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Association between NDRG2/IL-6/STAT3 signaling pathway and diabetic retinopathy in rats

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Abstract. – OBJECTIVE: The aim of this study was to observe the association between 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: The model of diabetes was successfully established in Sprague-Dawley rats. All rats were divided into diabetes model group (model group, n=10), pathway inhibitor group (CLT-005 group, n=10), and normal control group (control group, n=10). After successful modeling, blood and retinal tissues of rats were collected. The levels of blood glucose and serum IL-6 were detected. Meanwhile, oxidative and antioxidant indexes reactive oxygen species (ROS), superoxide dismutase (SOD), and malondialdehyde (MDA) were detected *via* enzyme-linked immunosorbent assay (ELISA). Morphological changes in retinal tissues were observed using hematoxylin-eosin (HE) staining, and the number of corneal nerve fibers was observed under a microscope. The expressions of vascular endothelial growth factor (VEGF) and NDRG2/IL-6/STAT3 pathway genes in tissues were determined *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Furthermore, the expressions of NDRG2/IL-6/STAT3 pathway proteins were determined *via* Western blotting.

RESULTS: The level of blood glucose in model group was significantly higher than that of control group ($p<0.05$), suggesting successful modeling. The levels of tumor necrosis factor-α (TNF-α), IL-6, and IL-1 in model group were significantly higher than those of control group ($p<0.05$). The content of ROS and MDA in tissues was significantly higher in model group than the other two groups ($p<0.05$). However, SOD increased markedly in CLT-005 group and was close to that of control group. Besides, the number of corneal nerve fibers decreased remarkably in model group. However, it increased significantly in CLT-005 group, but was still smaller than that in control group. According to HE staining, there were significant retinal edema and telangiectasia in model group. Mild retinal edema and more ganglion cells and inner nuclear layers were observed in CLT-005 group than model group. QRT-PCR demonstrated that the mRNA expressions of VEGF, NDRG2, IL-6, and STAT3 were remarkably higher in model group than those in control group ($p<0.05$). However, they decreased significantly in CLT-005 group ($p<0.05$). Model group exhibited remarkably higher protein expressions of NDRG2, IL-6, and STAT3 than control group ($p<0.05$). However, CLT-005 group had decreased protein expressions of these molecules ($p<0.05$), which were close to those in control group.

CONCLUSIONS: The activation of NDRG2/IL-6/STAT3 signaling pathway is positively correlated with the occurrence and development of DR in rats. Therefore, inhibiting the activation of NDRG2/IL-6/STAT3 signaling pathway can affect oxidation and antioxidation, thereby exerting a protective effect against retinal injury in diabetes rats.

Key Words:
NDRG2/IL-6/STAT3 signaling pathway, Rats, Diabetes, Retinopathy.

Introduction

Diabetes mellitus is one of the most common chronic metabolic diseases with many causes in the world[1]. It is characterized by high glucose concentration in the blood. This is caused by the body's inability to produce insulin or fully use insulin. Diabetes is a controllable disease that will not disappear. It has been reported that diabetes has become one of the diseases with high mortality rate worldwide. The traditional therapeutic methods for diabetes mainly include insulin preparations and oral hypoglycemic drugs. However, the wide use of drugs will

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lead to adverse effects such as lactic acidosis, pulmonary edema, etc.[2]. Diabetic patients need lifelong medication, which makes them unable to withstand the long-term economic pressure. Other complications of diabetes, which may result in cardiovascular diseases, such as kidney disease and myocardial fibrosis[3]. Moreover, it can cause renal insufficiency and decline in myocardial systolic and diastolic function, making it difficult to maintain the normal structure and functional components of myocardial tissues. Diabetes also threatens the vision. Therefore, cataract occurs earlier in diabetic patients. In many developed countries, diabetes is the major cause of non-invasive amputation, blindness, and visual impairment in adults. The risk of glaucoma is also significantly higher in diabetic patients[4]. In this lifelong disease, acute and cumulative long-term changes in persistent high concentration of circulating glucose can damage the retina. This may eventually lead to oxidative stress and inflammation, as well as reduce the integrity of vascular wall[5,6]. Dietary antioxidants are seemingly a kind of potential adjuvant therapy that can prevent or delay diabetic complications[7,8]. However, there have been no definite conclusions with great controversy.

Diabetic retinopathy (DR) is a retinal disease[9], whose adverse outcomes are irreversible changes caused by hyperglycemia in metabolic and biochemical pathways. DR is a persistent disease that develops in stages; it can rarely be detected within the first few years. However, the morbidity rate of DR will significantly increase many years after the occurrence of diabetes. DR has also been reported as the main cause of acquired blindness in adults, in which retinal microvessels are damaged with vascular swelling and exudation. If not prevented, new blood vessels will begin to grow, ultimately leading to retinal detachment[10]. Inflammation is considered as a key driver for DR pathophysiology. Furthermore, it can increase the expressions of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), thereby promoting the occurrence of retinopathy in diabetic rats[11].

Signal transducer and activator of transcription 3 (STAT3) is a member of the signal transducer family, which is involved in the pathogenesis of DR[12]. STAT3 exists in the form of an inactive monomer in the cytoplasm of most cell types. After phosphorylation, STAT3 monomers will aggregate and transfer to the nucleus. This can upregulate the transcription of numerous genes in multiple cellular signaling pathways in the nucleus[13]. Direct and indirect inhibition of STAT3 exerts a protective effect against a variety of inflammatory eye diseases. In addition, inhibiting vascular endothelial growth factor (VEGF) in a STAT3-dependent manner weakens the pathological retinal angiogenesis[14]. The activity of STAT3 is associated with the progression of DR. Both the messenger ribonucleic acid (mRNA) and protein levels of STAT3 are up-regulated in the retina of diabetic rats[15]. Inhibiting STAT3 can reduce retinal cell death in diabetic rats, which can also alleviate retinal inflammation in the rat model of type I diabetes[16]. The release of interleukin-6 (IL-6) activates the STAT3 pathway, further promoting the development of DR. In a variety of tissues, increased release of IL-6 activates the IL-6/STAT3 pathway at the injury site through autocrine[17]. N-myc downstream regulated gene 2 (NDRG2) is one of the discovered genes related to differentiation and stress. It is highly expressed in brain, heart, and muscle. Meanwhile, NDG2 is also a cytoplasmic protein containing multiple phosphorylation sites[18]. There is a correlation between NDRG2 and cell proliferation, retinopathy, or cancer cell invasion [19,20]. Therefore, it is of great importance to understand the association between NDRG2 and various downstream signaling molecules. As a switch or sensor for DR, NDRG2/IL-6/STAT3 produces the inflammatory response to various stimuli and initiates inflammatory genes. Many factors can activate the nuclear transcription factor, leading to activation of the pathway. Therefore, NDRG2/IL-6/STAT3 will become the driving force for disease in the case of dysregulation.

In recent years, few studies have focused on the effect of NDRG2/IL-6/STAT3 signaling pathway on DR. Deeply analyzing the molecular mechanism of DR and searching for new therapeutic targets are the key to effective treatment. In the present study, we proposed that the NDRG2/IL-6/STAT3 signaling pathway promoted the development of DR. The classical animal model of diabetes was first established in rats. The pathway inhibitor was used. Later, biochemical indexes were detected. Changes in NDRG2/IL-6/STAT3 pathway genes and proteins in tissues were determined using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and Western blotting, respectively. The aim of this investigation was to clarify the specific relation between

NDRG2/IL-6/STAT3 pathway and DR in rats. Our findings might provide a theoretical basis for the subsequent research on the NDRG2/IL-6/STAT3 pathway.

Materials and Methods

Animal Grouping and Modeling

After adaptive feeding under room temperature and normal humidity for 1 week, male Sprague-Dawley rats were divided into three groups, including normal control group (control group, n=10), diabetes model group (model group, n=10), and pathway inhibitor group (CLT-005 group, n=10). This study was approved by the Animal Ethics Committee of Maternity and Child Health Care of Zaozhuang Animal Center. The rat model of diabetes was successfully established *via* intraperitoneal injection of streptozotocin (STZ, 50 mg/kg). Rats in CLT-005 group were gavaged with CLT-005 (1 mL/kg) for 14 consecutive days. The clinical manifestations of rats were observed regularly every day, and detailed changes were recorded timely until successful modeling. After the trial period, blood samples were drawn from the caudal vein. After centrifugation, the serum was collected and stored at –80°C for subsequent detection of biochemical indexes. Next, the rats were anesthetized *via* injection of pentobarbital sodium. Two portions of retinal tissues were used, with one for enzyme-linked immunosorbent assay (ELISA) and the other one stored at –80°C for qRT-PCT and Western blot.

Detection of Blood Glucose Content

Successful establishment of the diabetes model is important for subsequent studies. Therefore, to observe whether the diabetes model was successfully established in rats, blood samples were drawn from the caudal vein and centrifuged. The serum was collected to detect the content of blood glucose. The raw data were recorded and analyzed. Finally, changes in blood glucose were analyzed to determine whether the diabetes model was successfully established.

Detection of Levels of TNF-α, IL-1, and IL-6 via ELISA

Serum inflammatory factors are important indexes of retinal injury, which can indicate the speed of injury repair. In this study, the content of serum inflammatory factors was detected *via* ELISA. Collected serum at –80°C were taken and slowly thawed at 4°C. The content of TNF-α, IL-1, and IL-6 was determined by ELISA. Absorbance of indexes in each group was detected using a micro-plate reader. Finally, standard curves were plotted and changes in content were analyzed.

Detection of Levels of Oxidative and Antioxidant Indexes Reactive Oxygen Species (ROS), Superoxide Dismutase (SOD), and Malondialdehyde (MDA) in Retinal Tissues Using ELISA

Collected tissues stored in a refrigerator at –80°C were taken out, and 150 mg of the tissues were weighed. After quickly ground in the mortar, lysis buffer was added, followed by centrifugation. The supernatant was then collected to detect changes in the levels of ROS, SOD, and MDA. Absorbance of indexes in each group was detected using a micro-plate reader. Finally, standard curves were plotted, and changes in content were analyzed.

Observation of Changes in Retinal Tissues Via Hematoxylin-Eosin (HE) Staining

Dissected retinal tissues were soaked in formalin and washed with running water for 24 h. Subsequently, the tissues were transparentized, immersed, and embedded in paraffin. Then, the embedding blocks were prepared into pathological sections (about 5 μm in thickness) and stained with hematoxylin for 10 min. After washing with water, the sections were counterstained with eosin for 3 min, dehydrated with ethanol, transparentized, and sealed with neutral resins. Finally, changes in retinal tissues were observed under a light microscope.

Microscopic Observation of Number of Corneal Nerve Fibers

The tissues were first washed with running water for 24 h, dehydrated with gradient ethanol, and routinely prepared into sections. Next, the sections were placed under a microscope. The number of nerve fibers was counted twice using the ocular micrometer in the most central field and 4 peripheral fields of view. The average value was finally calculated.

Table I. PCR primer sequences

Target gene	Primer sequence
IL-6	F: 5'-GAGTCCTTCAGAGAGATACAG-3' R: 5'-CTGTGACTCCAGCTTATCTG-3'
STAT3	F: 5'-TATCTTGGCCCTTTGGAATG-3' R: 5'-GTGGGGATACCAGGATGTTG-3'
β-actin	F: 5'-CTTCATTGACCTCAACTACATGG-3' R: 5'-CTCGCTCCTGGAAGATGGTGAT-3'
NDRG2	F: 5'-TACGTCGGCCGTGTCTAT-3' R: 5'-GAACTGTGATCCGTGTAGG-3'
VEGF	F: 5'-GCGGGCTGCAATGATG-3' R: 5'-TGCAACGCGAGTCTGTGTTT-3'

Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)

An appropriate number of retinal tissues frozen in the ultra-low temperature refrigerator was taken and added with liquid nitrogen. After homogenized under low temperature at 2000 rpm for 30 s, total RNA was extracted. RNA purity and concentration were then qualified. The primer amplification was performed using a 20 μL system, including 2 μL of cDNA, 10 μL of mix, 2 μL of primer, and 6 μL of ddH_2O, for a total of 40 cycles. Sequences of target genes and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed according to those in the GenBank (Table I). Relative expression levels of target genes were detected *via* qRT-PCR and calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

About 200 mg of tissues were taken into 10 mL Eppendorf (EP) tube (Hamburg, Germany) and placed on ice. After adding with lysis buffer prepared proportionally and incubation in a refrigerator, the tissues were fully lysed to release proteins. After centrifugation, the supernatant was collected. Protein concentration was measured according to the instructions of the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Western blotting was performed as follows: protein samples were separated by electrophoresis and transferred onto a membrane. Then, the membranes were incubated with primary antibody and the corresponding secondary antibody. Immuno-reactive bands were exposed using the gel imaging system. Finally, the gray value of protein band was analyzed using Image Lab, and the protein expression was calculated.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) software was used for all statistical analysis. Bar graph was plotted using GraphPad Prism 5.0 (La Jolla, CA, USA). Multiple comparisons were performed for the data. Experimental results were expressed as mean ± standard deviation ($\bar{x}$ ± SD). $p<0.05$ was considered statistically significant.

Results

Detection Results of Blood Glucose Content

To observe whether the diabetes model was successfully established in rats, blood glucose content was detected using a full-automatic biochemical analyzer. As shown in Figure 1, the level of blood glucose in model group was signifi-

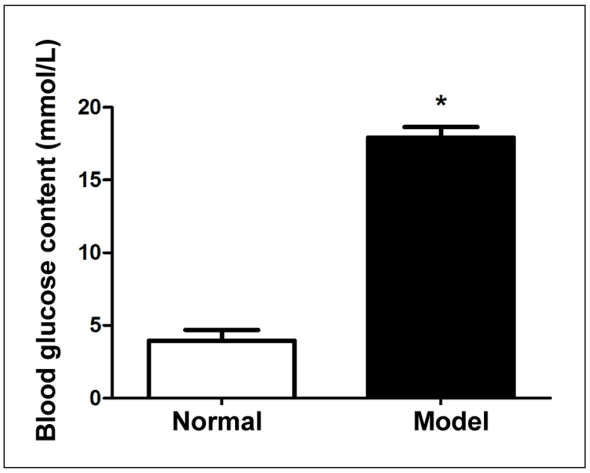

Figure 1. Blood glucose content. Blood glucose level in model group is significantly higher than that in control group ($p<0.05$). *$p<0.05$ *vs.* control group.

Table II. Content of serum TNF-α, IL-1 and IL-6

Group	IL-1 (mg/L)	TNF-α (fmol/mL)	IL-6 (mg/L)
Control group	18.35±1.23	14.38±2.58	16.39±4.25
Model group	99.63±7.02*	40.58±5.84*	87.41±2.58*
CLT-005 group	24.14±3.16#	22.35±5.74#	25.12±4.24#

Note: The content of inflammatory factors IL-1, IL-6 and TNF-α is increased in model group (p<0.05), while it declines in CLT-005 group (p<0.05). *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

Table III. Content of ROS, SOD and MDA

Group	ROS (U/L)	MDA (mmol/g)	SOD (μ/mg)
Control group	2.58±1.25	6.32±0.57	35.48±1.25
Model group	8.98±1.58*	45.64±2.47*	12.35±1.87*
CLT-005 group	4.31±0.89#	15.25±1.47#	24.87±0.87#

Note: The content of ROS and MDA is increased in model group (p<0.05) and declines in CLT-005 group (p<0.05), while that of SOD shows the opposite trends (p<0.05). *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

cantly higher than that of control group (p<0.05). This indicated that the rat model of DR was successfully established.

Content of Serum TNF-α, IL-1, and IL-6

As shown in Table II, the content of IL-1, IL-6, and TNF-α increased significantly in model group (p<0.05), while was markedly reduced in CLT-005 group (p<0.05).

Content of ROS, SOD, and MDA

DR has been found closely related to oxidation and antioxidation. In this study, detection results of ROS, SOD, and MDA showed that the content of ROS and MDA increased significantly in model group (p<0.05), while decreased markedly in CLT-005 group (p<0.05). However, the content of SOD showed the opposite trends (p<0.05; Table III).

Microscopic Observation Results of Number of Corneal Nerve Fibers

The number of corneal nerve fibers and the density of nerve plexuses were evidently reduced in model group (p<0.05). However, the number of nerve fibers increased significantly in CLT-005 group (p<0.05), but was still smaller than that of control group (Figure 2).

HE Staining Results

As shown in Figure 3, retinal edema, karyopyknosis, decreased number of ganglion cells, and evident telangiectasia were observed in model group (Figure 3A). In CLT-005 group (Figure 3B), there were mild retinal edema, and more ganglion cells and inner nuclear layers than model group.

QRT-PCR Results

The mRNA levels of VEGF, NDRG2, IL-6, and STAT3 were remarkably higher in model group than those in control group (p<0.05). However, they decreased in CLT-005 group (p<0.05) (Figure 4).

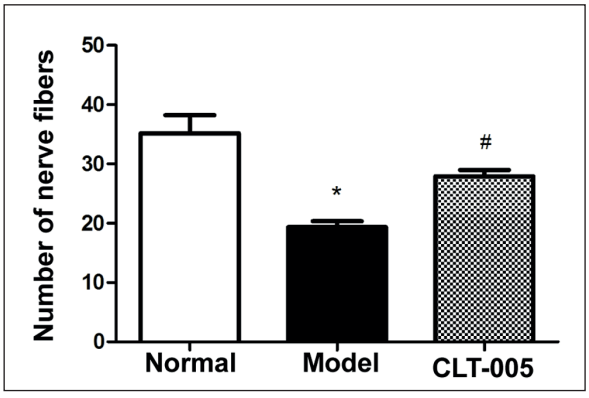

Figure 2. Number of nerve fibers. The number of corneal nerve fibers decreased significantly in model group (p<0.05). However, the number of nerve fibers increased significantly in CLT-005 group (p<0.05), but was still smaller than that in control group. *p<0.05 *vs.* control group, #p<0.05 *vs.* model group.

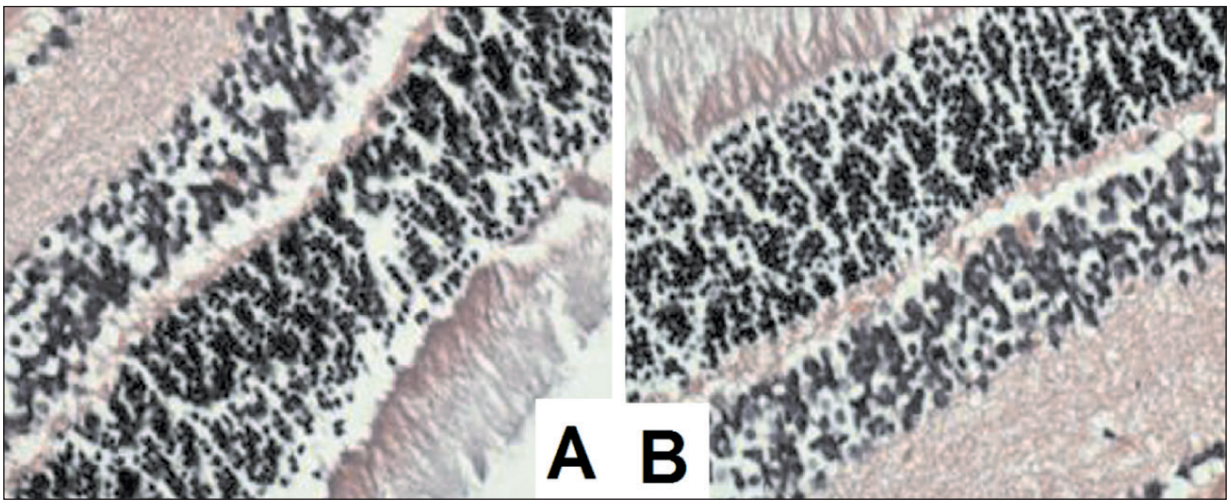

Figure 3. HE staining results. Retinal edema, karyopyknosis, decreased number of ganglion cells and significant telangiectasia were observed in model group (**A,** ×200). In CLT-005 group (**B,** ×200), there were mild retinal edema, and more ganglion cells, as well as inner nuclear layers than model group.

Western Blotting Results

The protein levels of NDRG2, IL-6, and STAT3 were significantly higher in model group than those in control group ($p<0.05$). However, they were significantly down-regulated in CLT-005 group ($p<0.05$; Figure 5).

Discussion

Diabetes is a lifelong chronic consumptive disease that causes many complications. Meanwhile, it is the major cause of non-invasive amputation, blindness, visual impairment, and end-stage renal disease in adults. DR is one of the leading causes of blindness in the world[21]. STZ results in a significant increase in fasting blood glucose level. It is also involved in the etiology of various diabetic complications, including DR[22]. Preventing the development of DR has always been a challenge in biomedical research. Inflammatory response characterized by chronic low-level inflammation and immune response disorders plays an important role in the pathogenesis of DR. In addition, inflammation is of great significance in DR-induced oxidative stress and the activation of inflammatory pathway[23]. TNF-α significantly increases vitreous hyperplasia in DR patients. Meanwhile, the elevated level of TNF-α promotes the occurrence of retinopathy[24]. In the present study, the rat model of DR was established to investigate the pathogenesis of DR. Our findings hoped to search for a potential therapeutic method for DR. The results revealed that blood glucose level in model group was significantly higher than that of control group. This indicated that the model of DR was successfully established in rats.

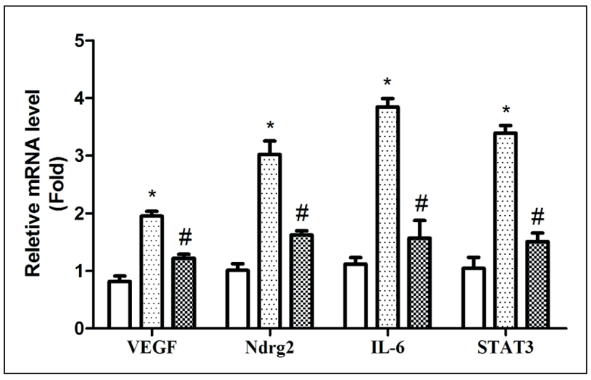

Figure 4. RT-PCR results. The mRNA levels of VEGF, NDRG2, IL-6, and STAT3 were remarkably higher in model group than those in control group ($p<0.05$). However, they remarkably declined in CLT-005 group ($p<0.05$). *$p<0.05$ *vs.* control group, #$p<0.05$ *vs.* model group.

Chronic inflammation is common in both type I and type 2 diabetes. It may be the result of increased ROS due to hyperglycemia[25]. Upregulation of specific inflammatory cytokines IL-1 and IL-6 in the acute phase of retinopathy in diabetic rats has been confirmed correlated with the development of the disease. High-level TNF-α has already been detected in the vitreous body of diabetic patients and animal models[26]. However, few researches have investigated whether the possible effect of CLT-005 on glucose level is

related to the mechanism of CLT-005 in reducing inflammation. In this study, the levels of IL-6, IL-1, and TNF-α increased significantly in model group. This suggested that elevated levels of IL-6 and TNF-α aggravated inflammatory response, further facilitating the development of DR. The levels of the molecules declined after the addition of the pathway inhibitor. TNF-α plays an indispensable role in the development of inflammation in DR rats. IL-6 can also stimulate excessive production of other inflammatory mediators[27]. Our results were consistent with previous findings, indicating that the addition of pathway inhibitor inhibited excessive inflammatory cytokines and prevented irreversible damage to cells caused by excessive production. Currently, the role of oxidative stress in DR has attracted extensive attention. Ubiquitous SOD prevents the nerve conduction abnormality in diabetes[28]. MDA can resist the effect of SOD, eventually producing cytotoxicity[29]. In this study, the levels of ROS and MDA were markedly higher in model group than those in the other two groups. However, the level of SOD showed the opposite trends, consistent with the above research results. In addition, according to morphological observation, retinal edema, evidently decreased number of corneal nerve fibers and significant telangiectasia were observed in model group. The number of nerve fibers increased significantly in CLT-005 group, but was still smaller than that in control group, which has also been observed by Schultz et al[30].

VEGF has become a key mediator of vascular rupture of eyeball and neovascularization in DR. Meanwhile, it plays an important role in leukocyte-mediated destruction of retinal neovascularization and has causality with its pathogenesis. The expression of VEGF has been found up-regulated in the retina of STZ-induced diabetic rats[31]. In addition, the expression of NDRG2 can induce the activation of the IL-6/STAT pathway in cells. NDRG2 can also serve as a regulator of differentiation of erythrocyte and megakaryocyte during hematopoiesis[32]. NDRG2 plays an important role in the positive regulation of early inflammatory response through activating the IL-6/STAT3 signaling pathway after cortical puncture[33]. Therefore, we determined the expressions of VEGF, NDRG2, IL-6, and STAT3 in retinal tissues of DR rats. The results found that the mRNA levels of VEGF, NDRG2, IL-6, and STAT3 were remarkably higher in model group than those in control group. However, they were significantly down-regulated in CLT-005 group. To determine

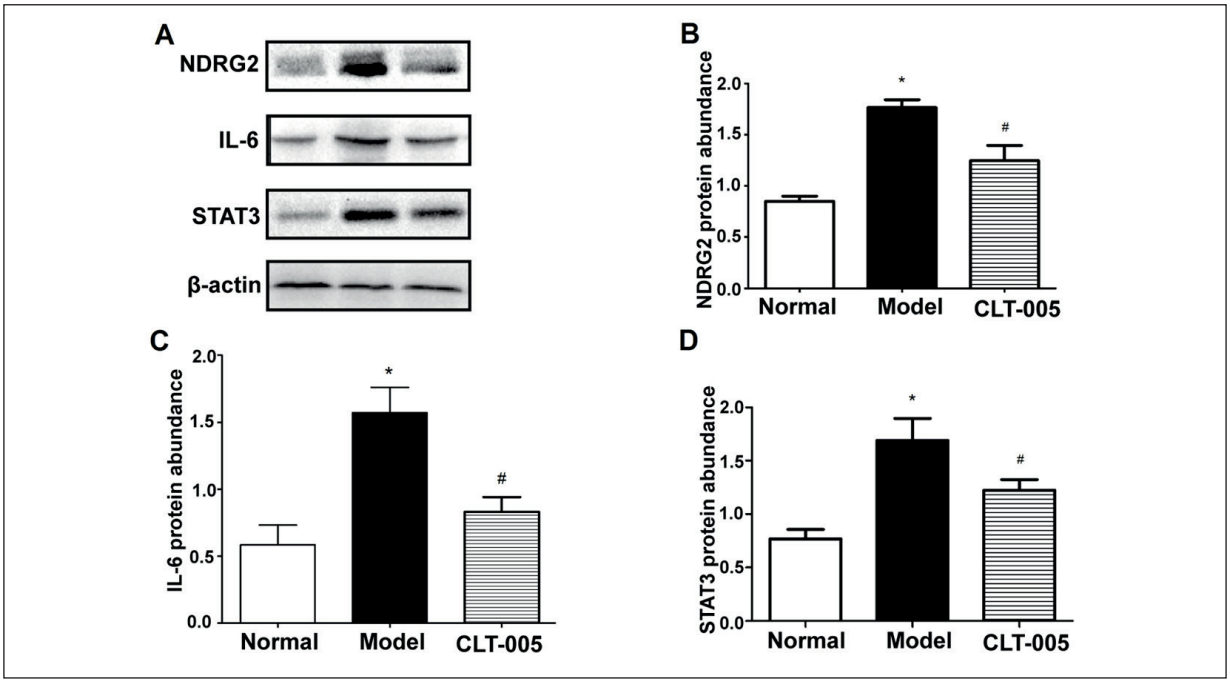

Figure 5. Western blotting results. The protein levels of NDRG2, IL-6, and STAT3 were significantly higher in model group than those in control group ($p<0.05$). However, they decreased remarkably in CLT-005 group ($p<0.05$). *$p<0.05$ *vs.* control group, #$p<0.05$ *vs.* model group.

whether the protein expressions were consistent with the mRNA expressions, changes in protein expressions of the above molecules were further observed. The results discovered that the protein levels of NDRG2, IL-6, and STAT3 were significantly higher in model group than those in control group. However, they decreased remarkably in CLT-005 group, consistent with previous studies. The above results demonstrated that activating the NDRG2/IL-6/STAT3 signaling pathway promoted the development of DR in rats. There were still some deficiencies in the present study. In the future, the mechanism of action should be further explored through multiple experiments.

Conclusions

We first found that the NDRG2/IL-6/STAT3 pathway inhibitor can alleviate inflammatory cell infiltration, reduce oxidative products, and affect the retinal neural structure, thereby inhibiting the occurrence and development of DR in rats. Our findings provide a theoretical basis for the prevention and treatment of DR, as well as new ideas for further research on the NDRG2/IL-6/STAT3 pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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