• Basic Research •

Lycium barbarum polysaccharide inhibits retinal neovascularization and inflammation *in vitro* and *in vivo*

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Received: 2024-10-09 Accepted: 2024-11-25

Abstract

● **AIM:** To explore the effect and mechanism of *Lycium barbarum* polysaccharide (LBP) inhibiting retinal neovascularization.

● **METHODS:** *In vitro* tests were performed on human retinal microvascular endothelial cells (HRECs) from three groups, including control group (normal oxygen), hypoxic group (hypoxia at 37°C, 1% O_2, 5% CO_2, and 94% N_2), and LBP group (hypoxic group with LBP 100 μg/mL). *In vivo* experiments, C57 mice were divided into three groups: control group (normal rearing group), the oxygen-induced ischemic retinopathy (OIR) group, and the OIR with 50 mg/kg LBP group. Retinal neovascularization was observed by fluorescein angiography and quantified. Retinal thickness was evaluated by Hematoxylin and eosin (HE) stain. The expression of epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), phosphorylated mammalian target of rapamycin (p-mTOR), protein kinase B (AKT), phosphorylated protein kinase B (p-AKT), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-α (TNF-α) in each group were analyzed by Western blot. IL-1β level in retina was analyzed using immunohistochemical staining.

● **RESULTS:** The increased area of neovascular clusters in OIR mice was significantly decreased by LBP. Retinal thickness of OIR mice was significantly thinner compared with normal oxygenated mice and was increased in LBP group. Compared with those in the hypoxic groups, Western blotting of HRECs and retinal tissues revealed that the expression of EGFR, PI3K, p-mTOR, p-AKT, IL-1β, iNOS, and TNF-α decreased in the LBP group but was still greater than that in control group. Moreover, IL-1β was reduced in retinal sections treated with LBP. In the scratch test, the cell migration of the hypoxic group was significantly greater than that of the control group, while LBP treatment attenuated this increase in migration.

● **CONCLUSION:** LBP reduces retinal neovascularization and inflammation *in vivo* and inhibits the migration of HRECs *in vitro* by regulating the EGFR/PI3K/Akt/mTOR signaling pathway.

● **KEYWORDS:** *Lycium barbarum* polysaccharide; neovascularization; epidermal growth factor receptor; retina

DOI:10.18240/ijo.2025.02.03

Citation: Wu B, Wang X, Zhang W, Wang X, Wang QM, Pang YJ, Kong YC. *Lycium barbarum* polysaccharide inhibits retinal neovascularization and inflammation *in vitro* and *in vivo*. *Int J Ophthalmol* 2025;18(2):216-221

INTRODUCTION

Neovascular retinopathy includes diabetic retinopathy, retinopathy of prematurity, and neovascular age-related macular degeneration. Patients may present with eye discomfort, and in severe cases, they may also experience vision loss or blindness. Neovascularization is one of the important pathological changes in the occurrence and development of these diseases[1-3]. Retinal neovascularization is a small vein between the optic disc surface and the retina, which grows on the surface of the retina. The new blood vessels can also grow into the vitreous body at the site of vitreous adhesion to form the new blood vessel membrane. This disease is mostly caused by large-scale capillary occlusion of the retina and chronic ischemia, which is related to the production and release of vascular endothelial growth factor (VEGF), as well as the occurrence and development of inflammatory response. Retinal inflammation is one of the central features of ocular neovascular diseases.

Lycium barbarum polysaccharide (LBP) is the main component of *Lycium* berries. A large number of literatures have reported that LBP has anti-radiation, anti-aging, anti-oxidation and other biological activities, and has protective effects on corneal injury, dry eye, optic nerve injury, diabetic retinopathy and other diseases[4-7].

PI3K/Akt/mTOR signaling pathway is a major signaling pathway that regulates cell survival, differentiation and development, and plays a catalytic role in a variety of vascular diseases. Many studies have shown that this signaling pathway plays an important role in senile macular degeneration[8], diabetic retinopathy[9], retinoblastoma[10], uveal melanoma[11], pterygium[12] and other ocular abnormalities. PI3K/Akt/mTOR signaling pathway participate in gene transcription and protein expression through phosphorylation of its downstream target proteins, thus affecting biological activities such as inflammation, oxidative stress, autophagy and apoptosis[13]. By inhibiting this pathway, diabetic retinopathy can be treated to a certain extent[14]. In this study, we investigated whether LBP can play a role in the inflammatory process of retinal neovascularization diseases by regulating PI3K/Akt/mTOR signaling pathway.

MATERIALS AND METHODS

Ethical Approval All the experimental animal protocols used in this study were reviewed and approved by the Animal Care and Use Committee of Tianjin Integrated Traditional Chinese and Western Medicine Hospital (Nankai Hospital, Tianjin, China). All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the Animal Welfare Act guidelines.

Animal Model Establishment C57 mice (GemPharmatech Co., Ltd., China) and their mothers were transferred from a normal environment to hyperoxic conditions (oxygen concentration of 75%-80%) to induce oxygen-induced ischemic retinopathy (OIR) group at 7d after birth. To ensure the survival of the young mice, we replaced the mother mouse once in the middle of the process. On day 12 after birth, the model was successfully generated, and the normoxic environment was restored.

Mice with OIR were evenly divided into two groups according to weight on the thirteenth to seventeenth days. There was a total of three groups together with the normoxia feeding group and at least six mice in each group. LBP was given at fixed times once a day. In one of the OIR groups, 50 mg/kg LBP solution (Solarbio Biotech, China) was administered by gavage. The solution was prepared by dissolving the corresponding weight of LBP powder in normal saline. In addition, the remaining was OIR group, and the normoxia feeding group was control group; animals in both groups were given equal amounts of normal saline. Mice were sacrificed on the eighteenth day after birth, and a portion of the retinal tissue was immediately dissected and stored at -80℃. Subsequently, the expression of epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), phosphorylated mammalian target of rapamycin (p-mTOR), protein kinase B (AKT), phosphorylated protein kinase B (p-AKT), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), and tumor necrosis factor-α (TNF-α) was determined by Western blotting. The remain parts of the eyeballs were enucleated and kept in ocular fixative at room temperature. Then, paraffin-embedded sections were cross-cut along the temporal nasal axis of the eyeball and subjected to hematoxylin and eosin (HE) stain. In this study, only sections containing the optic nerve stump were used to ensure comparability. Three retinal sections per mouse were obtained.

Cell Experiments human retinal microvascular endothelial cells (HRECs; Bnbio, Beijing, China) were subcultured. HRECs were cultured in endothelial cell medium (ECM) supplemented with 5% fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin solution (P/S; Epizyme Biotech, China) and 1% endothelial cell growth supplement (ECGS; Wuhan Pricella Biotechnology Co., Ltd., China), and the medium was replaced on alternate days.

The cells were divided into three groups: a normoxic blank group (37℃, 95% air, 5% CO_2), a hypoxia control group (37℃, 1% O_2, 5% CO_2, 94% N_2), and a hypoxia and LBP intervention group (100 μg/mL). After 24h, the cells were lysed and incubated on ice, and the expression levels of EGFR, PI3K, mTOR, P-mTOR, AKT, p-AKT, IL-1β, iNOS and TNF-α in each group were determined by Western blotting.

Western Blotting Western blotting was performed to determine the retinal expression of IL-1β, iNOS, TNF-α, EGFR, PI3K, mTOR, p-mTOR, AKT, p-AKT. Densitometry analyses of the bands were performed using Image J software.

Hematoxylin and Eosin Stain Eyeballs were fixed in 10% formalin, paraffin-embedded sections were cross-cut along the temporal nasal axis of the eyeball and cut into 4-μm sections. The sections were then deparaffinized, hematoxylin and eosin staining (H&E) and immunohistochemical analysis were performed. In this study, only sections containing the optic nerve stump were used to ensure comparability. Three retinal sections per mouse were obtained.

Fluorescein Angiography and Quantified Animals were anesthetized and perfused with fluorescein *via* retro-orbital injection of 2.5 mg/50 μL of FITC-dextran (Sigma-Aldrich, USA).The eyes were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 1h. The retina was then separated from the eyecup. The incisions were flat-mounted

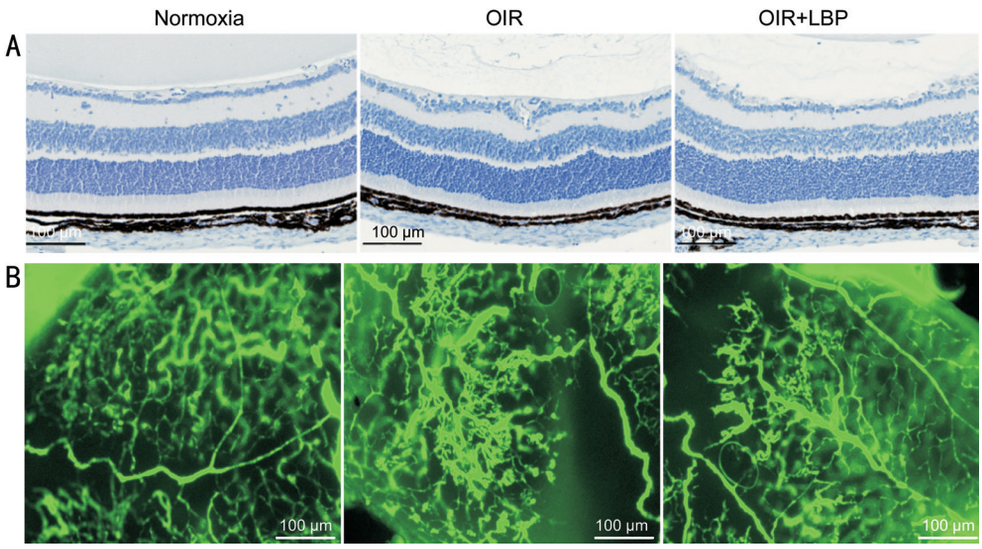

Figure 1 LBP improved retinal thickness and angiogenesis in OIR mice A: Compared with normal oxygenated mice, the retinal thickness of OIR mice was significantly thinner, and the intervention of LBP significantly increase the thickness of retinal layers in mice; B: The area of neovascular clusters in OIR mice was significantly increased compared with normal oxygenated mice, and LBP significantly reduce the area of retinal neovascular clusters. LBP: *Lycium barbarum* polysaccharide; OIR: Oxygen-induced ischemic retinopathy.

on a gelatin-coated slide. Retinas were examined under the fluorescent microscope (Nikon Eclipse TE20000-U, Nikon, Japan). The retinal neovascularization was quantified as a percentage of the total retinal area.

Cell Scratch Assay All six-well plates, straws, rulers and markers were sterilized for 30min. A marker was used to draw the horizontal line at an interval of 0.5 cm across the hole on the back of the six-well plate. Cells were subsequently added uniformly to the wells of three six-well plates; one plate was cultured in a normoxic incubator, and the other two were cultured in a low-oxygen environment. After 24h, the six-well plates were removed, and vertical lines were drawn. The cells were washed with sterile PBS, and the process was repeated three times to remove the floating cells. Serum-free medium (2.0 mL) was added to six-well plates subjected to hypoxia and normoxia, and an equal volume of the LBP solution in serum-free medium (100 μg/mL) was added to the other six-well plate. The samples were subsequently placed in the appropriate incubators for further cultivation. Samples were taken and photographed after 24h.

Statistical Analysis The results of immunoblot experiments were quantified using Image J (National Institutes of Health, USA). Data analyses were performed by SPSS version 13.0 software. Results were indicated as mean±standard deviation. Data were analyzed statistically using ANOVA for comparisons of multiple sets of data. Statistical significance was set at $P<0.05$. The cell experiments were repeated 3 times.

RESULTS

***Lycium barbarum* Polysaccharide Improved Retinal Thickness and Angiogenesis in OIR Mice** In the OIR model, newborn mice were exposed to 75% oxygen from day 7 (P7) to day 12 (P12), and the high oxygen environment blocked the blood vessels in the retina of the mice. After placing the animals back in the indoor air, abnormal neovascularization gradually proliferated from P12 until P17 after birth. Subsequently, from P17 to P21, the abnormal blood vessels gradually subsided. Retinal thickness was evaluated by HE staining. Compared with normal oxygenated mice, the retinal thickness of OIR mice was significantly thinner, and the intervention of LBP could significantly increase the thickness of retinal layers in mice (Figure 1A). Retinal blood vessel staining showed that the area of neovascular clusters in OIR mice was significantly increased compared with normal oxygenated mice, and the intervention of LBP could significantly reduce the area of retinal neovascular clusters (Figure 1B).

Lycium barbarum* Polysaccharides Inhibits the Inflammatory Response *in vivo* and *in vitro Compared with normal oxygenated mice, the retinal inflammatory response and inflammatory factors of OIR mice were increased, and the intervention of LBP could significantly inhibit the retinal inflammatory state of OIR mice (Figure 2A, 2B). In the experiment of HRECs, the secretion of inflammatory factors increased in the hypoxic intervention group, and the cell inflammation improved after the intervention of LBP (Figure 2C).

***Lycium barbarum* Polysaccharide Regulates EGFR/PI3K/AKT Pathway** Compared with normal oxygenated mice, the expression of EGFR and PI3K, p-AKT and p-mTOR levels in the retina of OIR mice were significantly increased and reduced by the intervention of LBP (Figure 3A). In HRECs, the expression of EGFR and PI3K, p-AKT and p-mTOR were

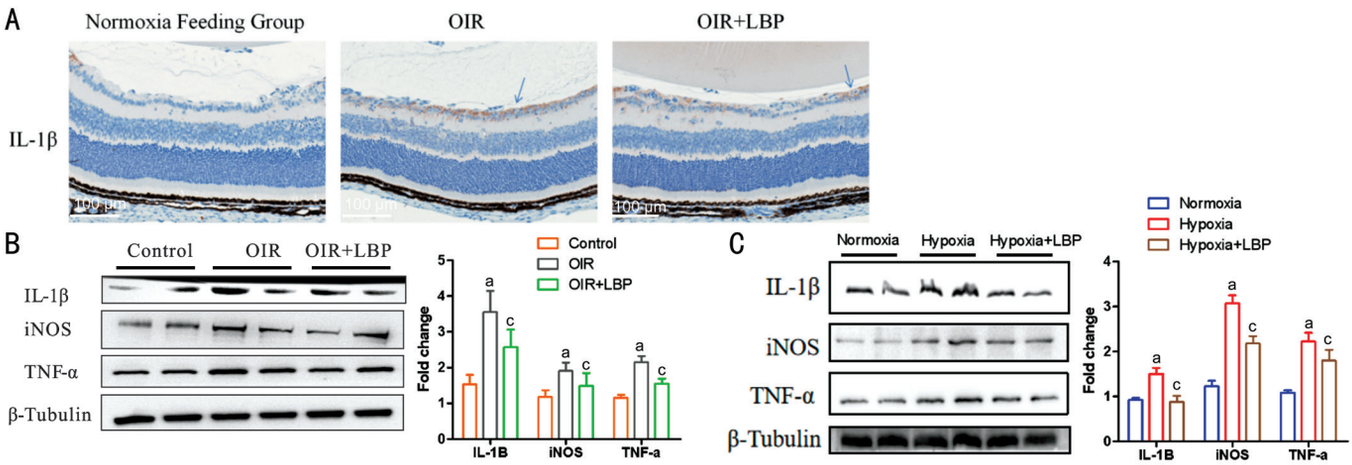

Figure 2 LBP inhibited the inflammatory response *in vivo* and *in vitro* A, B: The results of immunohistochemistry and Western blot in each group, the intervention of LBP significantly inhibit the retinal inflammatory of OIR mice; C: In HRECs, inflammatory factors increased in the hypoxic intervention group, and decreased after the intervention of LBP. [a]*P*<0.05 compared to the control or normoxia group; [c]*P*<0.05 compared to the OIR or hypoxia group. LBP: *Lycium barbarum* polysaccharide; OIR: Oxygen-induced ischemic retinopathy; HRECs: Human retinal microvascular endothelial cells; IL-1β: Interleukin-1β; iNOS: Inducible nitric oxide synthase; TNF-α: Tumor necrosis factor-α.

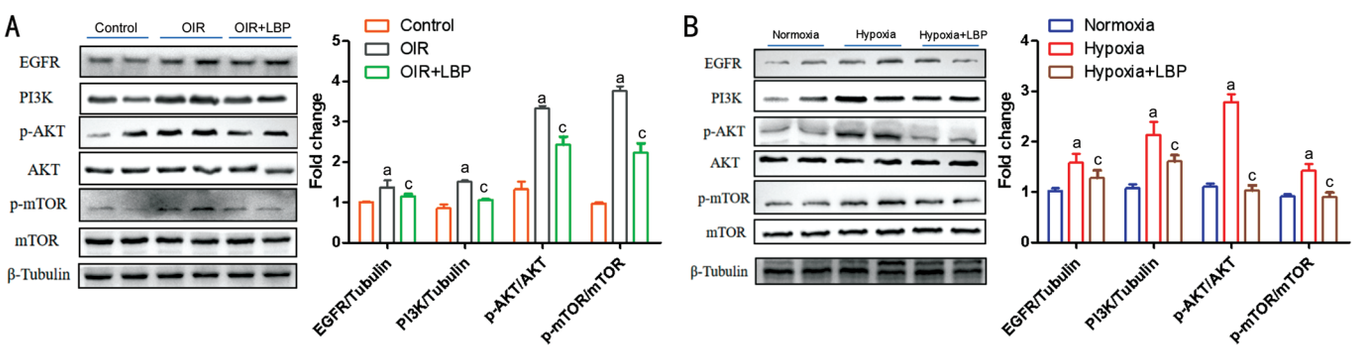

Figure 3 LBP regulates EGFR/PI3K/AKT pathway The expression levels of EGFR and PI3K in the retina of OIR mice (A) and HRECs (B) were significantly reduced by the intervention of LBP, and p-AKT and p-mTOR levels were inhibited. [a]*P*<0.05 compared to control or normoxia group; [c]*P*<0.05 compared to the OIR or hypoxia group. LBP: *Lycium barbarum* polysaccharide; OIR: Oxygen-induced ischemic retinopathy; HRECs: Human retinal microvascular endothelial cells; EGFR: Epidermal growth factor receptor; PI3K: Phosphatidylinositol 3-kinase; mTOR: Mammalian target of rapamycin; p-mTOR: Phosphorylated mammalian target of rapamycin; AKT: Protein kinase B; p-AKT: Phosphorylated protein kinase B.

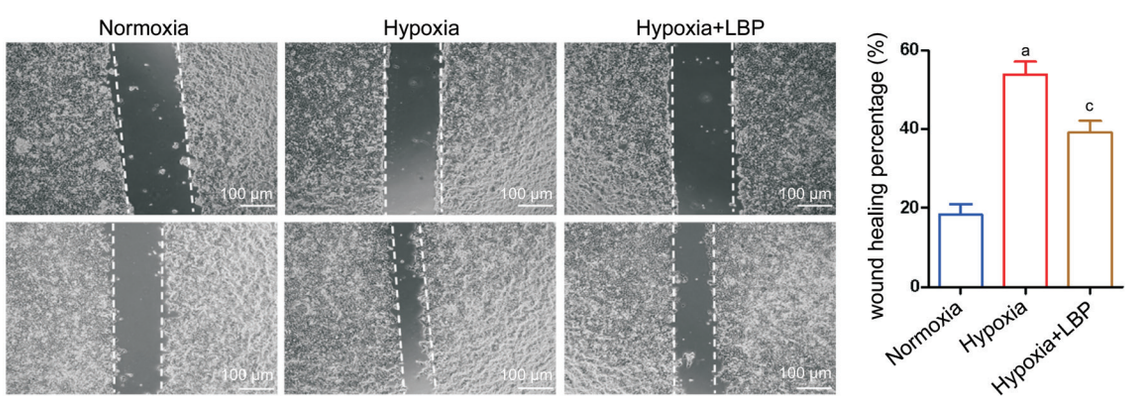

Figure 4 Cell migration of the hypoxia control group was significantly greater than that of the normoxic culture group, while LBP treatment attenuated this increase in migration [a]*P*<0.05 compared to normoxia group; [c]*P*<0.05 compared to hypoxia group. LBP: *Lycium barbarum* polysaccharide.

significantly increased in the hypoxic intervention group and decreased in LBP group (Figure 3B).

***Lycium barbarum* Polysaccharide Inhibits Cell Migration** Angiogenesis requires endothelial cell proliferation and migration. In the scratch test, the cell migration of the hypoxia control group was significantly greater than that of the normoxic culture group, while LBP treatment attenuated this increase in migration (Figure 4).

DISCUSSION

For normal metabolism, the retina can dynamically regulate the level of oxidative stress and the inflammatory response to avoid excessive cell damage and limit abnormal apoptosis in the surrounding circulatory system to maintain conduction of light signals in the visual system. However, during disease or aging, these regulatory mechanisms may be inactive or dysfunctional, making it difficult for the retina to maintain normal metabolic activity. Although some herbs and their active components, including LBP, may have no significant effect on the visual system under normal circumstances, these treatments have obvious beneficial effects on stress- or disease-induced retinal cell damage or apoptosis[15-16]. LBP can effectively clear reactive oxygen species (ROS) and delay the degeneration of retinal ganglion cells to protect retinal photoreceptor cells from light-induced damage[17].

OIR is a commonly used model for the study of pathological retinal neovascularization. This model can be used for the study of pathological retinal neovascularization-related diseases[18]. During the modeling process, a high-oxygen environment inhibits the regression of the initial immature blood vessels in the retina. When mice return to a normal air environment, the relative hypoxia of the retina promotes the rapid generation of pathological new blood vessels, leading to a large area of nonperfusion and a large number of new blood vessels in the retina.

Under hypoxia, the protein expression of the EGFR/PI3K/Akt/mTOR pathway in retinal tissue was greater than that in normal tissue, indicating that hypoxia activated the EGFR/PI3K/Akt/mTOR signaling pathway, while LBP reduced EGFR, PI3K, p-mTOR and p-AKT levels and played a role in inhibiting this pathway. Similar results were obtained in cell experiments *in vitro*. It is widely known that VEGF is closely related to the generation of retinal neovascularization. Studies have shown that the effect of VEGF on endothelial cells is partially mediated by the PI3K pathway, and sustained activation of AKT1 can induce the formation of structurally abnormal blood vessels[19]. At present, multiple scholars have found that drugs affect the expression of VEGF *via* the PI3K/Akt/mTOR pathway, thereby affecting the development or treatment in many diseases, such as coloretal cancer[20], gastric cancer[21], steoporotic fractures[22], ischemic stroke[23] *etc*. In ophthalmic research, the correlation between VEGF and PI3K/Akt/mTOR pathway has also been found. Husain *et al*[24] found that the PI3K/Akt/mTOR signaling pathway was activated on stiff substrates. It is amplified by VEGF-A stimulation, and correlations with enhanced endothelial cell proliferation, contrast, pro angiogenic secretion, and capillary like tube formation, providing a new treatment approach for retinal neovascularization.

Research on the effect of LBP in the treatment of retinopathy is constantly expanding and deepening. Yao *et al*[25] suggested that LBP exerts a positive effect on diabetic retinopathy and diabetic cataracts by activating the SIRT1-p53/SIRT1-FOXO1 signaling pathway. LBP can increase SIRT1 levels to reduce P53 and FOXO1 expression and inhibit apoptosis for preventive and therapeutic purposes. In their study of an optic nerve transection model, Li *et al*[26] suggested that LBP delayed the degeneration of retinal ganglion cells by inhibiting the JNK/c-jun pathway and simultaneously inhibiting the oxidative stress response.

LBP has also been shown to alleviate H_2O_2 and ischemia-reperfusion (I/R)-induced retinal cell damage by activating the Nrf2/HO1 pathway. LBP increases the nuclear translocation of Nrf2 and HO1 and antioxidant enzyme activity to exert its antioxidant effects, reducing oxidative stress damage and inhibiting apoptosis[27-28]. Moreover, LBP can significantly reduce VEGF levels and neoangiogenesis and improve the local hypoxic environment, thus preventing the occurrence of retinal microangiopathy[17].

Overall, LBPs may repair retinal cell degeneration and damage through multiple pathways rather than through a single pathway, and the factors of various pathways influence each other, forming a large and complex pathological network related to disease occurrence. The specific mechanism remains to be further investigated.

ACKNOWLEDGEMENTS

Foundations: Supported by the Tianjin Health Research Project (No.ZC20069; No.TJWJ2022MS040); the Foundation of the Committee of Integrated Traditional Chinese and Western Medicine (No.2021011); the Science and Technology Foundation of Tianjin Eye Hospital (No.YKYB1901).

Conflicts of Interest: Wu B, None; **Wang X,** None; **Zhang W,** None; **Wang X,** None; **Wang QM,** None; **Pang YJ,** None; **Kong YC,** None.

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