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Original Article

The effects of Lnc-NTF3-5 on oxidative stress in retinal vascular endothelial cells in diabetic retinopathy rat retinas and related mechanisms

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Abstract: Diabetic retinopathy (DR) is a common diabetes complication. The progression of DR is highly associated with oxidative stress. Many researches suggested that Lnc-NTF3-5 can participate in the occurrence and development of various diseases. However, the effect of Lnc-NTF3-5 on oxidative stress in DR remains unclear. Retinal vascular endothelial cells (RECs) from DR rats and normal rats were cultured before Lnc-NTF3-5 siRNA and Lnc-NTF3-5 plasmid were transfected into RECs, respectively followed by the analysis of Lnc-NTF3-5 expression by real time PCR, cell proliferation by MTT assay, Caspase 3 activity, SOD activity, MDA and ROS content, NF-κB expression by Western blot as well as level of IL-6 and TNF-α by ELISA. Compared with normal group, Lnc-NTF3-5 expression in RECs of DR rats was significantly increased (P<0.05). We draw the conclusion that compared with DR group, the transfection of Lnc-NTF3-5 plasmid significantly up-regulated Lnc-NTF3-5 expression in RECs, inhibited RECs proliferation, increased Caspase 3 activity, decreased SOD activity, increased MDA and ROS content, increased IL-6 and TNF-α levels as well as NF-κB expression. Transfection of Lnc-NTF3-5 siRNA down-regulated Lnc-NTF3-5 expression in DR rat RECs and reversed the above changes. Compared with DR group, the differences were statistically significant (P<0.05). We also observed that the expression of Lnc-NTF3-5 was increased in DR rat RECs. Lnc-NTF3-5 was used as a target to inhibit NF-κB expression, reduce the secretion of inflammatory factors, and regulate REC proliferation by regulating oxidative stress.

Keywords: Diabetic retinopathy, retinal vascular endothelial cells, Lnc-NTF3-5, ROS, SOD

Introduction

Diabetes is an endocrine and metabolic disease with a relatively high incidence. Its complications can eventually lead to multiple organ damage [1]. The most influential factor that impact on quality of life in patients with diabetes is its complications. Diabetic retinopathy (DR) is one of the most common complications [2, 3]. Pathological changes in DR include increased vascular permeability, retinal inflammation, and abnormal angiogenesis, which can lead to blindness [4]. The pathogenesis of DR has not been clearly elucidated yet. The occurrence and development of DR involve multiple factors, such as the production of protein kinase C, advanced glycation end products and vascular endothelial growth factor (VEGF) production [5]. Studies have shown that oxidative stress is highly associated with DR progression. In high glucose environment, oxidative stress generated a large amount of reactive oxygen species (ROS). While antioxidant scavenging ability is weakened, the increased ROS production led to disturbed dynamic balance between the production and clearance of ROS. Oxidative stress and other factors can lead to a thickening of the basement membrane of the retina and abnormal proliferation of vascular endothelial cells [6-8]. Retinal endothelial cells (RECs) are associated with the supply of retinal neurotrophic requirements, which is related to visual protection by maintaining the blood-retinal barrier, inhibiting inflammation, and eliminating toxins [4, 7]. The study found that diabetic retinopathy is closely related to the destruction of the microvascular system of the retina caused by high glucose environment [9]. The growth

and function of abnormal RECs are observed during DR lesions [10].

Studies have reported that long-chain non-coding RNAs (LncRNAs) are transcripts over 200 nucleotides in length but have little things to do with the function of encoding proteins [10]. LncRNAs play key roles in the regulation of various diseases and biological processes, including cell differentiation, proliferation, apoptosis, gene regulation, and cancer development [11]. Although the biological functions of most LncRNAs have not yet been fully elucidated, LncRNAs have been seen regulating gene expression through binding key transcription factors to promoters [12, 13]. Lnc-NTF3-5 can participate in the occurrence and development of various diseases, such as tumor and inflammation [14]. However, the effects of Lnc-NTF3-5 on oxidative stress in diabetic retina and related mechanisms have not been reported.

Materials and methods

Experimental animals

30 healthy female Sprague-Dawley rats (2 months old, SPF grade, body weight 250±20 g) were purchased from the experimental animal center of this unit and fed in SPF animal experiment center. Feeding conditions include maintaining the temperature at 21±1°C and maintaining relative humidity (50-70%) consistently for 12/day cycle every 12 hours. Animal experiments were performed in accordance with the experimental design and performed by experienced technicians to minimize animal suffering. This study was approved by the Ethics Committee of Qitaihe People's Hospital of Heilongjiang Province.

Reagents and instruments

Streptozotocin (STZ) was purchased from Sigma (USA). Western blot related chemical reagents were purchased from Biyuntian Biotechnology (China). ECL reagent was purchased from Amersham Biosciences (USA). Rabbit anti-mouse NF-κB monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP) labeled IgG secondary antibody were purchased from American cell signaling company. The α-MEM complete medium was purchased from Gibco (USA). The eukaryotic expression plasmid pCMV was purchased from Clontech (USA). Fetal bovine serum (FBS) and cyan chain double antibody were purchased from Hyclone Corporation (USA). Dimethyl sulfoxide and MTT powder were purchased from Gibco (USA); trypsin-EDTA digest was purchased from Sigma (USA). IL-6 and TNF-α ELISA kits were purchased from R&D (USA). The RNA extraction kit and the reverse transcription kit were purchased from Axygen (USA). The superoxide dismutase (SOD) content detection kit, the lactate dehydrogenase (LDH) content detection kit, and the ROS content test kit were purchased from the Nanjing Institute of Bioengineering (China). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (China). The ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (USA). The Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad Corporation (USA). The clean workbench was purchased from Suzhou Purification Equipment Factory (China). Surgical microscopy equipment was purchased from Suzhou Medical Instrument Factory (China). The Hera cell CO_2 incubator was purchased from Thermo Company (Germany).

Rat grouping and processing

SD rats were randomly divided into two groups i.e. control group and DR group, with n=15 rats in each group. Rats in DR group were used to prepare a diabetic model through intraperitoneal injection (60 mg/kg) of freshly prepared 1% STZ solution. After 48 hours, blood sampling in the tail vein was used to detect blood glucose. Blood glucose ≥16.5 mmol/L indicated that establishment of DM rats was successful. A micro-injector was used to inject 0.05 μg of VEGF into the vitreous cavity of the eye from 2 mm behind the temporal limbus, which completed the preparation of DR model [15].

RECs cell isolation and culture

Each group of rats were anesthetized with 1% pentobarbital and fixed, respectively. Rats were terminated with strict guidelines to reduce pain and suffering. The external iliac crest and bulbar conjunctiva were cut off. The surrounding soft tissue was separated to dislocate the eyeball. After the optic nerve was cut, the bilateral eyeballs were placed on ice, and the microscope was placed along the corner. The sclera

was cut open layer by layer to obtain retinal tissue. The RECs were cultured by the method of planting, and the tissue blocks were removed after the cells climbed out. After 80% of the cells were fused, the fused cells were digested and passaged for testing. The cells were cultured in a medium containing fetal bovine serum-free DMEM (containing 100 U/ml penicillin, 100 ug/ml streptomycin, 5.5 mmol/L glucose) at 37°C with 5% CO_2.

RECs cell grouping and processing

RECs cells were divided into control group, in which RECs cells were isolated from normal control rat retina and DR group. RECs cells isolated from DR rat retina Lnc-NTF3-5 siRNA group and Lnc-NTF3-5 plasmid group were also processed in which Lnc-NTF3-5 siRNA and Lnc-NTF3-5 plasmid were transfected into DR RECs respectively.

Lnc-NTF3-5 siRNA and Lnc-NTF3-5 plasmid transfection into DR rat RECs

Primers were designed from Primer Premier 6.0 according to the Lnc-NTF3-5 sequence and inserted into the pCMV vector. Lnc-NTF3-5 siRNA sequence: 5'-AGGTGACATGAATCTGCTC-3'; 5'-ATTTCACAGTCGGCTG-3'; primers were synthesized by Shanghai Yingjun Biotechnology Co., Ltd (China). The cloned rat cDNA library was used as a template, and the cDNA was constructed by pCMV-MT1M containing the restriction enzyme site. The PCR product was purified and digested before ligating into pCMV plasmid. The recombinant plasmid pCMV was constructed by double enzyme digestion and sequencing. The pCMV-MT1M plasmid was transfected into DR rat RECs using lipo2000 transfection reagent. The logarithmic growth phase RECs cells were collected. The cell suspension concentration was adjusted to 3×10^6 cells/m1, and then inoculated in a 6-well plate, placed in 5% CO_2 incubator culture at 37°C for 12 h, until cell density fusion reached 70-80%. We added 5 ul lipo2000 mixed into 200 ul serum-free medium, incubated for 15 min at room temperature, then added 200 μl serum-free medium, mixed thoroughly, incubated for 15 min at room temperature. The mixed lipo2000 was mixed with Lnc-NTF3-5 siRNA or Lnc-NTF3-5 plasmid and incubated for 30 min at room temperature. The cell serum in the 6-well plate was removed. PBS was used to wash the cells, and 1.6 ml of serum-free medium was added to all samples. The product was cultured in a 5% CO_2 incubator at 37°C for 6 hours.

Real-time PCR detection of Lnc-NTF3-5 expression in RECs cells

Under sterile conditions, RECs cells were washed with PBS followed by isolation of RNA using Trizol reagent. cDNA was synthesized by PCR according to the relevant primers (**Table 1**). Real-time PCR was used to detect the expression of the target gene. Reaction conditions: 52°C 1 min, 90°C 30 s, 58°C 50 s, 72°C 35 s, a total of 35 cycles. Fluorescence quantitative PCR reactor software was used to collect relevant data. According to the internal reference GAPDH, the cycle threshold (CT) was calculated, and the standard curve was drawn. The quantitative analysis was analyzed by 2-ΔCt method.

MTT assay analysis of growth of RECs cells

After 48 hours of cell culture, cells were digested, counted, and seeded into 96-well plates at 3000 cells/well. Five replicate wells were designed for each group, and 20 μl of 5 g/L MTT solution was added to each group of cells. After 4 hours of incubation in the incubator, the supernatant was completely removed. 150 μl/well of DMSO was added. The samples were shaken for 10 min. After the purple crystals were fully dissolved, the absorbance (A) value was measured at a wavelength of 570 nm by a microplate reader to calculate the cell proliferation rate.

Western blot analysis of NF-κB protein expression in RECs

Extract the RECs cell protein: add lysate, lyse the cells on ice for 15~30 min. Cell disruption was performed by sonication for 5 s × 4 times, centrifuge at 4°C, 10000 g for 15 min, transfer the supernatant to a new tube. After getting the quantification of protein, it was stored at -20°C for Western blot experiments. The isolated protein was electrophoresed on a 10% SDS-PAGE, transferred to a PVDF membrane by semi-dry transfer, blocked with 5% skim milk powder for 2 h to remove the non-specific background, and incubated with NF-κB protein monoclonal antibody (diluted 1:1000) at 4°C, overnight. After washing with PBST, 1:2000 goat anti-rab-

Table 1. Primer sequences

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTGCCAGCCTCGTCTCATAG	CGTTGAACTTGCCGTGGGTAG
Lnc-NTF3-5	AGCACAGCATCTGACTAATGG	GGTCTCGCAACCTTCCGTA

bit secondary antibody was added and incubated under dark for 30 min, followed by washing with PBST and X-ray exposure imaging after addition of enhanced chemiluminescence for 1 min. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity One software. The experiment was repeated 4 times (n=4).

ELISA detection of inflammatory factor levels

All the samples were tested for the expression changes of inflammatory factors IL-6 and TNF-α in the supernatant of each group by ELISA kit according to the ELISA kit instructions. The main operation steps include taking out a 96-well plate and adding 50 μl of the sequentially diluted standard to the corresponding reaction well to prepare a standard curve. 50 μl of the sample was added to the reaction well. We then washed the plate, dried it, and then filled each well with the diluted washing solution before shaking for 30 sec. We added 50 μl of the enzyme labeling reagent to each well, except for blank wells. Mix gently by shaking and incubate for 30 min at 37°C. Wash the plate 5 times. We added 50 μl of the developer A to each well, then added 50 μl of the developer B, gently shake and mix, and develop at 37°C for 10 min in the dark. The enzyme plate was taken out, 50 μl of the stop solution was added to each well to terminate the reaction, and the optical density value (OD value) of each well was measured by a microplate reader at a wavelength of 450 nm.

Caspase 3 activity analysis

The changes in Caspase3 activity in each group of cells were examined according to the kit instructions. Trypsin digested cells were centrifuged at 600 g and 4°C for 5 min, discard the supernatant, add cell lysate, lyse on ice for 15 min, centrifugation at 20000 g and 4°C for 5 min, and add 2 mM Ac-DEVD-pNA. OD value change was measured at 405 nm to calculate Caspase3 activity.

Oxidative stress index detection

The changes of superoxide dismutase (SOD) activity and MDA and ROS content in each group of cells were examined according to the kit instructions. The cell protein was extracted and washed in a 95°C water bath. After 40 min, it was taken out and rinsed with cold water. After cooling, it was centrifuged at 4000 rpm for 10 min. The ethanol phase in the tissue homogenate was extracted using an ethanol-chloroform mixture (5:3, v/v volume ratio 5:3) for detecting MDA and total SOD activity.

Changes in the levels of reactive oxygen species in each group of cells were examined. The treated cells were bathed in a 95°C water bath. After 40 min, they were taken out and rinsed with cold water. After cooling, they were centrifuged at 4000 rpm for 10 min. The tissue homogenate was incubated with 2', 7'-dichlorofluorescein diacetate (DCF-DA) for 15 min at 37°C, centrifuged at 10,000 rpm for 15 min, and the supernatant was discarded. We took the pellet, resuspend in sterile PBS phosphate buffer and incubated for 60 min at 37°C. The level of ROS was measured using a spectrophotometer.

Statistical analysis

Data were processed by SPSS 16.0 software. Measurement data were expressed as mean ± standard deviation (SD) Comparison of multiple groups of samples was performed using one-way ANOVA. $P<0.05$ indicated a statistically significant difference.

Results

Expression of Lnc-NTF3-5 in DR rat RECs

Compared with the normal group, the expression of Lnc-NTF3-5 in RECs of DR rats was increased. The difference was statistically significant ($P<0.05$). Transfection of Lnc-NTF3-5 plasmid significantly up-regulated Lnc-NTF3-5 expression in DR rat RECs ($P<0.05$). Transfection of Lnc-NTF3-5 siRNA significantly down-regulated Lnc-NTF3-5 expression in RECs in DR rat group ($P<0.05$) (**Figure 1**).

Effects of Lnc-NTF3-5 on the proliferation of RECs in DR rats

Compared with control group, the proliferation of RECs in DR rats was reduced, and the differ-

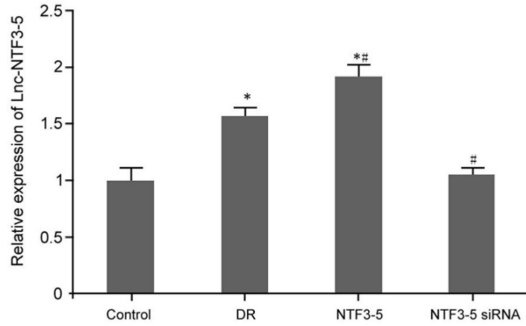

Figure 1. Expression of Lnc-NTF3-5 in DR rat RECs. Compared with the normal group, * P<0.05; compared with the DR group, # P<0.05.

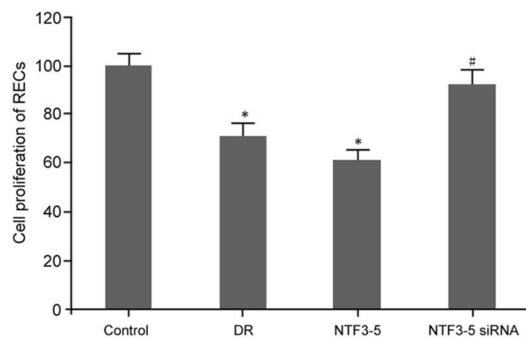

Figure 2. Effects of Lnc-NTF3-5 on the proliferation of RECs in DR rats. Compared with the normal group, * P<0.05; compared with the DR group, # P<0.05.

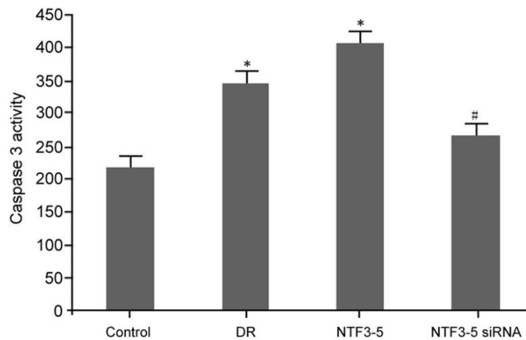

Figure 3. Effects of Lnc-NTF3-5 on Caspase 3 activity in DR rats. Compared with the normal group, * P<0.05; compared with the DR group, # P<0.05.

ence was statistically significant (P<0.05). Transfection of Lnc-NTF3-5 plasmid inhibited the proliferation of RECs. Compared with DR group, the difference was statistically significant (P<0.05). However, we also noticed that the transfection of Lnc-NTF3-5 siRNA significantly promoted the proliferation of RECs (P<0.05) (**Figure 2**).

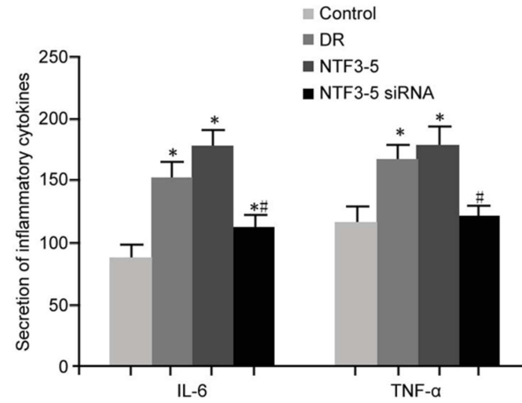

Figure 4. Effects of Lnc-NTF3-5 on the secretion of inflammatory factors in RECs of DR rats. Compared with the normal group, * P<0.05; compared with the DR group, # P<0.05.

Effects of Lnc-NTF3-5 on Caspase 3 activity in RECs of DR rats

Compared with control group, the activity of Caspase 3 in DR rat RECs cells was significantly increased (P<0.05). Transfection of Lnc-NTF3-5 plasmid promoted the increase of Caspase 3 activity. Compared with DR group, the difference was statistically significant (P<0.05). Transfection of Lnc-NTF3-5 siRNA inhibited Caspase 3 activity. Compared with DR group, the difference was statistically significant (P<0.05) (**Figure 3**).

Effects of Lnc-NTF3-5 on the secretion of inflammatory factors

The effect of Lnc-NTF3-5 on the secretion of inflammatory factors in RECs of DR rats was analyzed by ELISA. The secretion of IL-6 and TNF-α secreted by inflammatory factors in DR rat RECs cells was increased. The difference was statistically significant (P<0.05). Transfection of Lnc-NTF3-5 plasmid increased IL-6 and TNF-α secretion. Compared with DR group, the difference was statistically significant (P<0.05). However, transfection of Lnc-NTF3-5 siRNA inhibited IL-6 and TNF-α secretion. Compared with DR group, the difference was statistically significant (P<0.05) (**Figure 4**).

Effects of Lnc-NTF3-5 on oxidative stress in RECs of DR rats

The activity of SOD was decreased. The content of MDA and ROS was increased in DR rat RECs. Compared with the normal group, differences were statistically significant (P<0.05). Trans-

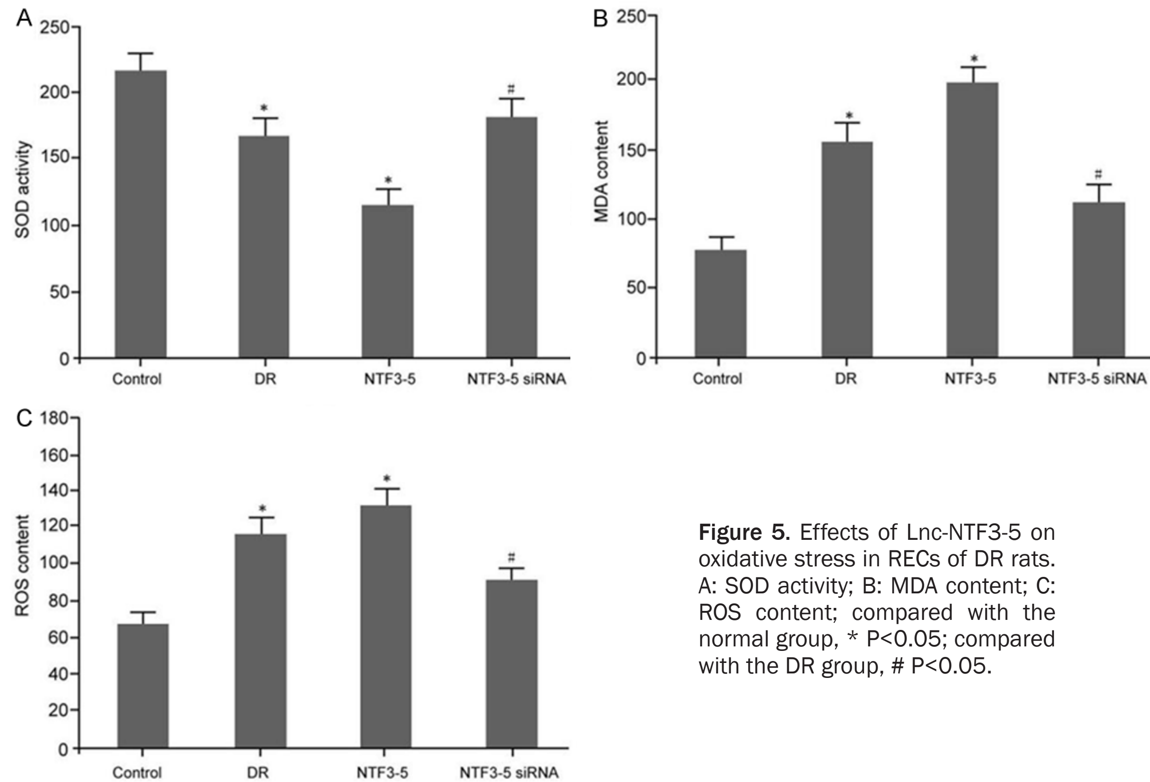

Figure 5. Effects of Lnc-NTF3-5 on oxidative stress in RECs of DR rats. A: SOD activity; B: MDA content; C: ROS content; compared with the normal group, * P<0.05; compared with the DR group, # P<0.05.

fection of Lnc-NTF3-5 plasmid reduced the activity of SOD, increase the content of MDA and ROS. Compared with DR group, the differences were statistically significant (P<0.05). However, Lnc-NTF3-5 siRNA transfection increased SOD activity, decreased MDA and ROS content. Compared with DR group, the differences were statistically significant (P<0.05) (**Figure 5**).

Effects of Lnc-NTF3-5 on the expression of NF-κB in DRC rat RECs

The expression of NF-κB in DR rat RECs cells was increased, compared with the normal group, the difference was statistically significant (P<0.05). Transfection of Lnc-NTF3-5 plasmid increased the expression of NF-κB. Compared with DR group, the difference was statistically significant (P<0.05). However, transfection of Lnc-NTF3-5 siRNA decreased the expression of NF-κB. Compared with DR group, the difference was statistically significant (P<0.05) (**Figure 6**).

Discussion

Diabetic retinopathy is a common ophthalmic disease that cause progressive damage to the patient's vision [16]. The therapeutic effect of DR has not yet achieved satisfactory results. The hyperglycemic environment of diabetic patients can affect the pathological changes and further cause damage of optic nerve function. Hyperglycemia is associated with the occurrence and development of complications in diabetic patients, which can eventually lead to abnormalities in the structure and function of the retina and other organs [17]. Changes in the structure and function of retinal endothelial cells are the primary cellular mechanisms of pathological changes during the chronic progression of diabetes. Hyperglycemia can lead to dysfunction of retinal endothelial cells, including inhibition of cell proliferation [18]. DR is a chronic inflammatory disease, and retinal ganglion cells are gradually oxidized by free radicals. Hyperglycemia can also cause dysfunction of retinal ganglion cells by regulating inflammatory factors. The neuronal damage releases a large number of inflammatory factors, which can cause the pro-inflammatory-anti-inflammatory balance to be disturbed, further aggravating neuronal damage [19, 20]. In this study, it was found that the proliferation of DR rat RECs was inhibited, the apoptotic activi-

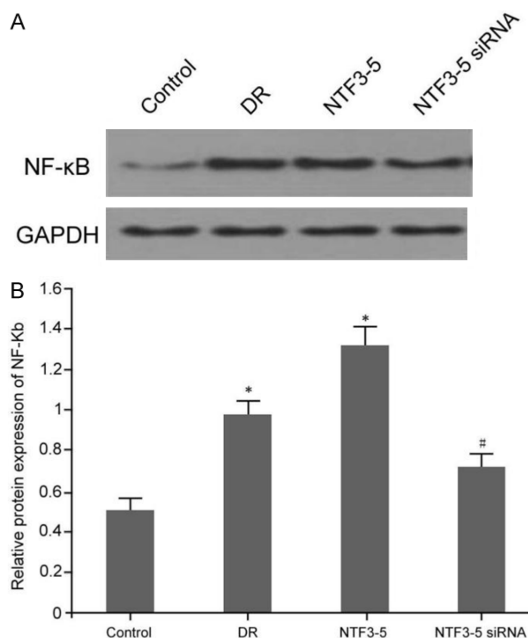

Figure 6. Effects of Lnc-NTF3-5 on the expression of NF-κB in DRC rat RECs. A. Western blot was used to detect NF-κB expression; B. NF-κB expression was statistically analyzed; compared with normal group, * P<0.05; compared with DR group, # P<0.05.

ty was increased, and the secretion of inflammatory factors was increased.

LncRNAs, as long-chain non-coding RNAs, are involved in the regulation of life processes, including cell development, proliferation, differentiation, apoptosis, and cell death. They are not involved in protein coding though [21]. Lnc-NTF3-5 has been shown to be expressed mainly in orthopedic diseases, and its role in DR has not been confirmed. This study confirmed that the expression of Lnc-NTF3-5 in RECs cells of DR model rats was increased, while the transfection of Lnc-NTF3-5 plasmid transfection up-regulated the expression of Lnc-NTF3-5 in DR rat RECs, inhibited the proliferation of RECs and promoted the increase of Caspase 3 and the secretion of inflammatory factors. Transfection of Lnc-NTF3-5 siRNA down-regulated the expression of Lnc-NTF3-5 in DR rat RECs and reversed the above changes, i.e. promoted the proliferation of RECs, inhibited the apoptotic activity and the secretion of inflammatory factors. Further analysis of its mechanism confirmed that high glucose can promote the increase of ROS and MDA production, and reduce SOD activity [22]. Regulation of Lnc-NTF3-5 expression in RECs of DR rats can change SOD activity, inhibit ROS content, reduce MDA content, and regulate oxidative stress. The expression of NF-κB activates the corresponding target genes, including adhesion molecules, inflammatory cytokines, and increased expression of acute phase response proteins, thereby modulating the immune response and amplifying the inflammatory response [23]. The results of this study confirmed that Lnc-NTF3-5 can affect the expression of NF-κB, which affects oxidative stress and inflammatory factors, thereby affecting the state of retinal vascular endothelial cells. In future studies, the expression and related effects of Lnc-NTF3-5 in DR patients are required to clarify the role of Lnc-NTF3-5 in DR as a reference for its related treatment.

Conclusion

The expression of Lnc-NTF3-5 in RECs of DR rats is increased. Lnc-NTF3-5 can inhibit the secretion of inflammatory factors and regulate the proliferation and inflammation by regulating oxidative stress, inhibiting the expression of NF-κB.

Disclosure of conflict of interest

None.

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