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Relationship between miR-375 regulating Ndrg2/IL-6/STAT3 signaling pathway and diabetic retinopathy in rats

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Abstract. – OBJECTIVE: To explore the relationship between micro ribonucleic acid (miR)-375 in regulating the N-Myc downstream-regulated gene 2 (Ndrg2)/interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling pathway and diabetic retinopathy (DR) in rats.

MATERIALS AND METHODS: Thirty Sprague-Dawley rats were randomly divided into Control group (n=10), Model group (n=10), and miR-375 inhibitor group [miR-375 small interfering RNA (siRNA) group, n=10]. The rats in Model group were injected with streptozotocin (STZ) *via* the tail vein to prepare into rat models of diabetes. The body weight, fasting blood glucose, and retinal barrier permeability of rats in each group were detected. The levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in rat serum were measured using kits. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay was performed to determine the apoptosis of optic ganglion cells in rat retinal tissues. Additionally, the messenger RNA (mRNA) and protein levels of Ndrg2, IL-6 and STAT3 in rat retinal tissues were detected *via* reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, respectively.

RESULTS: Compared with Control group, Model group had reduced body weight of rats, increased blood glucose and retinal permeability of rats, raised serum MDA content, decreased SOD activity, up-regulated apoptotic rate of optic ganglia, and notably elevated mRNA and protein levels of Ndrg2, IL-6 and STAT3 in retinal tissues. Compared with those in Model group, the body weight of rats declined, the blood glucose of rats rose, the retinal permeability of rats was decreased significantly, the serum MDA content was reduced, the SOD activity was raised, the apoptotic rate of optic ganglia was decreased, and the mRNA and protein levels of Ndrg2, IL-6 and STAT3 in retinal tissues were also decreased significantly in miR-375 siRNA group.

CONCLUSIONS: MiR-375 inhibitors are able to reduce blood glucose, retinal permeability, and optic ganglion apoptosis in rats with DR, and the mechanism of action may be related to the regulation on the Ndrg2/IL-6/STAT3 signaling pathway.

Key Words:
Rat diabetic retinopathy, Ndrg2/IL-6/STAT3 signaling pathway, Oxidative stress, Apoptosis, MiR-375.

Introduction

As the people's living standard continuously improves and the pace of life is accelerated, diabetes mellitus has an increasing incidence rate year after year due to the unhealthy high-fat and high-sugar diets[1,2]. Diabetic retinopathy (DR) caused by diabetes is one of the major microvascular diseases of diabetes and also one of the most important causes of blindness in diabetic patients[3]. Blindness in DR patients brings a great burden on the families and the society, and also severely affects the quality of life of patients. As to the treatment of DR, retinal photocoagulation and vitreous surgery are mainly employed at present, which block the progression of the disease to some extent, but, unfortunately, fail to cure the disease[4]. Given this, early prevention and diagnosis of patients with DR are of important significance in clinical practice.

The last few years have witnessed that the crucial role of the apoptosis of retinal optic ganglia in the pathogenesis and progression of DR, and therefore, the protection against retinal apoptosis is an important prerequisite for the treatment of DR[5,6]. Interleukin-6 (IL-6), an inflammatory cytokine mainly produced in the pancreas, plays

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an important role in apoptosis and inflammation. Increased IL-6 level is an independent risk factor for DR. Elevated blood glucose induces high expression of IL-6, triggering a series of inflammatory reactions and apoptosis[7,8]. As a downstream target of IL-6, signal transducer and activator of transcription 3 (STAT3) are abnormally activated after being stimulated by the IL-6 signal, which further facilitates apoptosis[9]. N-Myc downstream-regulated gene (Ndrg) is found by researchers to play a vital role in cell proliferation, differentiation, and apoptosis[10]. Recently, Ndrg2, one of the hot research topics, is located on chromosome 14q11.1 and widely distributed in various tissues and organs, whose expression is increased as the embryo matures[11]. Ndrg2 expression is up-regulated in mouse models of diabetes; however, the changes in Ndrg2 in the case of DR are rarely reported. This study, therefore, focused on whether Ndrg2 exerts an anti-apoptosis effect in DR by affecting the IL-6/STAT3 signaling pathway.

Micro ribonucleic acids (miRNAs), a class of endogenous gene-encoded non-coding single-stranded RNAs with about 22 nucleotides in length, are widely expressed in animals and plants. Tens of thousands of miRNAs have been discovered up to now, which play important regulatory roles in cells. Eliasson et al[12] found that the expression level of miR-375 is raised in diabetic patients and is positively correlated with the severity of diabetes. Hence, in this investigation, rat models of diabetes were established by injecting streptozotocin (STZ) through the tail vein to investigate the regulatory effect of miR-375 on DR rats and study the relation between miR-375 and Ndrg2/IL-6/STAT3 signaling pathway.

Materials and Methods

Reagents

MiR-375 small interfering RNA (siRNA; RiboBio, Guangdong, China), STZ and Evans blue (Sigma-Aldrich, St. Louis, MO, USA), malondialdehyde (MDA) and superoxide dismutase (SOD) kits (Beyotime Biotechnology, Shanghai, China), TRIzol solution (Shanghai Shiyi Biotech Inc., Shanghai, China), terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), polymerase chain reaction (PCR) SuperMix kit (TransGen Biotech, Beijing, China), Ndrg2, IL-6, STAT3 and β-actin primers (Invitrogen, Carlsbad, CA, USA), and rabbit anti-Ndrg2, IL-6 STAT3 and β-actin primary antibodies and FITC fluorescently labeled secondary antibodies (Jackson, West Grove, PA, USA).

Instruments

A fluorescence inverted microscope (Olympus, Tokyo, Japan), a microplate reader (DYNEX, St. Petersburg, FL, USA), a low temperature centrifuge (Shenzhen Anke High Technology Co., Ltd., Shenzhen, China), a gel imager (Invitrogen, Carlsbad, CA, USA), and an electrophoresis apparatus and a transfer box for Western blotting (Bio-Rad, Hercules, CA, USA).

Rats

A total of 30 healthy male Sprague-Dawley rats without eye diseases aged 8 weeks old and weighing 200-250 g [license number: SYXK (Sichuan, China) 2018-100] were purchased from Sichuan Academy of Traditional Chinese Medicine Sciences. The rats were generally in good health, with a shiny coat and normal eating, drinking, and action. They were adaptively fed for 7 d for related assays. This investigation was approved by the Animal Ethics Committee of The Affiliated Zhangjiagang Hospital of Soochow University Animal Center.

Establishment of Rat Models of DR

Rat models of diabetes were constructed by injecting STZ into the tail vein. Before modeling, the rats were fasted for 12 h and injected with STZ *via* the tail vein at a dose of 50 mg/kg. The rats in Control group were injected with the same volume of normal saline through the tail vein. Next, blood was drawn from the tail vein after 72 h, and the blood glucose level was measured using a blood glucose tester. Fasting blood glucose concentration >16.7 mmol/L indicated a successful modeling. According to the instructions, the rats in Model group were injected with miR-375 siRNA from the tail vein every three days. The experiment lasted for 3 months, and the body weight and blood glucose level were measured once a month. Besides, retinal barrier permeability was detected using the Evans blue solution.

Detection of MDA Content and SOD Activity in Rat Serum Via Kits

After the 90th day, the serum was collected from rats in each group, and relevant kits were used to determine the MDA level and SOD activ-

ity in rat serum. In accordance with the instructions, standard curves were plotted. Next, MDA and SOD working solution was prepared, and the sample and the standard were added to each well. Ultimately, the absorbance was measured using the microplate reader, and the MDA content and the SOD activity were calculated.

Determination of Apoptosis of Optic Ganglion Cells in Rat Retina Through TUNEL Staining Assay

On the 90th day, materials were taken. The ocular anterior segment and the vitreous body were removed with a pair of surgical scissors, and rat retinal tissues were isolated and soaked in 80% ethanol overnight and then 4% paraformaldehyde solution overnight. Then, they were embedded with paraffin, sectioned, permeabilized, and deparaffinized with xylene, rehydrated with ethanol solution with high to low concentration for 1 min each time. Next, the tissues were added with terminal deoxynucleotidyl transferase fluorescein-labeled deoxyuridine triphosphate reaction solution, incubated in a dark place, and added dropwise with anti-fluorescence quencher. Ultimately, the green fluorescence intensity was observed under the fluorescence microscope, and the apoptotic rate was detected.

Measurement of Ndrg2, IL-6 and STAT3 Messenger RNA (mRNA) Levels in Rat Retina by Reverse Transcription-PCR (RT-PCR) Assay

After the rat retinal tissues in each group were collected, 5 g tissues were taken, cut into pieces on ice, added with 1 mL of TRIzol extract (Invitrogen, Carlsbad, CA, USA) and ground, followed by centrifugation to collect the supernatant. After that, the supernatant was added with such reagents as chloroform and isopropanol and centrifuged. Then, the precipitate was re-suspended with 75% ethanol solution. Next, the RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) as per the instructions, and then PCR amplification was conducted. The designed primers are shown in Table I. The PCR products were subjected to gel electrophoresis, and the bands were ultimately observed under the gel imager.

Detection of Ndrg2, IL-6 and STAT3 Protein Levels in Rat Retina Via Western Blotting

The rat retinal tissues were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) containing protease inhibitors, and the protein concentration was determined using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). The samples prepared were separately loaded and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins. Next, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) and blocked with 5% skim milk powder for 1 h. Thereafter, corresponding rabbit anti-Ndrg2, IL-6, STAT3, and β-actin primary antibodies were added for incubation at 4°C overnight. Then, horseradish peroxidase (HRP)-labeled secondary antibodies were added. Eventually, the protein bands were detected through enhanced chemiluminescence (ECL), and the optical density values were analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Table I. Ndrg2, IL-6 and STAT3 primers sequence information.

Gene Name	Sequences
Ndrg2	5'-AGACTCACTCTGTGGAGACAC-3' 5'-CGTGGTAGGTAAGGATCGCTG-3'
IL-6	5'-ACTCACCTCTTCAGAACGAATTG-3' 5'-CCATCTTTGGAAGGTTCAGGTTG-3'
STAT3	5'-CAGCAGCTTGACACACGGTA-3' 5'-AAACACCAAAGTGGCATGTGA-3'
β-actin	5'-CTCCATCCTGGCCTCGCTGT-3' 5'-GCTGTCACCTTCACCGTTCC-3'

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was utilized for statistics and analysis. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed by the Student's *t*-test. Comparisons among multiple groups were done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Successful Modeling of Rat DR

In Control group, no significant difference was found in the body weight of rats in the three months, the body weight rose by the age, and the blood glucose level was normal. Compared with those in Control group, the body weight of

Table II. Body weight, blood glucose and retinal permeability of rats (*p<0.05, #p<0.05).

Group	Bodyweight (g)			Blood glucose (mmol/L)			Retinal permeability (ng/g)		
	30 d	60 d	90 d	30 d	60 d	90 d	30 d	60 d	90 d
Control group	22.4±1.6	23.7±3.1	25.3±2.8	9.6±0.8	9.2±1.2	8.7±0.9	0.0±0.0	0.2±0.0	0.1±0.1
Model group	23.5±2.9	20.3±1.4	18.5±3.6	23.4±2.7*	28.8±4.2*	32.9±6.4*	65.8±6.4	74.2±4.9	85.5±7.3
MiR-375 siRNA group	24.1±1.8	22.3±2.2	21.9±2.7	17.3±3.2#	19.5±3.4#	21.9±3.6#	37.6±4.3#	48.9±3.9#	52.8±4.4#

Note: *p<0.05: Model group vs. Control group, #p<0.05: miR-375 siRNA group *vs.* Model group.

rats declined in Model group (*p<0.05, *p<0.05, *p<0.05), and the blood glucose level was elevated (*p<0.05, *p<0.05, *p<0.05), with a chlorotic coat and low spirits. In comparison with retinal permeability of rats = 0 in Control group, the retinal permeability was notably raised in Model group (*p<0.05, *p<0.05, *p<0.05). Besides, miR-375 siRNA group exhibited larger body weight (#p<0.05, #p<0.05, #p<0.05), as well as reduced blood glucose (#p<0.05, #p<0.05, #p<0.05) and retinal permeability (#p<0.05, #p<0.05, #p<0.05) in comparison with Model group (Table II).

MiR-375 siRNA Decreased the Serum MDA Content and Increased the SOD Activity in DR Rats

The results of the MDA and SOD kits (Figure 1) revealed that the MDA content in rat serum was higher in Model group than that in Control group (*p<0.05), while the SOD activity was significantly lower in Model group than that in Control group (*p<0.05). These two indexes exhibited opposite tendencies in miR-375 siRNA group compared with Model group (Figure 1A-1B), suggesting that miR-375 siRNA ameliorates oxidative stress damage in DR rats.

MiR-375 siRNA Inhibited Apoptosis of Optic Ganglion Cells in Retinal Tissues of DR Rats

TUNEL staining assay results are shown in Figure 2A. Based on statistics, the apoptotic rate of optic ganglion cells in rat retina was remarkably elevated in Model group compared with that in Control group (*p<0.05), while it declined in miR-375 siRNA group compared with that in Model group (#p<0.05) (Figure 2B).

MiR-375 siRNA Repressed Ndrg2, IL-6 and STAT3 mRNA Levels in Retinal Tissues of DR Rats

Based on the results of RT-PCR (Figure 3A) and histogram (Figure 3B), the mRNA levels of Ndrg2, IL-6 and STAT3 in rat retinal tissues were overtly higher in Model group than those in Control group (*p<0.05, *p<0.05, *p<0.05), and they were evidently lower in miR-375 siRNA group than those in Model group (#p<0.05, #p<0.05, #p<0.05), implying that miR-375 siRNA is capable of downregulating the mRNA levels of Ndrg2, IL-6 and STAT3 in retinal tissues of DR rats.

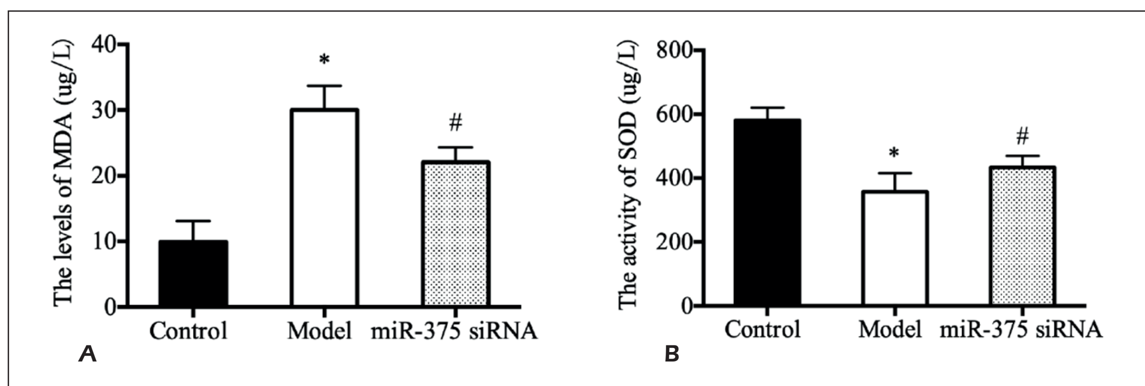

Figure 1. MDA content and SOD activity in rat serum. **A**, MDA content; **B**, SOD activity. (*p<0.05: Model group *vs.* Control group, #p<0.05: miR-375 siRNA group *vs.* Model group).

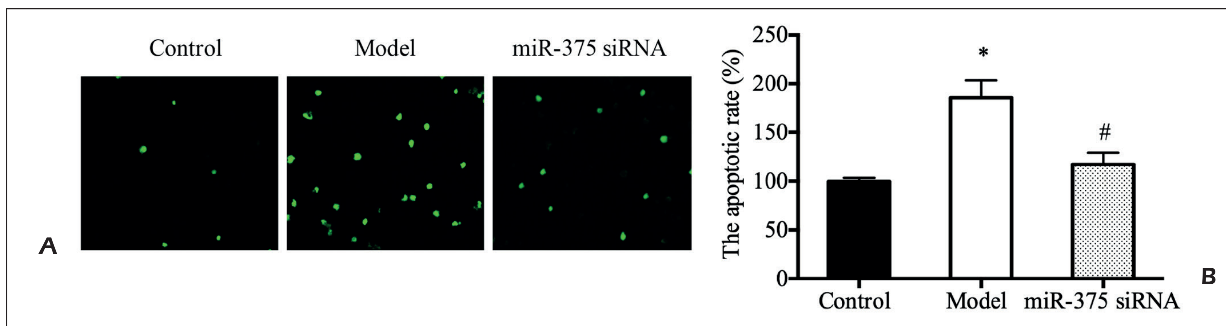

Figure 2. Apoptosis of optic ganglion cells in rat retina through TUNEL staining assay. **A**, TUNEL staining graph (magnification 20×), **B**, Apoptotic rate (*p<0.05: Model group *vs.* Control group, #p<0.05: miR-375 siRNA group *vs.* Model group).

MiR-375 siRNA Suppressed Ndrg2, IL-6, and STAT3 Protein Levels in Retinal Tissues of DR Rats

The results of Western blotting (Figure 4A) and histogram (Figure 4B) showed that the protein levels of Ndrg2, IL-6 and STAT3 in rat retinal tissues were distinctly higher in Model group than those in Control group (*p<0.05, *p<0.05, *p<0.05), and they were markedly lower in miR-375 siRNA group than those in Model group (#p<0.05, #p<0.05, #p<0.05), suggesting that miR-375 siRNA is able to decrease the protein levels of Ndrg2, IL-6 and STAT3 in retinal tissues of DR rats.

Discussion

As one of the common complications of diabetes, DR has a close relation to genetic background, lifestyle, and age, with complex pathogenesis. The classic theory is that DR is mainly caused by the apoptosis of ganglion cells, inflammatory abnormal vascular nerve injury, and damage to the blood-retinal barrier in the retina[13]. Simo-Servat et al[14] proved that the abnormal apoptosis of the optic ganglia plays an important role in the pathogenesis of DR. The growth inhibitor Nogo, one of the regulators of cell repair and regeneration, plays a crucial role in apoptosis. Ndrg is a receptor for Nogo, whose functions have attracted more and more attention. The Ndrg family mainly includes Ndrg1, Ndrg2, Ndrg3, and Ndrg4, which have a high amino acid sequence homology, but are significantly different in the distribution in tissues[15]. Many studies have demonstrated that Ndrg2 participates in various physiological and pathological processes, including tumorigenesis, cellular oxidative stress, metabolism, inflammation, and apoptosis[16]. Zuo et al[17] found that the mechanical allodynia in rats observed at 28 d after the onset of diabetes is associated with the expression of Ndrg2 in abundance in activated astrocytes, which indicates that repressing Ndrg2 expression may be a strategy for the treatment of diabetic tactile allodynia. However, the investigations on the regulatory effect of Ndrg2 in rat models of DR are rare.

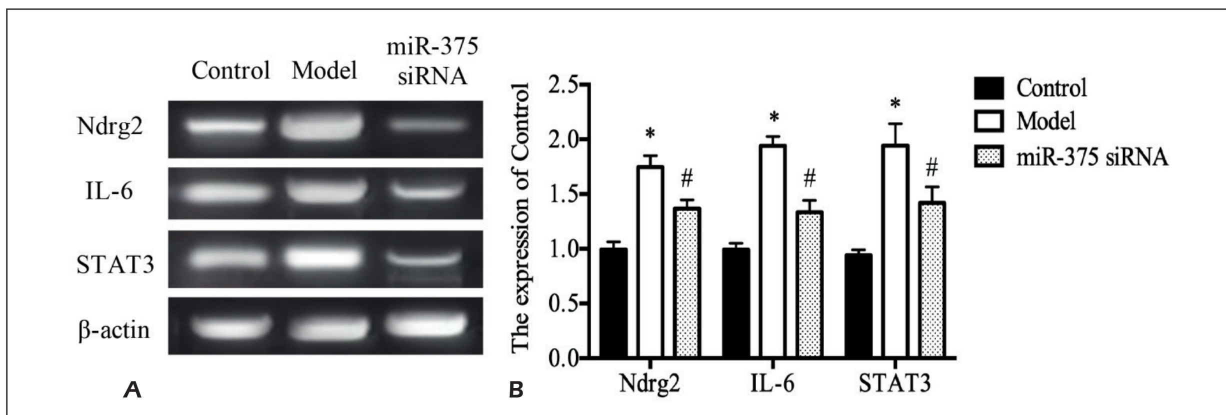

Figure 3. MRNA levels of Ndrg2, IL-6 and STAT3 in rat retinal tissues measured by RT-PCR assay. **A**, RT-PCR band graph. **B**, Band histogram. (*p<0.05: Model group *vs.* Control group, #p<0.05: miR-375 siRNA group *vs.* Model group).

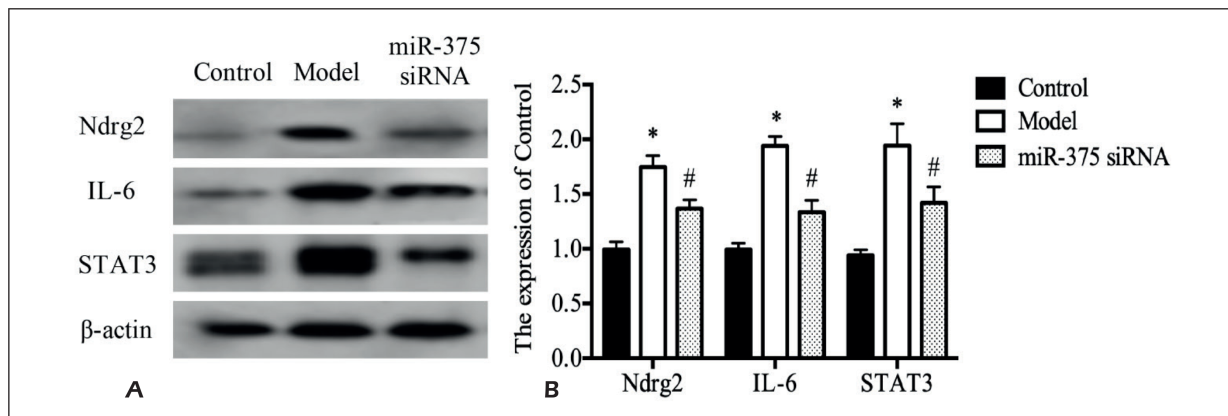

Figure 4. Protein levels of Ndrg2, IL-6 and STAT3 in rat retinal tissues detected via Western blotting. **A**, Western blotting band graph. **B**, Band histogram. (*p<0.05: Model group *vs*. Control group, $^{\#}p<0.05$: miR-375 siRNA group *vs*. Model group).

As molecular biology research develops, miRNAs become a hotspot in the studies of diabetes. MiRNAs complement the target mRNAs and regulate the expression of genes at the post-transcriptional level, facilitating their degradation or inhibiting their translation into proteins. Latreille et al[18] discovered that miR-375 is a key regulator in the biological function of β cells, and the glucose metabolism in diabetic mice is normalized if miR-375 is knocked out, indicating that miR-375 level is a momentous biological marker of islet β cells. Therefore, in this study, diabetes was firstly induced in rats by injecting streptozotocin into the tail vein to investigate the regulatory role of miR-375 in DR rats. STZ, a glucosamine-nitrosourea, is toxic to and selectively destructs islet β cells, which can induce diabetes in many animals. The body weight, blood glucose and retinal permeability of rats were measured at 30 d, 60 d, and 90 d, respectively, and it was uncovered that compared with Control group, Model group had a decreased body weight, slow action, a significantly increased blood glucose level and damaged retinal barrier. Next, the MDA content and SOD activity in rat serum were examined. The MDA content reflects the degree of lipidation in the body. The higher content suggests that the body undergoes an excessive oxidation reaction, which will lead to the aggregation of molecules like proteins and nucleic acids, causing cytotoxicity[19]. The results showed that miR-375 siRNA down-regulated the MDA content in serum of DR rats (Figure 1A). The SOD activity reflects the ability of the body to resist oxidation, and the higher the activity is, the stronger the body's antioxidant capacity will be[20]. The results of the assay revealed that the SOD activity in serum of DR rats was clearly decreased, and miR-375 siRNA significantly modulated the SOD activity, implying that abnormal redox reaction occurs in the rats with DR, with remarkably lowered antioxidant capacity of the rats, and miR-375 siRNA markedly attenuates oxidative stress damage. Thereafter, TUNEL staining assay was carried out to detect the apoptosis of optic ganglion cells in rat retina in each group, and the results (Figure 2) showed that the number of green fluorescent cells was significantly larger in Model group than that in Control group, while it overtly declined in miR-375 siRNA group. To investigate the regulatory mechanism of miR-375 in the signaling pathways affecting the redox reaction and apoptosis in DR rats, the expression of key targets on the Ndrg2/IL-6/STAT3 signaling pathway was examined at the gene and protein levels, respectively. The results (Figure 3-4) revealed that the expression levels of Ndrg2, IL-6 and STAT3 in the retinal tissues of DR rats were evidently increased, while they were clearly suppressed by miR-375 siRNA, suggesting that the inhibition of miR-375 siRNA on the development of DR is closely correlated with the repression on the activation of the Ndrg2/IL-6/STAT3 signaling pathway, and that inhibiting the Ndrg2/IL-6/STAT3 signaling pathway may be a new way to treat DR.

Conclusions

In summary, the results of this study indicate that miR-375 siRNA is able to decrease the body weight and the SOD activity, lower the blood glucose level, increase the retinal barrier permeability and the MDA content in the serum of DR rats, and inhibit the apoptotic rate of optic ganglion cells in the retinal tissues. The mechanism of action may be

related to the regulation of the Ndrg2/IL-6/STAT3 signaling pathway. The results provide a potentially new perspective for the treatment of DR.

Conflict of Interests

The authors declare that they have no conflict of interests.

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