, which can be used to track alterations in ROS concentration over time. Intracellular ROS expression levels were analyzed by an Oxiselect Intracellular ROS Assay kit (Cell Biolabs, Inc.), which measures ROS by employing the cell-permeable fluorogenic probe DCFH-DA.

**Assessment of oxidative stress and inflammation.** Mice were anaesthetized using 35 mg/kg pentobarbital sodium (Sigma-Aldrich; Merck KGaA) and sacrificed using decapitation. Serum was collected. The supernatants were separated by centrifugation at 500 x g for 5 min at 4°C and stored at -80°C. Superoxide dismutase (SOD; cat. no. A001-3), malondialdehyde (MDA; cat. no. A003-1), nuclear factor (NF)-κB (cat. no. H202), tumor necrosis factor (TNF)-α (cat. no. H052), interleukin (IL)-6 (cat. no. H007) and IL-1β (cat. no. H002) levels were measured using rat ELISA kits (Nanjing Institute of Biological Engineering) at a wavelength of 450 nm using a microplate reader (BMG Labtech GmbH, Ortenberg, Germany), according to the manufacturer's protocol.

**Western blot analysis.** Total proteins were lysed from renal tissue in radioimmunoprecipitation buffer (Beyotime Institute of Biotechnology) and protein concentrations (collected by centrifugation at 12,000 x g for 10 min at 4°C) and determined using an Enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Proteins (50 µg) were separated by 8–12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Membranes were blocked in TBS with Tween-20 (TBST) buffer containing 5% non-fat dry milk. Following this, they were probed with Nox4 (cat. no. sc-55142), vascular cell adhesion molecule (VCAM)-1 (cat. no. sc-1504), intracellular adhesion molecule (ICAM)-1 (cat. no. sc-1511), all at a 1:500 dilution and purchased from Sigma-Aldrich; Merck KGaA, and GAPDH (cat. no. AG019, 1:5,000, Beyotime Institute of Biotechnology) primary antibodies at 4°C overnight. Blots were washed with TBST buffer and then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. no. sc-2030; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Protein

bands were detected using an Enhanced Chemiluminescence Hyperfilm (GE Healthcare Life Sciences, Little Chalfont, UK) and quantified using Bio-Rad gel imaging system 4.6 software (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and Student's Newman-Keuls test for comparisons were used to determine differences between control and experimental groups.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of microRNA-146a and Nox4 in the DN model.** As presented in Fig. 1A, the expression of microRNA-146a in DN model mice was reduced compared with the control group. However, the expression of Nox4 mRNA in DN model mice was increased compared with the control group (Fig. 1B).

**Overexpression of miR-146a on Nox4 expression in the DN model.** The overexpression of miR-146a on the effect on Nox4 expression in DN mice was evaluated. As presented in Fig. 2A, there was a significant inhibition of miR-146a expression in the negative group, compared with the control group. An miR-146a mimic effectively increased miR-146a expression in HK-2 cells exposed to D-glucose. Then, there was a significant increase in Nox4 protein expression levels in HK-2 cells exposed to D-glucose, compared with the control group (Fig. 2B and C). Overexpression of miR-146a significantly inhibited Nox4 protein expression in HK-2 cells exposed to D-glucose, compared with the D-glucose group (Fig. 2B and C).

**Overexpression of miR-146a on ROS generation and oxidative stress in the DN model.** Whether overexpression of miR-146a affected ROS generation and oxidative stress in the DN model was investigated. As presented in Fig. 3A and B, there was a significant increase of ROS generation and MDA activity, respectively, in HK-2 cells exposed to D-glucose, compared with the control group. However, there was a significant decrease in SOD activity in HK-2 cells exposed to D-glucose, compared with the control group (Fig. 3C). However, overexpression of miR-146a significantly reversed these effects (Fig. 3).

**Overexpression of miR-146a on inflammation in the DN model.** To confirm whether overexpression of miR-146a affected inflammation in the DN model, NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  activity were measured using ELISA kits. There was a significant increase of NF- $\kappa$ B (Fig. 4A), TNF- $\alpha$  (Fig. 4B), IL-6 (Fig. 4C) and IL-1 $\beta$  (Fig. 4D) activity in HK-2 cells exposed to D-glucose, compared with the control group. Overexpression of miR-146a significantly reversed this effect (Fig. 4).

**Overexpression of miR-146a on VCAM-1 protein expression in the DN model.** Overexpression of miR-146a on VCAM-1 protein expression in the DN model was examined by western blot analysis. As presented in Fig. 5, VCAM-1 protein expression levels were significantly increased in the negative group

compared with the control group. Overexpression of miR-146a significantly reversed this effect.

**Overexpression of miR-146a on ICAM-1 protein expression in the DN model.** Overexpression of miR-146a on ICAM-1 protein expression in the DN model was examined by western blot analysis. As presented in Fig. 6, ICAM-1 protein expression levels were significantly increased in the negative group compared with the control group. Overexpression of miR-146a significantly reversed this effect.

**Inhibition of Nox4 on ROS generation and oxidative stress in DN following overexpression of miR-146a.** Whether inhibition of Nox4 affects ROS generation and oxidative stress in DN following overexpression of miR-146a was examined. ROS generation was significantly increased in the negative group compared with the control group; however, overexpression of miR-146a ameliorated this effect. NAC significantly decreased ROS generation (Fig. 7A), increased SOD activity (Fig. 7B) and decreased MDA activity (Fig. 7C) in HK-2 cells exposed to D-glucose following overexpression of miR-146a, compared with the miR-146a group.

**Inhibition of Nox4 on inflammation in the DN model following overexpression of miR-146a.** Whether inhibition of Nox4 would affect inflammation in the DN model following overexpression of miR-146a was examined. Inhibition of Nox4 by NAC significantly inhibited NF- $\kappa$ B (Fig. 8A), TNF- $\alpha$  (Fig. 8B), IL-6 (Fig. 8C) and IL-1 $\beta$  (Fig. 8D) activity in HK-2 cells exposed to D-glucose following overexpression of miR-146a, compared with the compared with the miR-146a group.

**Inhibition of Nox4 on VCAM-1 protein expression in the DN model following overexpression of miR-146a.** Whether inhibition of Nox4 would affect VCAM-1 protein expression in the DN model following overexpression of miR-146a was examined. As presented in Fig. 9, inhibition of Nox4 significantly inhibited VCAM-1 protein expression in HK-2 cells exposed to D-glucose following overexpression of miR-146a, compared with the miR-146a group.

**Inhibition of Nox4 on ICAM-1 protein expression in the DN model following overexpression of miR-146a.** Whether inhibition of Nox4 would affect ICAM-1 protein expression in the DN model following overexpression of miR-146a was examined. As presented in Fig. 10, inhibition of Nox4 significantly suppressed the protein expression of ICAM-1 in HK-2 cells exposed to D-glucose following overexpression of miR-146a, compared with compared with the miR-146a group.

## Discussion

DN is one of the most common chronic microvascular complications of diabetes, of which the incidence rate increases with diabetes duration (2). Patients with partial DN may develop into end-stage renal disease, causing kidney failure and requiring a kidney transplant, and may lead to mortality (19). The results demonstrated that overexpression of miR-146a significantly decreased NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  activities in HK2 cells exposed to D-glucose.

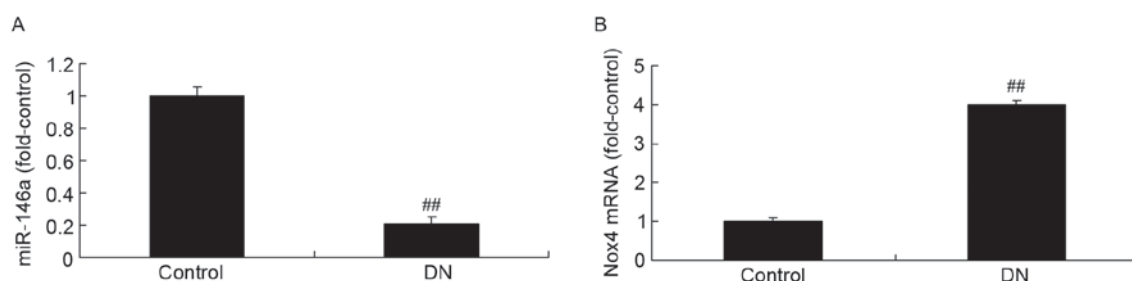


Figure 1. Expression of miR-146a and Nox4 in a DN model. mRNA expression levels of (A) miR-146a and (B) Nox4 in a DN model. Data are presented as the mean  $\pm$  standard deviation. ##P<0.01 vs. control group. DN, diabetic nephropathy; miR, microRNA; Nox4, NADPH oxidase 4.

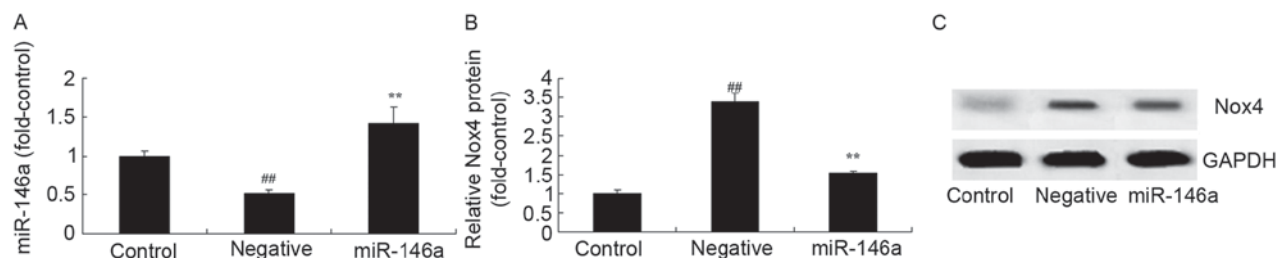


Figure 2. Overexpression of miR-146a on Nox4 expression in a DN model. (A) miR-146a expression levels. (B) Quantification and (C) representative western blot images of Nox4 protein expression levels. Data are presented as the mean  $\pm$  standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group. DN, diabetic nephropathy; miR, microRNA; Nox4, NADPH oxidase 4.

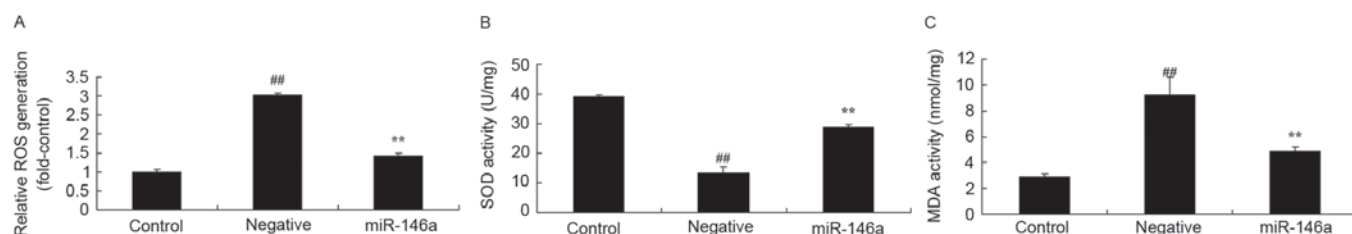


Figure 3. Overexpression of miR-146a on ROS generation and oxidative stress in a DN model. (A) ROS generation, and (B) SOD and (C) MDA activity in a DN model. Data are presented as the mean  $\pm$  standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group. DN, diabetic nephropathy; miR, microRNA; ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde.

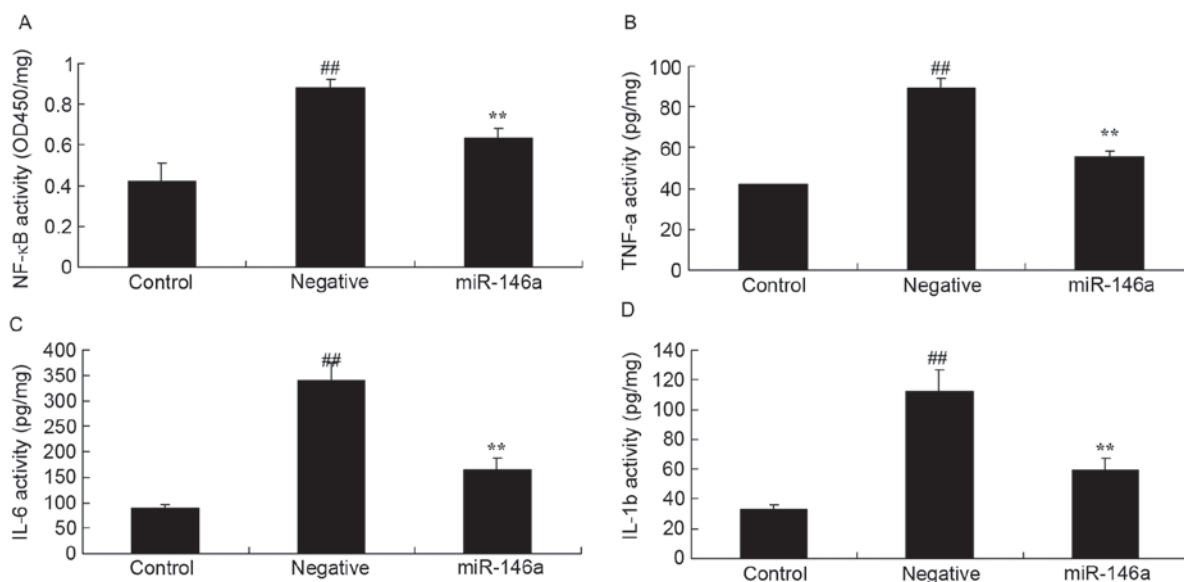


Figure 4. Over-expression of miR-146a affect on inflammation in DN model. Activities of (A) NF- $\kappa$ B, (B) TNF- $\alpha$ , (C) IL-6 and (D) IL-1 $\beta$  in a DN model. Data are presented as the mean  $\pm$  standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group. DN, diabetic nephropathy; miR, microRNA; NF- $\kappa$ B, nuclear factor  $\kappa$ B; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin.



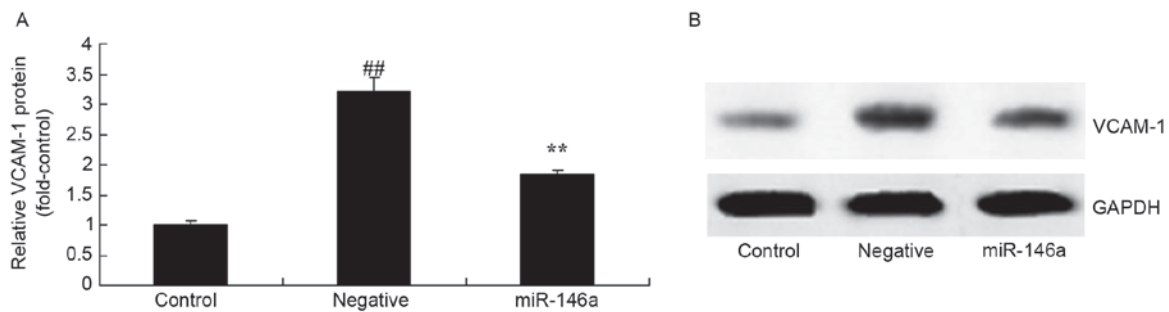


Figure 5. Overexpression of miR-146a on VCAM-1 protein expression in a DN model. (A) Quantification and (B) representative western blot images of VCAM-1 protein expression levels in a DN model. Data are presented as the mean  $\pm$  standard deviation. ## $P$ <0.01 vs. control group; \*\* $P$ <0.01 vs. negative group. DN, diabetic nephropathy; miR, microRNA; VCAM-1, vascular cell adhesion molecule-1. (VCAM)-1 (sc-1504), intracellular adhesion molecule.

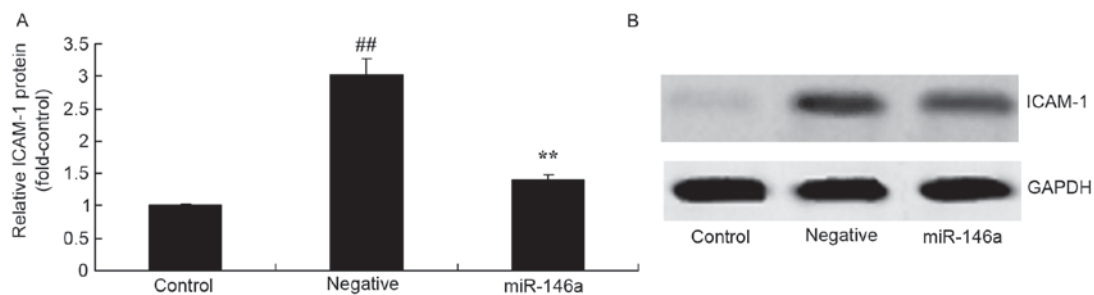


Figure 6. Overexpression of miR-146a on ICAM-1 protein expression in a DN model. (A) Quantification and (B) representative western blot images of ICAM-1 protein expression levels in a DN model. Data are presented as the mean  $\pm$  standard deviation. ## $P$ <0.01 vs. control group; \*\* $P$ <0.01 vs. negative group. DN, diabetic nephropathy; miR, microRNA; VCAM-1, vascular cell adhesion molecule-1.

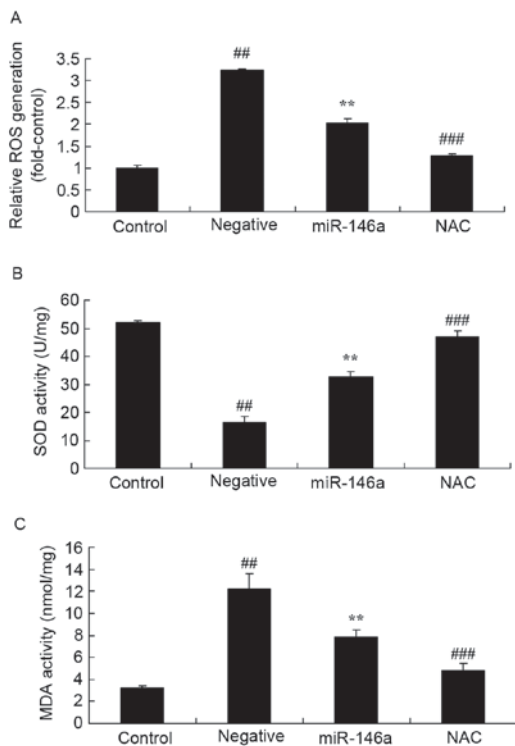


Figure 7. Inhibition of Nox4 on ROS generation and oxidative stress in DN following overexpression of miR-146a. Effect of treatment with the Nox4 inhibitor, NAC, on (A) ROS generation, and (B) SOD and (C) MDA activity in a DN model following overexpression of miR-146a. Data are presented as the mean  $\pm$  standard deviation. ## $P$ <0.01 vs. control group; \*\* $P$ <0.01 vs. negative group; ### $P$ <0.01 vs. miR-146a group. DN, diabetic nephropathy; miR, microRNA; NAC, N-acetylcysteine; ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde; Nox4, NADPH oxidase 4.

Increasing evidence suggests that there is an obvious enhancement in oxidative stress response under DM state (20). ROS produced in the oxidative stress reaction may be a major cause of vascular complications and DM (21). The excessive generation of ROS destroys the dynamic equilibrium of normal oxidation/reduction reaction, which results in oxidative damage of biological macromolecules, including proteins, lipids and nucleic acids, and interferes with regular biological activities (21). Certain studies have suggested that excessive generation of ROS is a direct result of hyperglycemia, and ROS generated from mitochondria by the induction of chronic hyperglycemia is the main initiator of multiple pathological pathways for DN (22,23). The results of the present study suggested that overexpression of miR-146a significantly reversed ROS generation, SOD and MDA activity in HK-2 cells exposed to D-glucose.

NADPH oxidase is originally found in neutrophils, and also exists in other cell types such as endothelial, vascular smooth muscle, mesangial and renal tubular epithelial cells, similar to phagocytic cells, which also produce ROS under hyperglycemic conditions. In physiological conditions, NADPH oxidase activity is reduced, resulting in small amounts of ROS, and kidneys can tolerate this without injury (24). However, under the stimulation of various factors, including diabetes DNA oxidative damage, its activity is increased significantly. When hemodynamic alterations occur, excessive ROS can cause kidney cell dysfunction and even more serious injuries (25). Nox is a homolog of the catalytic subunit gp91phox, mainly including Nox1, Nox3, Nox4 and Nox5, and exist in a variety of non-phagocytic cells, with cell and tissue specificity, and can

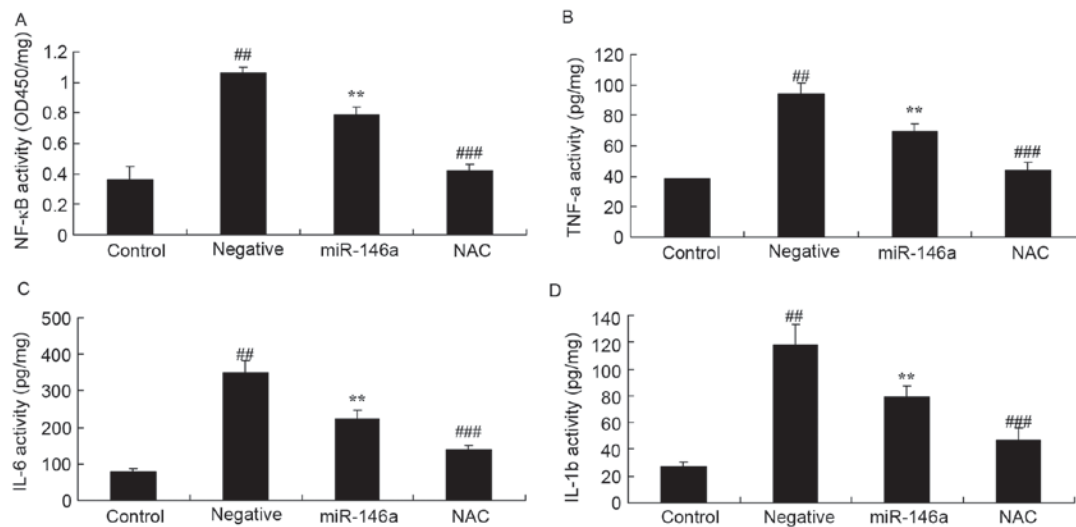


Figure 8. Inhibition of Nox4 affect on inflammation in a DN model following overexpression of miR-146a. Effect of treatment with the Nox4 inhibitor, NAC, on (A) NF-κB, (B) TNF-α, (C) IL-6 and (D) IL-1β activity in a DN model following overexpression of miR-146a. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group; ###P<0.01 vs. miR-146a group. DN, diabetic nephropathy; miR, microRNA; NAC, n-acetylcysteine; NF-κB, nuclear factor κB; TNF-α, tumor necrosis factor-α; IL, interleukin; OD, optical density.

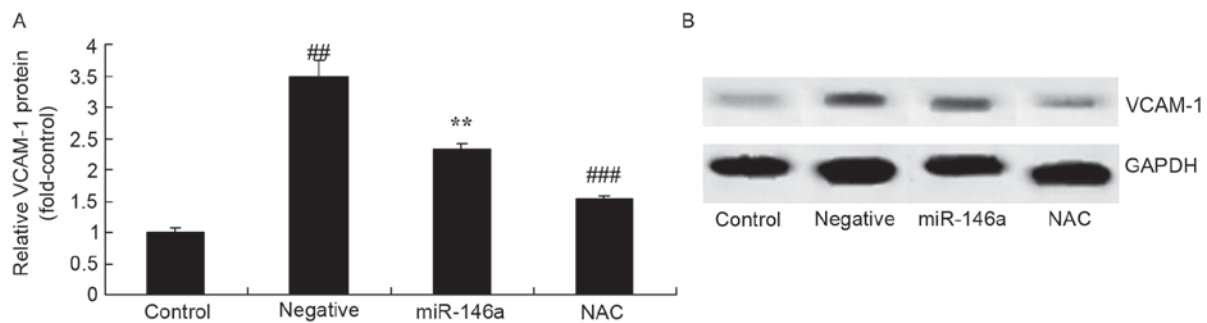


Figure 9. Inhibition of Nox4 on VCAM-1 protein expression in DN model following overexpression of miR-146a. (A) Quantification and (B) representative western blot images of VCAM-1 protein expression levels in a DN model, following treatment with the Nox4 inhibitor, NAC. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group; ###P<0.01 vs. miR-146a group. DN, diabetic nephropathy; miR, microRNA; VCAM-1, vascular cell adhesion molecule-1; Nox4, NADPH oxidase 4.

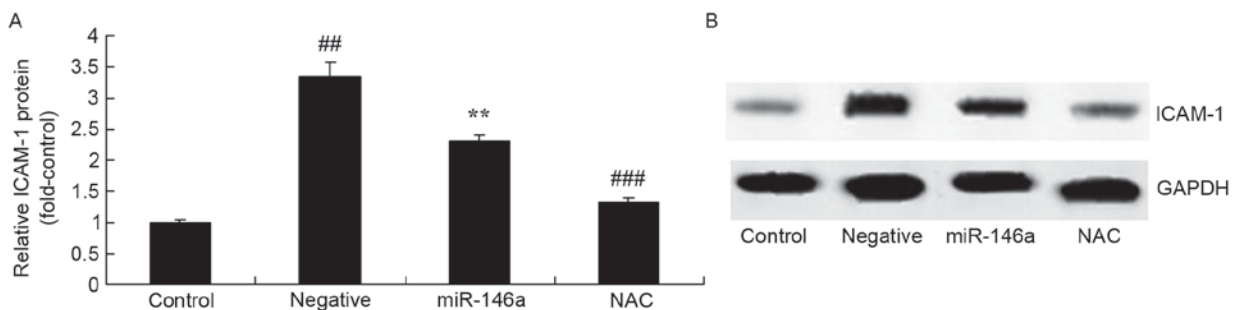


Figure 10. Inhibition of Nox4 on ICAM-1 protein expression in DN model following overexpression of miR-146a. (A) Quantification and (B) representative western blot images of ICAM-1 protein expression levels in a DN model, following treatment with the Nox4 inhibitor, NAC. Data are presented as the mean ± standard deviation. ##P<0.01 vs. control group; \*\*P<0.01 vs. negative group; ###P<0.01 vs. miR-146a group. DN, diabetic nephropathy; miR, microRNA; ICAM-1, intracellular adhesion molecule-1; Nox4, NADPH oxidase 4.

generate ROS through catalysis (26). As a second messenger, ROS generated by Nox is involved in the regulation of cell proliferation, differentiation and apoptosis. Nox4 is the main type present in MCs in Nox subunits (27). The endogenous ROS of Nox4 is closely associated with glomerular injury

associated with diabetes, and upregulation of Nox4 expression can induce glomerular hypertrophy in diabetic rat models (9). The results of the present study demonstrated that overexpression of miR-146a significantly inhibited Nox4 mRNA expression in HK-2 cells exposed to D-glucose.

As the members of the immunoglobulin superfamily, VCAM-1 and ICAM-1 have increased expressions in DN, which are important in the DN immune response and inflammatory reaction, and their effect in DN has gained increasing attention (28). VCAM-1 and ICAM-1 are a class of glycoproteins distributed on the cell surface or extracellular matrix, with adhesive functions. They act by ligand-receptor interactions, which serves an important role in many physiological and pathological processes, including embryonic differentiation, maintenance of healthy structure, inflammation and immune response, blood coagulation and thrombosis, wound healing, and tumor spread and metastasis (29,30). In the present study, overexpression of miR-146a significantly suppressed upregulated VCAM-1 and ICAM-1 protein expression levels in HK-2 cells exposed to D-glucose.

The early stage of DN primarily includes monocyte/macrophage infiltration, in which monocytes gathered in the kidney are activated and proliferate into macrophages, further inducing the production of the inflammatory cytokines, including TNF- $\alpha$ , IL-6 and matrix metalloproteinase (15). Therefore, a series of circulating inflammatory markers such as TNF- $\alpha$  and IL-6 have increased expression levels in DN, which are closely associated with disease progression and proteinuria (31). In addition, hyperglycemia and angiotensin II stimulate vascular endothelial growth factor production, causing the release of endothelial nitric oxide, blood vessel dilation and increased glomerular filtration. Hyperglycemia also induces the synthesis of TGF- $\beta$ 1, IV collagen and fibronectin, so as to increase the extracellular matrix (32). The results of the present study indicated that NAC, a ROS inhibitor, suppressed VCAM-1 and ICAM-1 protein expression, and decreased oxidative stress and inflammation of HK-2 cells following overexpression of miR-146a.

In conclusion, the present study demonstrated that overexpression of miR-146a significantly decreased NF- $\kappa$ B, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  activities, inhibited ROS generation and MDA activity, and increased SOD activity via Nox4/VCAM-1 and ICAM-1 expression in HK-2 cells exposed to D-glucose. Therefore, miR-146a/Nox4 may represent a potential therapeutic target for patients with DN.

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