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ORIGINAL RESEARCH

Effect of Shuangdan Mingmu Capsule on Diabetic Retinopathy in Rats via Regulation of miRNAs

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Purpose: To evaluate the effects of Shuangdan Mingmu (SDMM) capsule on diabetic retinopathy in rats by regulating miRNAs.

Materials and Methods: Streptozotocin (STZ) (50 mg/kg) was successfully used to induce diabetes in male Sprague-Dawley rats, which were randomly assigned to a group taking SDMM capsules ("diabetic+SDMM") or a control group ("diabetic"), and the normal group (n=10/group). The diabetic+SDMM capsule group received 1.89g/kg/d of SDMM capsule by gavage, whereas the other groups received the same amount of distilled water. After 12-weeks of gavage, the retina was removed from all rats for histopathological analysis, and miRNA sequencing experiments were carried out to identify the differential expression of miRNAs. These results were then confirmed by quantitative real-time polymerase chain reaction (qRT-PCR).

Results: SDMM capsules improved retinal morphology, restored the number of cells in the ganglion cell layer ($p<0.0001$) and reduced apoptosis in all retinal layers (p values in the outer nuclear layers, inner nuclear layers and ganglion cell layers 0.0001, 0.0147, 0.0034, respectively). In addition, miRNA expression was changed in rats taking SDMM capsules. Compared with the diabetic group, six miRNAs were up-regulated and four miRNAs were down-regulated in the diabetic+SDMM capsule group. The qRT-PCR validation results showed that the expression levels of miR-450b-5p, miR-1249 and miR-155-5p were consistent with the trend of miRNA sequencing results, and were all up-regulated after SDMM capsule treatment. Target gene prediction and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differentially expressed miRNAs showed that these pathways were mainly concentrated in the focal adhesions and PI3K/Akt, MAPK, and neural factor signaling pathways.

Conclusion: SDMM capsules may prevent and treat diabetic retinopathy by regulating the expression of miR-450b-5p, miR-1249 and miR-155-5p.

Keywords: SDMM capsule, diabetic retinopathy, traditional Chinese medicine, miRNAs, bioinformatics analysis

Introduction

Diabetic retinopathy (DR) is one of the most common complications of diabetes mellitus and one of the predominant blinding eye diseases. The incidence of DR increases with the duration of diabetes, affecting over 50% of patients with a history of diabetes of more than 10 years.[1,2] Retinopathy occurs in almost all patients with type 1 diabetes and in up to 75% of patients with type 2 diabetes with disease duration of more than 15 years.[3] DR poses a serious threat to human visual health and quality of life. The pathogenesis of DR is complex and involves multiple factors, such as inflammation, oxidative stress, epigenetics, and others. Despite the clinical emphasis on strict control of blood glucose, blood lipids, and blood pressure, many patients have progressive retinopathy. Research into the effective prevention and treatment of DR is therefore urgently needed.

Traditional Chinese medicine (TCM) has a long history and extensive role in the treatment of diabetes and its complications, and may provide an alternative treatment for prevention or delay of progression of DR. In addition to traditional Chinese medicine tonics, various forms of Chinese medicine, such as proprietary Chinese medicine (granules,

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tablets, capsules, and liquids),[4] Chinese herbal extracts,[5] or combined Western and Chinese medicine therapies,[6] have been widely used in clinical practice. SDMM capsule (SDFA approval number: Z20080062), a proprietary Chinese medicine for the treatment of DR, manufactured by Beijing Qihuang Pharmaceutical Co., Ltd., is produced from clinical practice experience and has undergone a clinical Phase IV trial. It has shown great potential in treating polyuria, polydipsia, polyphagia, irritability and other symptoms of diabetes. Extensive research has been conducted on the mechanism by which SDMM capsule may treat DR in its early stages and has found that it can lower blood glucose, improve retinal microvascular damage, protect islet function and reduce the effects of apoptosis in the retinal pericyte.[7–9]

MicroRNAs are a class of non-coding RNAs between 21 to 25 nucleotides in length that are involved in almost all physiological and pathological processes, such as cell growth, development, proliferation, differentiation and apoptosis.[10] Research has shown that miRNAs play key roles in the proliferation, migration and apoptosis of retinal cells,[11] as well as critical regulatory functions in the developmental stages of DR, such as the regulation of inflammation, oxidative stress, neovascularization and other mechanisms.[12–15] Traditional Chinese medicine can prevent and treat DR by regulating miRNA expression.[16–18]

Therefore, we evaluated the protective effect of SDMM capsules on the retinas of diabetic rats and explored the miRNA effects of SDMM capsules. Firstly, a diabetic rat model was established via a single intraperitoneal injection of STZ, and then the effects of SDMM capsules on retinopathy in diabetic rats were observed by hematoxylin-eosin (HE) staining, Periodic Acid-Schiff (PAS) staining, and the TdT-mediated dUTP nick end labeling (TUNEL) method. Finally, the pharmacological mechanism of action of SDMM capsules was revealed by miRNA sequencing, bioinformatics methods, and quantitative real-time polymerase chain reaction (qRT-PCR). The results suggest that SDMM capsules may regulate miRNAs to prevent DR. This study may provide a scientific basis for the clinical application of SDMM capsules and suggests avenues for further in-depth study on the molecular mechanism of DR.

Materials and Methods

Animals

Sprague-Dawley male rats (body weight 200 ± 25 g, 6 weeks old) were purchased from Hunan Slike Jingda Experimental Animal Co., Ltd. (Changsha, China, certificate number: SCXK (Xiang) 2019–0004). The rats were housed in a Specific Pathogen Free grade animal laboratory maintained at 24°C and 65% temperature and humidity and were provided with regular feed and tap water. The disposal of animals during the experiment was carried out in accordance with the Ministry of Science and Technology's Guidelines for the Protection and Utilization of Laboratory Animals. The animal protocol was approved by the Institutional Animal Ethics Committee of Hunan University of Traditional Chinese Medicine, Changsha, China (approval number LL2021051901).

Model Induction and Treatment

Rats were allocated into three groups: the normal group, the diabetic group, and the diabetic+SDMM capsule group (n=10/group). Rats in the diabetic group and diabetic+SDMM capsule group were injected with a single intraperitoneal injection of STZ at a dose of 50 mg/kg.[19] Blood glucose levels (Mm) were measured 3d and 7d after dosing, and rats with blood glucose levels higher than 16.7 mmol/L were selected for subsequent experiments and randomly divided into a diabetic group and diabetic+SDMM capsule group. Rats in the normal group received an equal amount of citrate buffer. Dosage for rats[20] = X mg/kg*70kg*0.018/200g=6.3 X mg/kg (X: the daily dose for a normal person; 70: the standard body weight of an adult; 200g: the body weight of a rat; 0.018: the equivalent dose ratio between humans and rats based on body surface area conversion is 0.018). The effective dose of SDMM capsules in humans is about 0.3 g/kg/d. Therefore, SDMM capsules were given to rats in the diabetic+SDMM group at a dose of 1.89 g/kg/d for 12 weeks, once daily. (We assessed the safety of the drug at this dose by evaluating tissue sections of vital organs. See Supplementary Figure 1) The SDMM capsules were dissolved in distilled water to make a suspension with a concentration of 189mg/mL, and the volume of the gavage was 1mL/100g. Equal volumes of distilled water were given to the normal and diabetic groups. After 12 weeks of treatment, the rats were euthanized by isoflurane inhalation overdose and the eyeballs were collected.

Sample Preparation and Component Identification of SDMM Capsules

The SDMM capsules were manufactured and supplied by Beijing Qihuang Pharmaceutical Co., Ltd. (batch number: Z20080062). The capsules were composed of 11 medicinal ingredients, including Ligustrum lucidum, Eclipta, Panax notoginseng, Smilax china L, Achyranthes, Salvia, Dogwood, Chinese yam, Peony bark, Poria, and Alisma. HPLC chromatograms characteristic of SDMM capsules have been established and used for their quality control.[21,22] One hundred and two chemical constituents were identified, including phenolic acids, flavonoids and their glycosides, terpenes, coumarins, lignans, and others. Thirty-six prototypal components of blood were detected, mainly derived from Eclipta and Dogwood. Three metabolites were also identified, namely the glucuronic acid conjugate of protocatechuic acid, the glycine conjugate of Danshensu, and the D-ring-opening metabolite of cryptotanshinone.

Hematoxylin and Eosin (HE) Staining

Once the eyes were removed, they were fixed with eye fixative, embedded in paraffin and sectioned (5μm thick). The sections were then subjected to HE staining and changes in retinal microstructure were observed under a light microscope. The total retinal thickness from the retinal pigment epithelium (RPE) to the ganglion cell layer (GCL) was determined and the cell counts of the GCL was calculated. Images of the retina were captured using an optical microscope at 400x magnification. Measurements were made using Image-Pro Plus analysis software (Media Cybernetics, USA), with two measurements were taken for each section at the two reference lines, which were 1 mm away from the optic nerve on both the superior and inferior sides. Retina cell counts in GCL was measured at 400x magnification. All the cell nuclei within a fixed 25-mm column and centered with the 1-mm reference lines were counted. Results were taken from five slices per eye, with three retinas in each group.

Periodic Acid-Schiff (PAS) staining

After paraffin-embedding and sectioning, tissue was stained with PAS. The retinal microstructural changes were observed under a light microscope.

TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay

Following the instructions of the TUNEL assay kit (cat. NO 40306ES50), the steps of sectioning, dewaxing, fluorescein labeling, incubation, color development, counterstaining, dehydration and transparency were completed, then the dried sections were observed under a fluorescence microscope to calculate the retinal Apoptotic Index (AI). The number of cells in the ganglion cell layer (GCL), inner nuclear layer (INL), and outer nuclear layer (ONL) were counted at 1000x magnification, and six randomly selected fields of view from each section were analyzed to determine the number of apoptosis-positive cells in the three layers.

RNA Extraction, Library Preparation and Deep Sequencing

Extraction of total RNA from rat retinal lesions was conducted using TRIzol Reagent (Invitrogen, cat. NO 15596026). DNA digestion was carried out after RNA extraction by using DNaseI (Invitrogen, cat. NO AM9784). The A260/A280 was tested using a Nanodrop™ OneCspectrophotometer (Thermo Fisher Scientific Inc) to determine RNA quality. RNA Integrity was confirmed by 1.5% agarose gel electrophoresis. Verified RNAs were finally quantified by Qubit 3.0 with QubitTM RNA Broad Range Assay kit (Life Technologies, Q10210).

For miRNA library preparation, three microliters of total RNA were input to the KC-Digital™ small RNA Library Prep Kit for Illumina® (Catalog NO. DR08602, Wuhan Seqhealth Co., Ltd., Wuhan, China) following the manufacturer's instructions. The kit eliminates duplication bias in PCR and sequencing steps by using a unique molecular identifier (UMI) of eight random bases to label the pre-amplified small RNA molecules. The eluted cDNA library was separated on a 6% PAGE gel. Bands of approximately 160 bp were isolated, purified and quantified using Qubit 3.0 and finally sequenced on the Novaseq6000 sequencer (Illumina) using the PE150 model.

MiRNA-Seq Data Analysis

To calculate miRNA expression levels, read lengths of sequences in each sample were compared with the existing miRNA database and predictions of new miRNAs, and were screened using CPM. Differences in expressed miRNAs between groups were identified using the Edger package (version: 3.12.1). The criteria $p<0.05$ and |Log2Fold-change|>1 were used to determine statistical significance of miRNA expression differences. The target mRNA of the differentially expressed miRNA was predicted using miRanda v3.3a. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of targeted mRNA were implemented using KOBAS software (version: 2.1.1) with a corrected P-value criterion of 0.05 for statistically significant enrichment.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

To verify the miRNA sequencing results, miRNA levels were detected using qRT-PCR. Total RNA was extracted using TRIzol (Cat. No. DP405-02). The yield of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and its integrity was assessed using 1% agarose gel electrophoresis. A PrimeScript™ RT reagent Kit with gDNA Eraser (Cat. No.: RR047B) was used for reverse transcription of cDNA, and the procedure was conducted according to the product instructions. qRT-PCR analysis was performed according to the instructions of the TB Green TM Ex Taq TM II Kit. U6 was used as an internal control for miRNA, and the primers are shown in Table 1.

Statistical Analysis

Statistical analysis was performed using SPSS version 23.0. Data are presented as mean ± standard error (SEM). The significance of differences between groups was determined using a one-way ANOVA, and $p < 0.05$ was considered statistically significant.

Results

SDMM Capsule Has Protective Effect on Retinal Structure of Diabetic Rats

As shown in Figure 1, HE staining showed that in the normal group, the retinal structure was clear, with closely arranged cells, structural integrity, and normal morphology, and with no tissue edema. In the diabetic group, the cells in each layer were loose, irregular, and structurally disturbed, with marked edema of the retinal tissue; the cells in the ganglion cell layer were disorganized and reduced in number (p=0.0049). PAS staining showed that in the normal group the retinal inner boundary membrane was intact with no capillary dilatation. In the diabetic group, the inner boundary membrane was swollen and thickened with an uneven surface. After treatment, the SDMM capsules effectively alleviated these abnormalities; the retinal tissue was slightly edematous, with intact cellular arrangement and clear structural layers. The number of cells in the GCL was increased in the diabetic+SDMM capsule group (p<0.0001).

SDMM Capsules Can Reduce Retinal Apoptosis

As shown in Figure 2, TUNEL fluorescence staining was used to detect the effect of the SDMM capsule on retinal cell apoptosis. The rate of apoptosis in the retinal layers of rats in the diabetic group was significantly higher than that in the

Table 1 Primer Sequence

Gene	Sequence (5'-3')	
miR-450b-5p	Upstream primer	TTTTGCGATGTGTTCCTAATA
miR-450b-5p	Downstream primer	GGCCAACCGCGAGAAGATG
miR-1249	Upstream primer	CCCTTCCCCCCCTTCTTC
miR-1249	Downstream primer	GGCCAACCGCGAGAAGATG
miR-155-5p	Upstream primer	TTAATGCTAATTGTGATAGGGGT
miR-155-5p	Downstream primer	GGCCAACCGCGAGAAGATG
U6	Upstream primer	CTGCGCAAGGATGACACGCAAATT
U6	Downstream primer	GGCCAACCGCGAGAAGATG

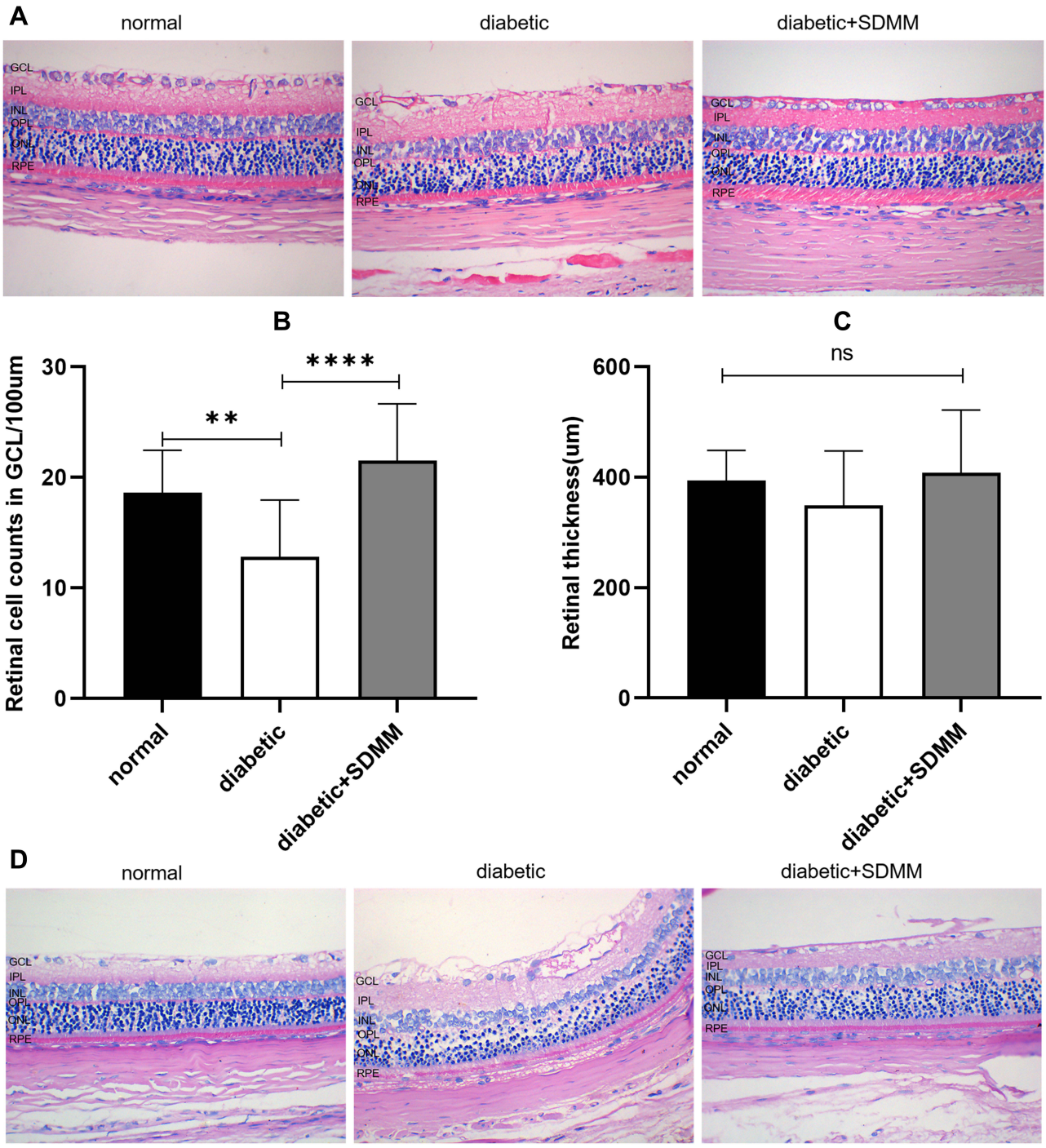

Figure 1 Effects of Shuangdan Mingmu (SDMM)capsules on retinal structure in STZ-induced diabetic rats. (**A**) retinal HE-stained images; (**B**) retinal cell counts in GCL; (**C**) retinal thickness from RPE to GCL; (**D**) Images of retinal PAS staining. χ±s, n=3, **p<0.01, ****p<0.0001, scale bar 100um.

Abbreviations: GCL, ganglion cell layer; IPL, internal plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

normal group. (p-$_{ONL}$<0.0001, p-$_{INL}$=0.0089, p-$_{GCL}$=0.0001). After treatment with SDMM capsules, retinal apoptosis was significantly decreased compared with the diabetic group (p-$_{ONL}$=0.0001, p-$_{INL}$=0.0147, p-$_{GCL}$=0.0034). The above results suggest that SDMM capsules have a protective effect on retinal cells in diabetic rats.

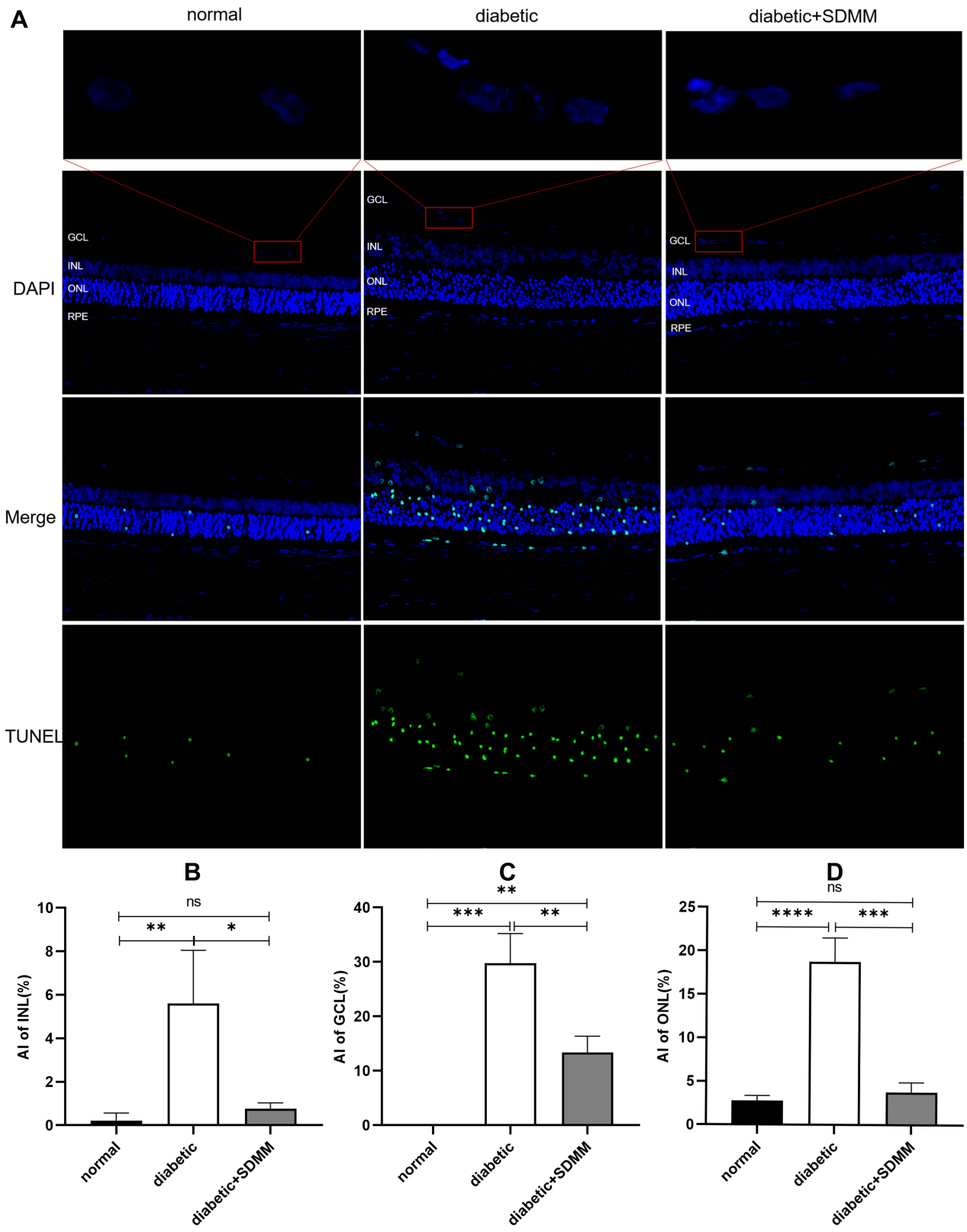

Figure 2 Effects of Shuangdan Mingmu (SDMM) capsules on STZ-induced retinal apoptosis in diabetic rats. (**A**) Images of retinal TUNEL staining; (**B-D**) AI of INL, GCL and ONL, respectively, in each group. Positive signal is green fluorescence, blue is nuclear staining signal, AI = number of apoptotic cells/number of total cells×100%. $\chi \pm s$, n=3, *p<0.05, **p<0.01, ***p=0.0001, ****p<0.0001, scale bar 100um.

Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium; AI, Apoptotic Index.

SDMM Capsules Altered miRNA Expression Levels

As shown in Figure 3, analysis of the sequencing results showed that two miRNAs were up-regulated and five were down-regulated in the normal group compared with the diabetic group, with miR-122-5p up-regulated and miR-205 down-regulated. Six miRNAs were up-regulated and four miRNAs were down-regulated after SDMM capsule intervention. The up-regulated miRNAs were miR-451-5p, miR-484, miR-450b-5p, miR-1249, miR-452-5p, miR-1247-5p, while miR-122-5p was down-regulated.

Bioinformatics Analysis of Differentially Expressed miRNAs Between SDMM Capsule Group and Model Group

As shown in Figure 4, bioinformatics analysis showed that the target gene functions in the diabetic+SDMM capsule group and the diabetic group were mainly divided into three aspects: biological process, cellular components, and molecular function.[23] The main signaling pathways included the PI3K/Akt pathway, MAPK pathway, focal adhesions, and neural factor signaling pathways, which are involved in cell differentiation, proliferation, apoptosis, and angiogenesis.

Validation of miRNAs Sequencing Data Using qRT-PCR Technology

To verify the sequencing results, qRT-PCR was used to detect the expression of miR-450b-5p, miR-1249, and miR-155-5p. As shown in Figure 5, these three miRNAs were down-regulated in the diabetic group compared with the normal group, and SDMM capsules eliminated this effect.

Discussion

DR remains a major problem that threatens the vision of tens of millions of people around the world. Blood glucose is a key modifiable risk factor for the development of DR and its control can help delay the development of DM and related complications. For DR that has already occurred, treatment options include retinal laser photocoagulation, anti-Vascular Endothelial Growth Factor drug therapy, hormone therapy, and surgery. The scope of application and therapeutic effect of these methods are limited, and alleviating DR damage still requires adequate prevention and early diagnosis and treatment.

Several scholars have conducted extensive research on the mechanisms of herbal medicine in the treatment of DR. For example, Ma et al studied the effect and mechanism of Bushen Huoxue Recipe on the secretion of Vascular Endothelial Growth Factor(VEGF) and Pigment Epithelium Derived Factor (PEDF) by Müller cells under advanced glycation end products or hypoxia and concluded that Bushen Huoxue Recipe could alleviate the imbalance between VEGF and PEDF.[24] Gao et al found that Danggui Buxue Decoction (Astragalus, Angelica, Panax notoginseng) reduced the levels of IL-1β, IL-6, TNF-α, NF-κB, MCP-1, ICAM-1, and VCAM-1 in the DR rat, and reversed the blood glucose-related inhibition of endothelial cell migration and proliferation.[25] However, the mechanism of herbal medicine in the treatment of DR lack of systematic and standardized DR models and in-depth research on molecular study.

In this study, after establishing the experimental DR rat model, the ccrats were further randomly allocated into normal, diabetic and diabetic+SDMM capsule groups. We first assessed the retinal structure of rats and then performed a comparison between groups. The results showed that diabetic rats had reduced retinal thickness and GCL cell numbers and increased numbers of apoptotic cells, while SDMM capsules alleviated retinal edema, apoptosis, and other signs. The SDMM capsule is based on the ancient formula Erzhi Pill and Liuwei Dihuang Pill, combined with years of clinical experience and modern research results, into the formula “SDMM Capsule”. It is composed of 11 medicinal ingredients: Ligustrum lucidum, Eclipta, Panax notoginseng, Smilax china L, Achyranthes, Salvia, Dogwood, Chinese yam, Peony bark, Poria, and Alisma. Relevant studies have shown that Ligustrum lucidum has important therapeutic significance for diabetes and its complications, lowering blood glucose, antioxidants and inhibiting retinal neovascularisation.[26–28] Eclipta is a traditional herbal medicine that has long been used in the treatment of bleeding disorders, skin diseases, diabetes, and coronary heart disease. Its isolated extracts and compounds have hypoglycemic, anti-inflammatory, antihyperlipidemic and neuroprotective effects.[29–31] Panax notoginseng is an important component of many clinical

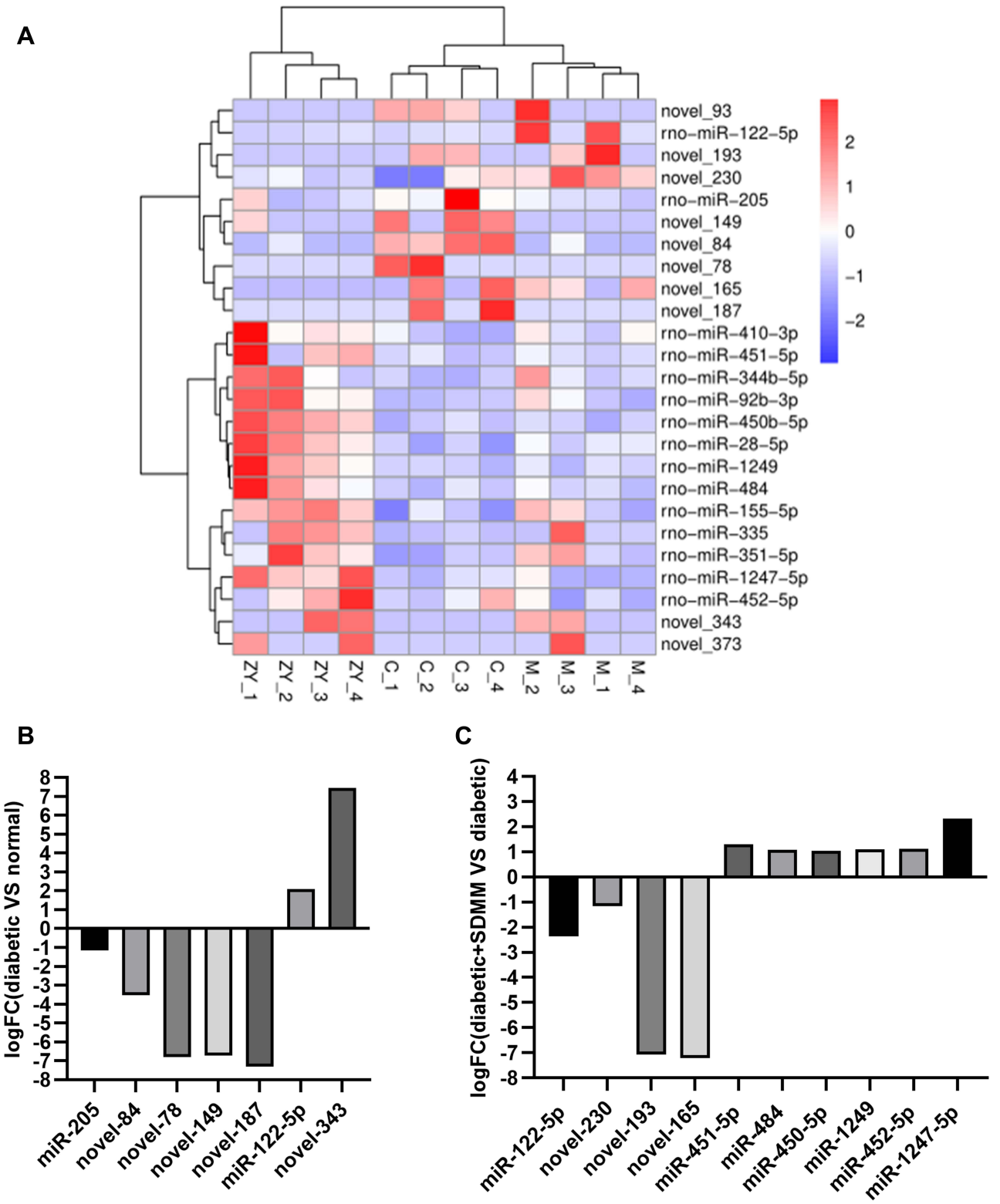

Figure 3 MiRNA expression profiling was performed using miRNA sequencing. (**A**) Heatmap of differentially expressed miRNAs. (C is the normal group, M is the diabetic group, ZY is the diabetic+SDMM group). miRNA expression levels are depicted in red (up-regulated) and blue (down-regulated); (**B** and **C**) The logFC value of different miRNAs compared between the diabetic group and the normal group (**B**) and between the diabetic+SDMM group and the diabetic group (**C**).

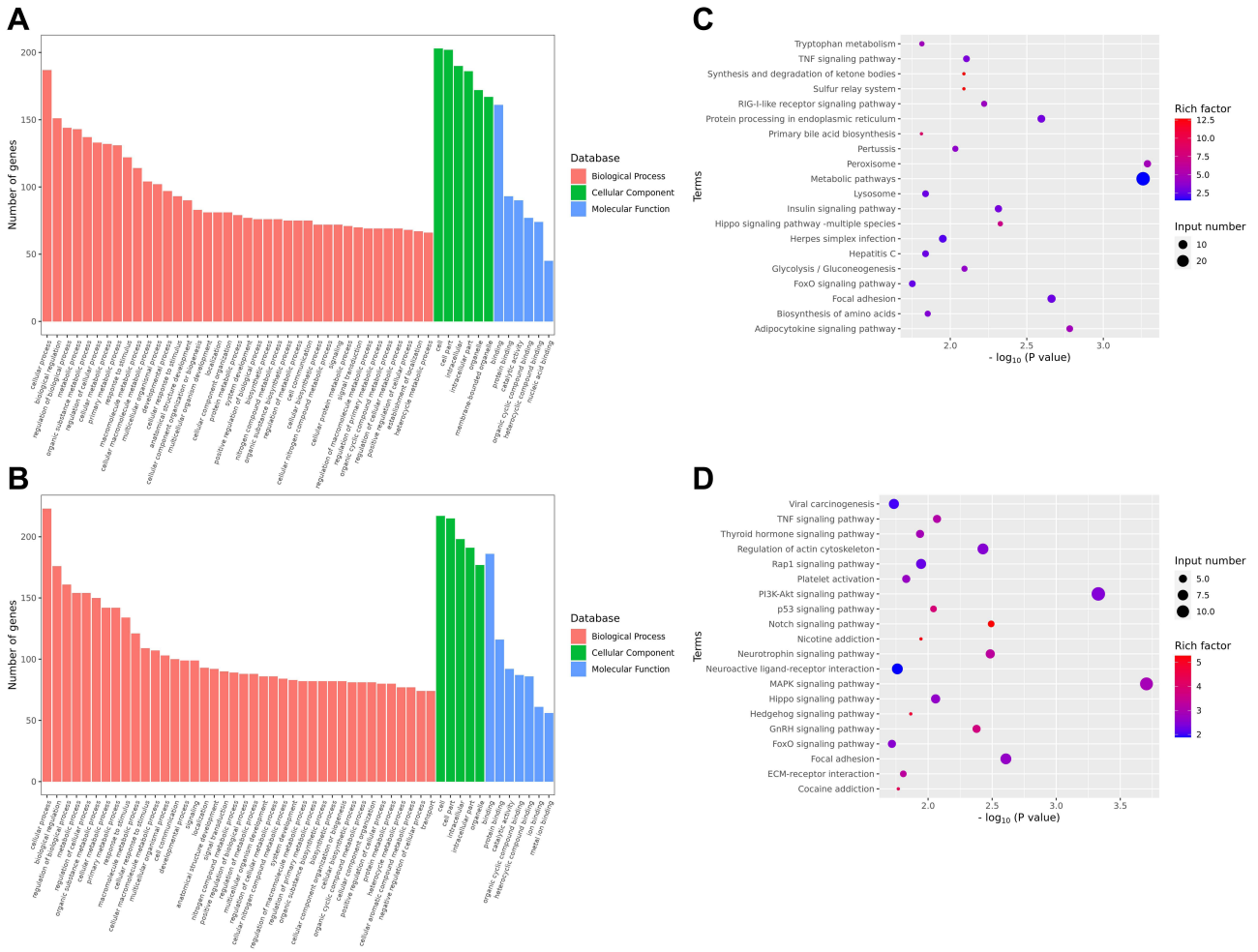

Figure 4 GO and KEGG analysis. Functional enrichment analysis (**A** and **B**) and KEGG analysis (**C** and **D**) of differentially expressed miRNA target genes between the diabetic group and the normal group, the diabetic group and the diabetic+SDMM capsule group.

drugs, such as Compound Danshen Drops, Xueshuantong and Xuesaitong. Panax notoginseng extract Panax notoginseng saponins (PNS) are widely used in cardiovascular disease and have pharmacological effects such as anti-cancer, neuroprotection, anti-inflammatory, and prevention of diabetic complications,[32,33] while the combination of Salvia and Panax notoginseng has significant antidiabetic efficacy.[34] Salvia can improve endothelial function,[35] resist oxidative stress,[36] inhibit inflammation,[37] and regulate lipid metabolism in the treatment of diabetic microangiopathy. Smilax china L., a dicotyledonous medicinal plant of the Liliaceae family, and its extract have anti-inflammatory, analgesic, and antioxidant effects.[38] Studies have shown that anti-hyperglycemic properties are also found in Achyranthes, Dogwood, Chinese yam, Peony bark, Poria, and Alisma, and that SDMM capsules have a protective effect on retinal damage caused by diabetes.[39–42]

MiRNAs exist widely in various organisms as non-coding RNAs, and research has shown that miRNAs have multiple biological functions and complex mechanisms. As key upstream genes, miRNAs play an important role in promoting or inhibiting the occurrence and development of diabetic retinopathy.[43,44] In this study, we used miRNA second-generation sequencing technology to obtain differential miRNA expression profiles in three different groups of rat retinal tissues. The results showed that compared with the normal group, two miRNAs were up-regulated and five miRNAs were down-regulated in the diabetic group. Six miRNAs were up-regulated and four miRNAs were down-regulated after SDMM capsule intervention, including miR-450b-5p, miR-1249, and miR-155-5p.

MiR-1249 may inhibit the angiogenesis of human umbilical vein endothelial cells by targeting VEGF-A,[45] which is involved in endothelial cell proliferation and apoptosis. Furthermore, miR-1249-3p has attenuated insulin resistance and

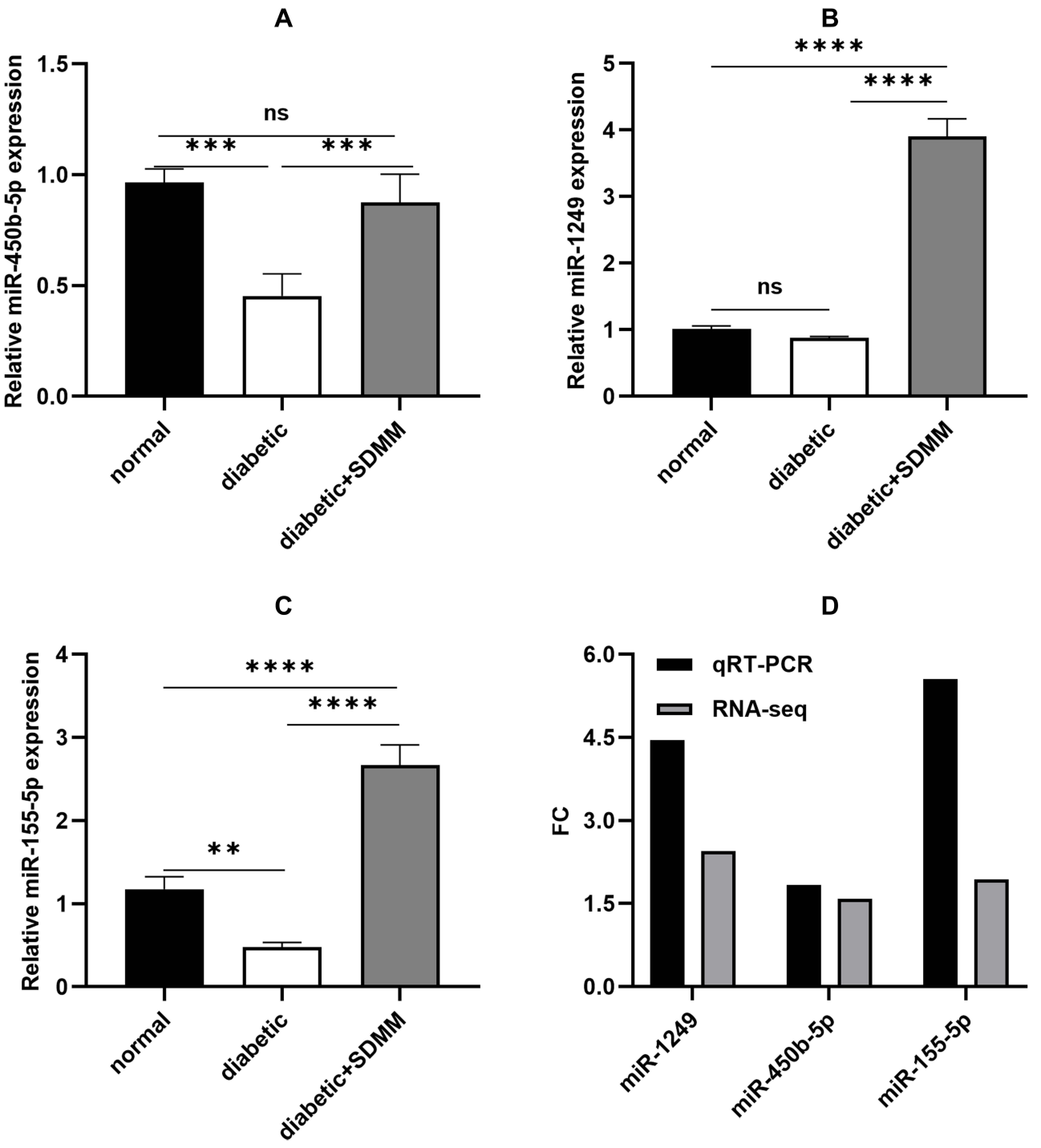

Figure 5 qRT-PCR validation of miRNA expression changes. qRT-PCR detection of (**A**) the relative expression levels of miR-450b-5p, (**B**) miR-1249, (**C**) miR-155-5p, and (**D**) FC (Fold Change) values of 3 miRNAs in qRT-PCR and RNA sequencing, FC>1.5 indicates up-regulation ($\bar{x}$±s, n=4, **p<0.01, ***p=0.0001, ****p<0.0001).

inflammation in a mouse model of type 2 diabetes,[46] is involved in the development of diabetes and its complications,[47–49] and can be used as a biomarker for diabetes and a potential target for the treatment of diabetes. Numerous studies have shown that miR-155-5p plays an important role in the progression of diabetes.[50,51] It regulates the proliferation and angiogenesis of HRMECs (human retinal endothelial cells) and simultaneously affects the protein level of VEGF-A in HRMECs. Our previous findings indicated that SDMM capsules inhibit angiogenesis by suppressing the expression of the

VEGF family, including VEGF-A.[9] Therefore, we speculated that SDMM capsules may regulate retinal cell proliferation, apoptosis, and differentiation by regulating miR-1249 and miR-155-5p. At present, miR-450b-5p is mainly found in malignant tumors and ischemic diseases, such as hepatocellular carcinoma, transient cerebral ischemia, hepatic ischemia,[52] and detailed reports are lacking in diabetes and DR. However, miR-450b-5p can inhibit the apoptosis of human lung microvascular endothelial cells (HPMECs) and reduce the inflammatory response.[53] Our study found that the expressions of miR-450b-5p, miR-1249, and miR-155-5p were up-regulated in qRT-PCR and miRNA sequencing after SDMM capsule treatment compared to the diabetic group. This suggests that miR-450b-5p, miR-1249, and miR-155-5p may be targeted by the SDMM capsule to prevent DR.

The classification of genes involved in the same function or pathway using GO and KEGG analysis is of great significance for studying the mechanism by which the SDMM capsule regulates DR pathogenesis and progression. Analysis of the KEGG pathway in this study showed that the main signaling pathways included the PI3K/Akt pathway, MAPK pathway, focal adhesions, and neural factor signaling pathways. PI3K/Akt is one of the important regulatory mechanisms for maintaining normal physiological function, and is involved in biological processes such as metabolism, inflammatory responses, cell proliferation, apoptosis and others. Numerous studies have found that the PI3K/Akt signaling pathway plays a role in the occurrence and development of DR by regulating retinal neovascularization, insulin resistance, oxidative stress, retinal nerve damage, and inflammatory responses.[54–57] MAPKs are Ser/Thr protein kinases that convert extracellular stimuli into diverse cellular responses and are involved in a variety of physiological processes, including gene expression, cell proliferation, differentiation, death, and survival.[58,59] MAPK family members are regulated by a series of phosphorylation and are activated only in response to extracellular stimulation.[60] Classical MAPKs include ERK1/2, JNK1/2/3, p38 isoforms, and ERK5, and atypical MAPKs include ERK3/4, ERK7, and NLK.[58] Studies have shown that STZ-induced increased expression of ICAM-1, VEGF, inflammatory factors, and phosphorylation of p38 MAPK in the retina of DM rats have led to increased retinal cell death and vascular permeability.[61] Based on the results of KEGG enrichment analysis of differentially expressed miRNA target genes after SDMM capsule intervention, we speculate that SDMM capsules may play a role in the prevention and treatment of DR by modulating PI3K/Akt and MAPK signaling pathways through the regulation of differentially expressed miRNAs.

In this study, we identified several differentially expressed miRNAs that may be associated with DR progression, suggesting a potential treatment effect of SDMM capsules and related signaling pathways involved in regulation. The limitations of this study are the single approach to assess the improvement of DR by SDMM capsules, the selection of only three miRNAs for validation, and the use of only Sprague Dawley rats. In future studies, more miRNAs could be investigated, the study design could be extended to the DR population, and the mechanism could be validated in more in-depth in vitro experiments.

Conclusion

MiR-450b-5p, miR-1249, and miR-155-5p may be potential biomarkers for DR. SDMM capsules may effectively treat DR by regulating differentially expressed miRNAs to inhibit or activate the expression of target genes or their related signaling pathways, providing a basis for the application of SDMM capsules and new insights into the mechanism and prevention of DR. However, further studies are still needed to explore miRNA targets.

Abbreviations

SDMM, Shuangdan Mingmu; KEGG, Kyoto Encyclopedia of Genes and Genomes; DR, Diabetic retinopathy; TCM, Traditional Chinese medicine; HE, Hematoxylin-Eosin; PAS, Periodic Acid-Schiff; TUNEL, TdT-mediated dUTP nick end labeling; qRT-PCR, quantitative real-time polymerase chain reaction; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; VEGF, Vascular Endothelial Growth Factor; PEDF, Pigment Epithelium Derived Factor.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no competing financial interests.

References

1. Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet*. 2010;376(9735):124–136. doi:10.1016/S0140-6736(09)62124-3
2. Yau JW, Rogers SL, Kawasaki R, et al. Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care*. 2012;35(3):556–564. doi:10.2337/dc11-1909
3. Stitt AW, Curtis TM, Chen M, et al. The progress in understanding and treatment of diabetic retinopathy. *Prog Retin Eye Res*. 2016;51:156–186. doi:10.1016/j.preteyeres.2015.08.001
4. Chaoxiu Q, Xuhua T, Qigen L, Xiaoli W. Clinical study of compound Danshen dripping pills in the treatment of diabetic retinopathy. *Chine Materia Medica*. 2007;3:375–377. doi:10.13863/j.issn1001-4454.2007.03.045
5. Min Z. Effects of Ginkgo biloba extract tablets on diabetic retinopathy and pharmacological analysis. *Chine Med Guide*. 2020;18(12):197–198. doi:10.15912/j.cnki.gocm.2020.12.095
6. Guoqiang G, Wenzhong Y. Clinical study of Yiqi Yangyin Huoxue Tang assisting Conbercept for diabetic retinopathy complicated with vitreous hemorrhage. *New Chine Med*. 2022;54(3):75–79. doi:10.13457/j.cnki.jncm.2022.03.016
7. Nie F, Yan J, Ling Y, et al. Effect of Shuangdan Mingmu capsule, a Chinese herbal formula, on oxidative stress-induced apoptosis of pericytes through PARP/GAPDH pathway. *BMC Complement Med Ther*. 2021;21(1):118. doi:10.1186/s12906-021-03238-w
8. Chaojun F, Yongwang Z, Yanjun L, et al. Effects of Shuangdan Mingmu Capsule on the expressions of Spred-1 and VEGF in the retina of rats with diabetic retinopathy. *China J New Drugs*. 2019;28(23):2893–2898. doi:10.3969/j.issn.1003-3734.2019.23.017
9. Jun P, Kun P, Zheng-Rong L, Yu-Hui Q, Qing-Hua P. Effects of Shuang Dan Ming Mu Capsule on expression of VEGF-a, VEGF-b, VEGF-c and the VEGF receptor, Flk-1, in diabetic retinopathy rats. *Digital Chine Med*. 2018;1(3):548.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281–297. doi:10.1016/S0092-8674(04)00045-5
11. Gong Q, Su G. Roles of miRNAs and long noncoding RNAs in the progression of diabetic retinopathy. *Biosci Rep*. 2017;37(6):BSR20171157. doi:10.1042/BSR20171157
12. Xiong F, Du X, Hu J, Li T, Du S, Wu Q. Altered retinal microRNA expression profiles in early diabetic retinopathy: an in silico analysis. *Curr Eye Res*. 2014;39(7):720–729. doi:10.3109/02713683.2013.872280
13. Regazzi R. MicroRNAs as therapeutic targets for the treatment of diabetes mellitus and its complications. *Expert Opin Ther Targets*. 2018;22 (2):153–160. doi:10.1080/14728222.2018.1420168
14. Satari M, Aghadavod E, Mirhosseini N, Asemi Z. The effects of microRNAs in activating neovascularization pathways in diabetic retinopathy. *J Cell Biochem*. 2019;120(6):9514–9521. doi:10.1002/jcb.28227
15. Ye EA, Steinle JJ. miR-146a attenuates inflammatory pathways mediated by TLR4/NF-κB and TNFα to protect primary human retinal microvascular endothelial cells grown in high glucose. *Mediators Inflamm*. 2016;2016:3958453. doi:10.1155/2016/3958453
16. Li HL, Hao GM, Tang SJ, et al. HuoXue JieDu formula improves diabetic retinopathy in rats by regulating microRNAs. *J Ethnopharmacol*. 2021;268:113616. doi:10.1016/j.jep.2020.113616
17. Deng W, Huang D, Xie H, et al. Danhong injection represses diabetic retinopathy and nephropathy advancement in diabetic mice by upregulating microRNA-30d-5p and targeting JAK1. *Bioengineered*. 2022;13(4):8187–8200. doi:10.1080/21655979.2021.2006964
18. Dai C, Jiang S, Chu C, Xin M, Song X, Zhao B. Baicalin protects human retinal pigment epithelial cell lines against high glucose-induced cell injury by up-regulation of microRNA-145. *Exp Mol Pathol*. 2019;106:123–130. doi:10.1016/j.yexmp.2019.01.002
19. Li L, Li YL, Zhou YF, et al. Jiangtang Xiaozhi Recipe prevents diabetic retinopathy in streptozotocin-induced diabetic rats. *Chin J Integr Med*. 2017;23(6):425–432. doi:10.1007/s11655-016-2595-x
20. Shuyun X, Rulian B, Xiu C. The second edition of "Pharmacological Experiment Methodology" was published. *Chin Pharmacological Bulletin*. 1992;1:19.
21. Hongping L, Yuhui Q, Zhengrong L, Yuhong W, Rong Y. Analysis of chemical constituents of Shuangdan Mingmu capsule by UPLC-Q-TOF. *Chine Patent Med*. 2017;39(07):1527–1531. doi:10.3969/j.issn.1001-1528.2017.07.046)
22. Hongping L, Yuhui Q, Zhengrong L, Yuhong W, Rong Y. UPLC-Q-TOF-MS analysis of blood components of Shuangdanmingmu capsule. *Chin Proprietary Med*. 2017;39(10):2204–2206. doi:10.3969/j.issn.1001-1528.2017.10.049
23. Gene T. The Gene Ontology Resource: 20 years and still GOing strong. *Nucleic Acids Res*. 2019;47(D1):D330–D338. doi:10.1093/nar/gky1055
24. Dianwei M, Xuejun X, Jing L, Mei Z. Effects of Bushen Huoxue Recipe on PEDF and VEGF in retinal Müller cells under AGEs/hypoxic conditions. *China J Traditional Chine Med Ophthalmol*. 2021;31(03):157–163. doi:10.13444/j.cnki.zgzyykzz.2021.03.001

25. Gao D, Guo Y, Li X, et al. An aqueous extract of Radix Astragali, Angelica sinensis, and Panax notoginseng is effective in preventing diabetic retinopathy. *Evid Based Complement Alternat Med*. 2013;2013:578165. doi:10.1155/2013/578165
26. Gao D, Li Q, Li Y, et al. Antidiabetic and antioxidant effects of oleanolic acid from Ligustrum lucidum Ait in alloxan-induced diabetic rats. *Phytother Res*. 2009;23(9):1257–1262. doi:10.1002/ptr.2603
27. Lee SI, Oh SH, Park KY, Park BH, Kim JS, Kim SD. Antihyperglycemic effects of fruits of privet (Ligustrum obtusifolium) in streptozotocin-induced diabetic rats fed a high fat diet. *J Med Food*. 2009;12(1):109–117. doi:10.1089/jmf.2007.0000
28. Wu J, Ke X, Fu W, et al. Inhibition of hypoxia-induced retinal angiogenesis by Specnuezhenide, an effective constituent of Ligustrum lucidum Ait., through suppression of the HIF-1α/VEGF signaling pathway. *Molecules*. 2016;21(12):1756. doi:10.3390/molecules21121756
29. Feng L, Zhai YY, Xu J, et al. A review on traditional uses, phytochemistry and pharmacology of Eclipta prostrata (L.). *L J Ethnopharmacol*. 2019;245:112109. doi:10.1016/j.jep.2019.112109
30. Ananthi J, Prakasam A, Pugalendi KV. Antihyperglycemic activity of Eclipta alba leaf on alloxan-induced diabetic rats. *Yale J Biol Med*. 2003;76(3):97–102.
31. Jaiswal N, Bhatia V, Srivastava SP, Srivastava AK, Tamrakar AK. Antidiabetic effect of Eclipta alba associated with the inhibition of alpha-glucosidase and aldose reductase. *Nat Prod Res*. 2012;26(24):2363–2367. doi:10.1080/14786419.2012.662648
32. Yang CY, Wang J, Zhao Y, et al. Anti-diabetic effects of Panax notoginseng saponins and its major anti-hyperglycemic components. *J Ethnopharmacol*. 2010;130(2):231–236. doi:10.1016/j.jep.2010.04.039
33. Xu C, Wang W, Wang B, et al. Analytical methods and biological activities of Panax notoginseng saponins: recent trends. *J Ethnopharmacol*. 2019;236:443–465. doi:10.1016/j.jep.2019.02.035
34. Xie Z, Loi Truong T, Zhang P, Xu F, Xu X, Li P. Dan-Qi prescription ameliorates insulin resistance through overall corrective regulation of glucose and fat metabolism. *J Ethnopharmacol*. 2015;172:70–79. doi:10.1016/j.jep.2015.05.041
35. Qian S, Huo D, Wang S, Qian Q. Inhibition of glucose-induced vascular endothelial growth factor expression by Salvia miltiorrhiza hydrophilic extract in human microvascular endothelial cells: evidence for mitochondrial oxidative stress. *J Ethnopharmacol*. 2011;137(2):985–991. doi:10.1016/j.jep.2011.07.018
36. Lu TC, Wu YH, Chen WY, Hung YC. Targeting oxidative stress and endothelial dysfunction using Tanshinone IIA for the treatment of tissue inflammation and fibrosis. *Oxid Med Cell Longev*. 2022;2022:2811789. doi:10.1155/2022/2811789
37. Ma S, Zhang D, Lou H, Sun L, Ji J. Evaluation of the anti-inflammatory activities of tanshinones isolated from Salvia miltiorrhiza var. alba roots in THP-1 macrophages. *J Ethnopharmacol*. 2016;188:193–199. doi:10.1016/j.jep.2016.05.018
38. Feng H, He Y, La L, et al. The flavonoid-enriched extract from the root of Smilax China L. inhibits inflammatory responses via the TLR-4-mediated signaling pathway. *J Ethnopharmacol*. 2020;256:112785. doi:10.1016/j.jep.2020.112785
39. Akhtar MS, Iqbal J. Evaluation of the hypoglycaemic effect of Achyranthes aspera in normal and alloxan-diabetic rabbits. *J Ethnopharmacol*. 1991;31(1):49–57. doi:10.1016/0378-8741(91)90143-2
40. Wang D, Li C, Fan W, Yi T, Wei A, Ma Y. Hypoglycemic and hypolipidemic effects of a polysaccharide from Fructus Corni in streptozotocin-induced diabetic rats. *Int J Biol Macromol*. 2019;133:420–427. doi:10.1016/j.ijbiomac.2019.04.160
41. Alharazi WZ, McGowen A, Rose P, Jethwa PH. Could consumption of yam (Dioscorea) or its extract be beneficial in controlling glycaemia: a systematic review. *Br J Nutr*. 2021;128(4):1–12.
42. Lau CH, Chan CM, Chan YW, et al. Pharmacological investigations of the anti-diabetic effect of Cortex Moutan and its active component paeonol. *Phytomedicine*. 2007;14(11):778–784. doi:10.1016/j.phymed.2007.01.007
43. Torus B, Korkmaz H, Ozturk KH, et al. Downregulation of plasma microRNA-29c-3p expression may be a new risk factor for diabetic retinopathy. *Minerva Endocrinol*. 2020. doi:10.23736/S0391-1977.20.03278-2
44. Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. *Invest Ophthalmol Vis Sci*. 2011;52(7):4402–4409. doi:10.1167/iovs.10-6879
45. Chen X, Zeng K, Xu M, et al. P53-induced miR-1249 inhibits tumor growth, metastasis, and angiogenesis by targeting VEGFA and HMGA2. *Cell Death Dis*. 2019;10(2):131. doi:10.1038/s41419-018-1188-3
46. Wang Y, Li M, Chen L, et al. Natural killer cell-derived exosomal miR-1249-3p attenuates insulin resistance and inflammation in mouse models of type 2 diabetes. *Signal Transduct Target Ther*. 2021;6(1):409. doi:10.1038/s41392-021-00805-y
47. Massaro JD, Polli CD, Costa E Silva M, et al. Post-transcriptional markers associated with clinical complications in Type 1 and Type 2 diabetes mellitus. *Mol Cell Endocrinol*. 2019;490:1–14. doi:10.1016/j.mce.2019.03.008
48. Yan S, Wang T, Huang S, et al. Differential expression of microRNAs in plasma of patients with prediabetes and newly diagnosed type 2 diabetes. *Acta Diabetol*. 2016;53(5):693–702. doi:10.1007/s00592-016-0837-1
49. Peipei N, Yanan G. Relationship between hsa-miR-1249 and hsa-miR-486-5p and type 2 diabetes mellitus. *Acta Laser Biol*. 2016;25(3):240–243.
50. Wang G, Wu B, Zhang B, Wang K, Wang H. LncRNA CTBP1-AS2 alleviates high glucose-induced oxidative stress, ECM accumulation, and inflammation in diabetic nephropathy via miR-155-5p/FOXO1 axis. *Biochem Biophys Res Commun*. 2020;532(2):308–314. doi:10.1016/j.bbrc.2020.08.073
51. He J, Zhang R, Wang S, et al. Expression of microRNA-155-5p in patients with refractory diabetic macular edema and its regulatory mechanism. *Exp Ther Med*. 2021;22(3):975. doi:10.3892/etm.2021.10407
52. Huang Z, Mou T, Luo Y, et al. Inhibition of miR-450b-5p ameliorates hepatic ischemia/reperfusion injury via targeting CRYAB. *Cell Death Dis*. 2020;11(6):455. doi:10.1038/s41419-020-2648-0
53. Gong X, Zhu L, Liu J, et al. MIR3142HG promotes lipopolysaccharide-induced acute lung injury by regulating miR-450b-5p/HMGB1 axis. *Mol Cell Biochem*. 2021;476(12):4205–4215. doi:10.1007/s11010-021-04209-y
54. Cao YL, Liu DJ, Zhang HG. MiR-7 regulates the PI3K/AKT/VEGF pathway of retinal capillary endothelial cell and retinal pericytes in diabetic rat model through IRS-1 and inhibits cell proliferation. *Eur Rev Med Pharmacol Sci*. 2018;22(14):4427–4430. doi:10.26355/eurrev_201807_15493
55. Ao H, Li H, Zhao X, Liu B, Lu L. TXNIP positively regulates the autophagy and apoptosis in the rat müller cell of diabetic retinopathy. *Life Sci*. 2021;267:118988. doi:10.1016/j.lfs.2020.118988
56. Zeng J, Zhao H, Chen B. DJ-1/PARK7 inhibits high glucose-induced oxidative stress to prevent retinal pericyte apoptosis via the PI3K/AKT/mTOR signaling pathway. *Exp Eye Res*. 2019;189:107830. doi:10.1016/j.exer.2019.107830

57. Qiu F, Tong H, Wang Y, Tao J, Wang H, Chen L. Recombinant human maspin inhibits high glucose-induced oxidative stress and angiogenesis of human retinal microvascular endothelial cells via PI3K/AKT pathway. *Mol Cell Biochem*. 2018;446(1–2):127–136. doi:10.1007/s11010-018-3280-5
58. Cargnello M, Roux PP. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev*. 2011;75(1):50–83. doi:10.1128/MMBR.00031-10
59. Pearson G, Robinson F, Beers GT, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev*. 2001;22(2):153–183. doi:10.1210/edrv.22.2.0428
60. Cervellini I, Galino J, Zhu N, Allen S, Birchmeier C, Bennett DL. Sustained MAPK/ERK activation in adult Schwann cells impairs nerve repair. *J Neurosci*. 2018;38(3):679–690. doi:10.1523/JNEUROSCI.2255-17.2017
61. El-Remessy AB, Al-Shabrawey M, Khalifa Y, Tsai NT, Caldwell RB, Liou GI. Neuroprotective and blood-retinal barrier-preserving effects of cannabidiol in experimental diabetes. *Am J Pathol*. 2006;168(1):235–244. doi:10.2353/ajpath.2006.050500