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Protective Effect and Autophagy Mechanism of *Lycium barbarum* Polysaccharides on Retinal Pigment Epithelial Cells Under High-Glucose Conditions

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Abstract: *Objective:* To study the effects of *Lycium barbarum* polysaccharide (LBP) on the proliferation, apoptosis, and autophagy of retinal pigment epithelial (RPE) cells cultured under high-glucose conditions. *Methods:* The ARPE-19 cell line was randomly divided into a control group (normally cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 [DMEM/F-12] medium), a high-glucose group (HG; 50 mmol/L glucose added to DMEM/F-12 medium), and a HG+LBP group (incubated in DMEM/F-12 medium containing 1 mg/mL LBP for 24 h, and then treated with 50 mmol/L glucose for 24 h). Following Ad-mCherry-GFP-LC3B infection, cell proliferation, apoptosis, mammalian target of rapamycin (mTOR) expression, and autophagic flux were determined by Cell Counting Kit-8 (CCK-8), AnnexinV-APC/7-AAD Apoptosis Detection Kit, Western blot, and laser confocal microscopy, respectively. *Results:* The proliferation rate of ARPE-19 cells in the HG group was significantly lower than that in the control group ($P < 0.05$), while the proliferation rate of ARPE-19 cells in the HG+LBP group was significantly higher than that in the HG group ($P < 0.05$). The apoptosis rate of ARPE-19 cells in the HG group was significantly higher than that in the control group ($P < 0.05$), while the apoptosis rate of ARPE-19 cells in the HG+LBP group was significantly lower than that in the HG group ($P < 0.05$). The relative expression of phosphorylated mTOR (p-mTOR) of ARPE-19 cells in the HG group was significantly lower than that in the control group ($P < 0.05$), with enhanced autophagic flux; when compared with the HG group, the HG+LBP group had significantly higher expression of p-mTOR ($P < 0.05$), with diminished autophagic flux. *Conclusion:* LBP has a protective effect on RPE cells with high glucose-induced injury, and its mechanism may be related to LBP inhibition of high glucose-induced abnormal autophagy.

Keywords: *Lycium barbarum* polysaccharides; High glucose; Retinal pigment epithelial cell; Autophagy; Cell culture

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1. Introduction

Retinal pigment epithelium (RPE) is an important component of the blood-ocular barrier, and the cells play an important role in maintaining the normal metabolism of the retina [1]. Although RPE is crucial to neural retinal cell activity, the neural retina is still the core of diabetic retinopathy (DR) research as it is the main site of lesion in DR [2] and RPE layer changes are not easily detected in DR [3]. With the research progress in recent years, it has been found that the structural and functional abnormalities of RPE are involved in the pathogenesis of DR. For example, high glucose acting on RPE cells will cause intracellular metabolic disorders, which will lead to the destruction of the local microenvironment of the retina and the occurrence of retinal damage [4]. However, the pathophysiological regulation mechanism of RPE is not widely understood. Therefore, it is necessary to further study the mechanisms involved in RPE damage and explore strategies to alleviate the pathological damages to RPE. As an intracellular catabolic pathway, autophagy plays a role in protecting cells and repairing cell functions under physiological conditions. However, the abnormal activation of autophagy under pathological conditions can cause cell damage and apoptosis [5]. A large number of studies have suggested that autophagy dysregulation is involved in the pathological process of DR, and RPE cells are the key cells to maintaining retinal function; hence, regulating autophagy in RPE cells is a potential target in the treatment of DR [6].

Lycium barbarum polysaccharide (LBP) is a water-soluble polysaccharide extracted from *Lycium barbarum* fruit, with various biological activities, including anti-inflammatory, anti-oxidation, anti-aging, anti-tumor, neuroprotective, and immune regulation functions [7]. Existing research results have shown that LBP is a promising therapeutic drug, especially in hyperlipidemia, diabetes, liver disease, *etc*. [8,9]. In diabetic animals and cell models, LBP can protect retinal function and morphology, protect the blood-retinal barrier, and reduce retinal neovascularization, thereby alleviating retinal damage caused by diabetes [10-12]. Although autophagy dysregulation in RPE cells plays an important role in the pathological mechanism of DR, whether or not LBP can play a protective role in DR by regulating autophagy in RPE cells remains unclear. In this study, a high glucose-induced RPE cell injury model was used to study the role of LBP in DR and the mechanism of autophagy so as to provide an experimental basis for clarifying the role of LBP in the treatment of DR.

2. Materials and methods

2.1. Main reagents and instruments

The cell line, main reagents, and instruments used were as follows: ARPE-19 cell line (ATCC cell bank, USA); LBP standard (Shanghai Hengfei Biotechnology Co., Ltd.), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) cell culture medium, 0.25% trypsin, and fetal bovine serum (Gibco, USA); cell proliferation detection kit (Cell Counting Kit-8 [CCK-8], MCE, USA) and AnnexinV-APC/7-AAD Cell Apoptosis Kit (Tianjin Sanjian Biotechnology Co., Ltd.); rabbit mammalian target of rapamycin (mTOR) polyclonal antibody, rabbit phosphorylated mTOR (p-mTOR) monoclonal antibody (CST, USA), rabbit glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Hangzhou Xianzhi Biotechnology Co., Ltd.), horseradish peroxidase-labeled secondary antibody (Affinity, USA), and adenovirus expressing autophagy protein LC3B (Ad-mCherry-GFP-LC3B, Shanghai Biyuntian Biotechnology Co., Ltd.); carbon dioxide (CO_2) constant temperature cell incubator (SANYO, Japan), flow cytometer (Beckman Coulter, USA), microplate reader (MD, USA), inverted microscope (Nikon Corporation, Japan), and laser scanning confocal microscope (Nikon Corporation, Japan).

2.2. Cell culture and grouping

The cryopreserved ARPE-19 cell line was routinely resuscitated and passaged [13]. Cells in the logarithmic

growth phase and in good growth state were selected to prepare a single cell suspension, and cell density was adjusted to 1×10^5 cells/mL. The cell suspension was inoculated at 100 μL/well in a 96-well plate and then placed in an incubator at 37°C, 5% CO_2 for culture. The cells were randomly divided into a control group, a high-glucose (HG) group, and a HG+LBP group. All cells were starved in serum-free medium for 24 h before treatment, and the original culture medium in the 96-well plate was discarded the following day. Cells in the control group were treated once with complete medium, cells in the HG group were treated with complete medium containing 50 mmol/L glucose [14] for 24 h, and cells in the HG+LBP group were treated with 1 mg/mL LBP [13] after pretreatment with complete medium for 24 h , followed by 50 mmol/L glucose for 24 h.

2.3. Cell proliferation detected by Cell Counting Kit-8

After the cells were cultured for 24 h, we replaced the medium with serum-free DMEM medium, added 10 μL of CCK-8 solution to each well, incubated the culture plate in an incubator at 37°C in the dark for 4 h, and used a microplate reader to detect the absorbance value of each well at a wavelength of 450 nm. The experiment was repeated 3 times independently.

2.4. Detection of cell apoptosis by flow cytometry

The cells were treated with trypsin digestion solution (without ethylenediaminetetraacetic acid [EDTA]), carefully collected, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded, and the cells were resuspended with phosphate-buffered saline (PBS). Then, 5 μL of 7-AAD staining solution was added to 50 μL of binding buffer and mixed well. The reaction was incubated in the dark for 15 min at room temperature. After adding 450 μL of binding buffer, 5 μL of Annexin V-APC was added, and the reaction was incubated in the dark at room temperature for 15 min. Within 1 hour following the completion of staining, flow cytometry was performed.

2.5. Detection of the expression of autophagy pathway protein by Western blot

We centrifuged the collected ARPE-19 cell suspension, discarded the supernatant, extracted the total protein, and placed it into a 10 mL Eppendorf (EP) tube, in which sodium dodecylsulfonate was added. After mixing, it was placed into boiling water at 95~100℃, boiled, and denatured for later use. The extracted 40 μg sample of total protein was subject to electrophoresis, blocking, and transmembrane operation. After transfer, mTOR (1:1000), p-mTOR (1:1000), and GAPDH (1:1000) antibodies were incubated overnight. We incubated polyvinylidene difluoride (PVDF) membrane with horseradish peroxidase-labeled secondary antibody (1:50000) diluted in tris-buffered saline and Tween 20 (TBST) for 2 h. The membrane containing protein markers was washed, enhanced chemiluminescence was developed, the film was exposed, and the relative gray value of different factors in the cells of each group of the film was analyzed with BandScan5.0.

2.6. Detection of autophagy flux by Ad-mCherry-GFP-LC3B transfected cells

Cells in the logarithmic growth phase were taken and digested with 0.25% trypsin to prepare a single-cell suspension. ARPE-19 cells were evenly seeded into a 12-well plate at 1×10^5/well and placed in 5% CO_2 with saturated humidity overnight in a constant-temperature incubator at 37°C. The cells were incubated for 48 h, and 50 μL of Ad-mCherry-GFP-LC3B virus solution was added to each well at the same time. Confocal laser microscopy was used to observe the expression of autophagy fluorescent proteins in cells. In the early stage of autophagy, yellow fluorescent dots (LC3B located on autophagosomes) formed by the fusion of green fluorescent protein (GFP) and red fluorescent protein (mCherry) representing autophagosomes are usually seen. In the late stage of autophagy, due to the formation of autolysosomes, red fluorescent spots are mainly observed

(GFP is rapidly quenched in acidic environment, and mCherry remains relatively stable).

2.7. Statistical analysis

SPSS 22.0 was used for statistical analysis, and data were expressed in mean ± standard deviation. The comparison between multiple groups was performed by analysis of variance, while the comparison between two groups was performed by the least significant difference (LSD) t-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of *Lycium barbarum* polysaccharide on the proliferation of ARPE-19 cells under high-glucose conditions

The CCK-8 results showed that the proliferation rate (%) of ARPE-19 cells in each group was as follows: 100 ± 0% in the control group, 66.23 ± 3.58% in the HG group, and 85.80 ± 1.54% in the HG+LBP group. There was statistically significant difference in the cell proliferation rate among the three groups ($F = 170.43$, $P < 0.05$). Compared with the control group, the HG group had significantly lower cell proliferation rate ($P < 0.05$); compared with the HG group, the HG+LBP group had significantly higher cell proliferation rate ($P < 0.05$), as shown in **Figure 1**. The results suggested that the proliferation activity of ARPE-19 cells decreases under high-glucose culture conditions and LBP can promote cell proliferation under high-glucose conditions.

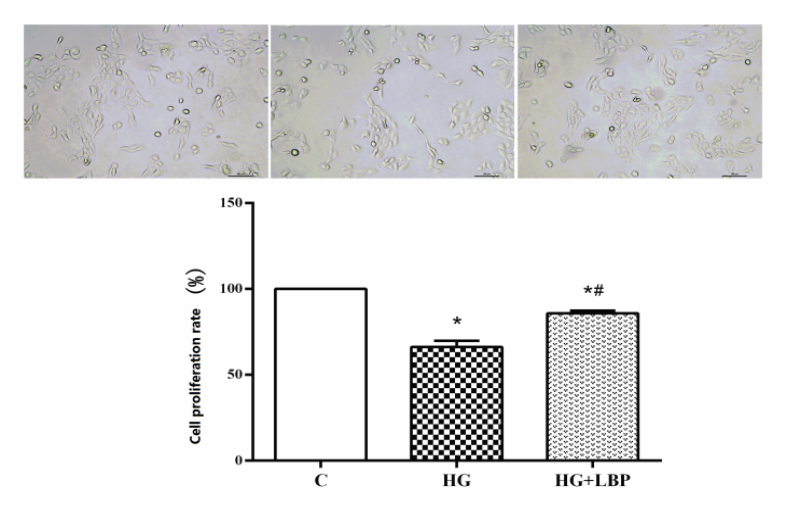

Figure 1. Comparison of ARPE-19 cell proliferation rate in each group. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the high glucose group. Abbreviations: C, control; HG, high glucose; LBP, *Lycium barbarum* polysaccharide

3.2. Effect of *Lycium barbarum* polysaccharide on ARPE-19 cell apoptosis under high-glucose conditions

The apoptosis rate (%) of ARPE-19 cells in each group after treatment for 24 h was as follows: 5.85 ± 0.25% in the control group, 28.10 ± 0.93% in the HG group, 14.24 ± 0.32% in the HG+LBP group. The difference among the three groups was statistically significant ($F = 1092.31$, $P < 0.05$). The apoptosis rate in the HG group was significantly higher than that in the control group ($P < 0.05$) but lower than that in the HG+LBP group ($P < 0.05$), as shown in **Figure 2**. The results suggested that the apoptosis of ARPE-19 cells increases under high-glucose conditions and LBP has an inhibitory effect on cell apoptosis under high-glucose conditions.

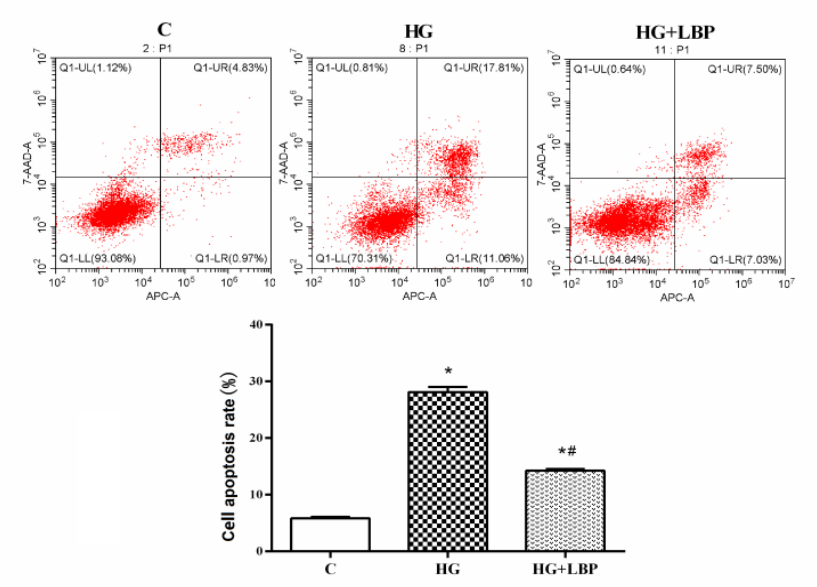

Figure 2. Comparison of ARPE-19 cell apoptosis rate in each group. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG group. Abbreviations: C, control; HG, high glucose; LBP, *Lycium barbarum* polysaccharide

3.3. Effect of *Lycium barbarum* polysaccharide on autophagy in ARPE-19 cells under high-glucose conditions

The results of Western blot experiments showed that the relative expression of mTOR in ARPE-19 cells in each group was as follows: 0.30 ± 0.03 in the control group, 0.29 ± 0.02 in the HG group, and 0.28 ± 0.03 in the HG+LBP group; there was no statistical difference among the three groups ($F = 0.45$, $P > 0.05$). The relative expression of p-mTOR in each group was as follows: 0.30 ± 0.03 in the control group, 0.06 ± 0.02 in the HG group, and 0.13 ± 0.03 in the HG+LBP group; there was statistical difference among the three groups ($F = 74.00$, $P < 0.05$), as shown in **Figure 3**. Weaker green and red fluorescent spots were observed in the cells of the control group; there were significantly more green fluorescent spots in the cells of the HG group than in the cells of the control group, but less green fluorescent spots were observed in the cells of the HG group than the cells of the HG+LBP group (**Figure 4**). The results suggested that autophagy in ARPE-19 cells enhances under high-glucose culture conditions and LBP has an inhibitory effect on cell autophagy under high-glucose conditions.

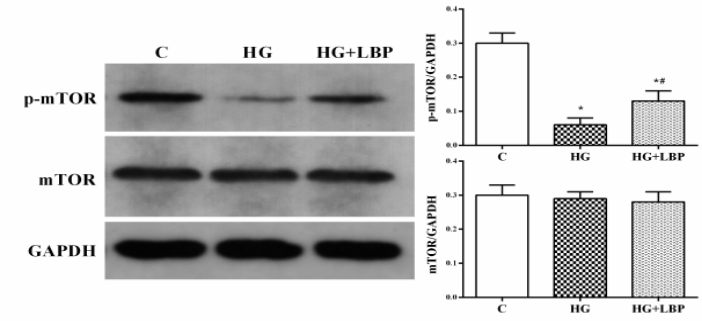

Figure 3. Comparison of autophagy proteins mTOR and p-mTOR expression in ARPE-19 cells in each group. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the HG group. Abbreviations: C, control; HG, high glucose; LBP, *Lycium barbarum* polysaccharide; mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated mammalian target of rapamycin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

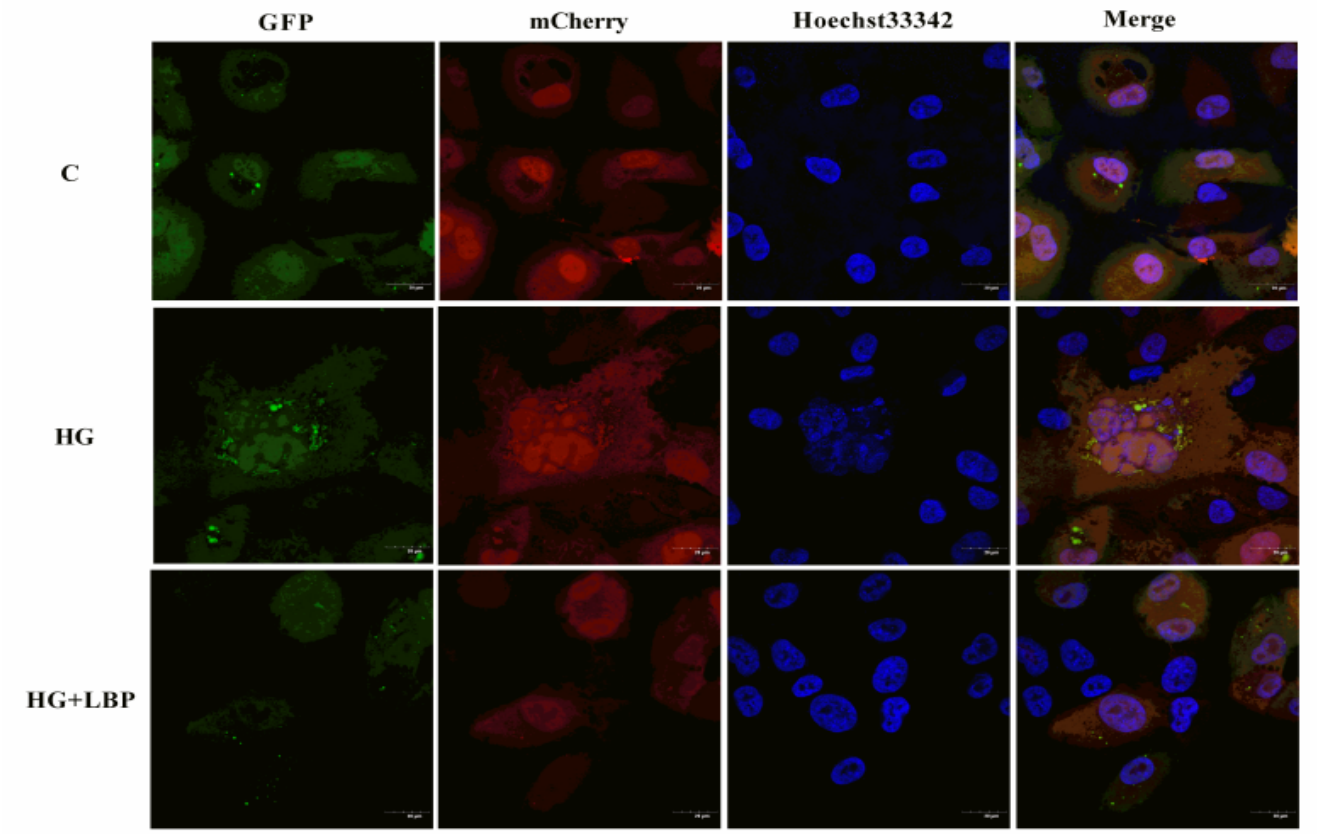

Figure 4. Autophagic flux in ARPE-19 cells in each group (×1000, scale bar = 20μm). Abbreviations: C, control; HG, high glucose; LBP, *Lycium barbarum* polysaccharide; GFP, green flourescent protein; mCherry, red fluorescent protein.

4. Discussion

According to the International Diabetes Federation, the number of people with diabetes will reach 693 million by 2045 worldwide, and the prevalence of diabetes in China will rank first in the world [15]. As the most common microvascular complication of diabetes, DR has become the main cause of visual impairment and blindness in the working-age population [16]. Although retinal laser treatment and vitreous surgery can preserve the vision of some patients to a certain extent, the safety and long-term efficacy of these therapies are still less than ideal. In terms of drug treatment, although anti-vascular endothelial growth factor (anti-VEGF) is very effective in the treatment of macular edema and proliferative DR (PDR), the cost of maintenance therapy, which takes several months to years, is often high [17], and it may not be effective for chronic patients [18]. Therefore, further exploring the pathogenesis of DR and finding new therapeutic targets has far-reaching significance for improving the visual prognosis of patients. With the deepening of research, many other drug treatments have emerged in recent years, aiming at inhibiting or weakening the oxidative stress response and signaling pathways in the pathogenesis of DR, such as aldose reductase pathway and protein kinase C cascade. However, the development of these drugs is still in the experimental stage, and their efficacy has yet to be clinically verified [19]. The strong antioxidant capacity and neuroprotective effect of LBP render it a hotspot in retinal protection research in recent years. In the present study, we simulated a high-glucose environment *in vitro* and found that LBP has a protective effect on RPE damage under high-glucose conditions and its pretreatment can significantly increase cell viability and reduce cell apoptosis rate. The results obtained are similar to other studies on LBP, suggesting that LBP can prevent and treat glaucoma, retinal ischemia-reperfusion, age-related macular degeneration, retinitis pigmentosa, and DR, thereby preserving visual function [20].

Lycium barbarum has always been a traditional nourishing Chinese medicinal material in China and is also commonly used in traditional Chinese medicine for ophthalmology cases. Its rich polysaccharide LBP has attracted much attention because of its wide range of biological activities. There have been very few reports on the role of LBP in preventing and/or protecting DR [10-12], and its specific molecular mechanism has not been fully elucidated. Using monkey retinal vascular endothelial cells (RF/6A), a study has found that LBP treatment can alleviate high glucose-induced retinal angiogenesis by promoting the expression of miR-15a-5p and reducing the expression of vascular endothelial growth factor (VEGF) and its receptors VEGFR2 and angiotensin 2 (ANG2) protein [12]. In a diabetic rat model, LBP treatment can completely or partially reverse a series of changes caused by diabetes, including the decrease of electroretinogram (ERG) a-wave, b-wave amplitude and oscillation potential, retinal blood flow, retinal thickness, and the increase in thickness of retinal capillary basement membrane. In addition, LBP can inhibit the messenger RNA (mRNA) and protein expression of diabetes-induced pro-angiogenic factors glial fibrillary acidic protein (GFAP) and VEGF as well as increase the level of pigment epithelium-derived factor (PEDF), an anti-angiogenic factor. Accordingly, it is believed that the protective effect of LBP on retinal function and morphology in diabetic rats may be through the restoration of balance between pro-angiogenic factors and anti-angiogenic factors, thereby reducing the formation of new blood vessels [10]. Another study has also found that LBP treatment in diabetic rats can thicken the entire retina, prevent disorders of photoreceptor discs, and inhibit pathological angiogenesis in diabetes. Decreased expression of P-Occludin and increased expression of RhoA-related protein kinase (ROCK) or phosphorylated myosin light chain (P-MLC) were observed in high glucose-induced RF/6A cells and retinal tissues of diabetic rats, which could be rescued by LBP treatment. It is believed that LBP has a protective effect on the blood-retinal barrier of diabetic rats by regulating the Rho/ROCK signaling pathway [11]. Increased oxidative stress is associated with a common pathological process in many eye diseases, such as glaucoma, ischemic optic neuropathy, and DR. Studies have shown that LBP can protect against cobalt (II) chloride ($CoCl_2$)-induced retinal ganglion cell apoptosis by reducing mitochondrial membrane potential and reactive oxygen species levels [21].

Normal levels of autophagy can effectively maintain the physiological function of RPE, but in the occurrence and development of DR, it is often accompanied by excessive activation of autophagy [6]. The regulation of autophagy is a multi-step and extremely complex process, and the mTOR signaling pathway is an important regulatory link in it. mTOR is a cytoplasmic kinase that plays an important regulatory role in controlling cell growth and metabolism [22]. It is also a key protein in the process of autophagy induction. Pathways that positively regulate mTOR, such as AKT and MAPK signaling pathways, can inhibit autophagy, while pathways that negatively regulate mTOR, such as AMPK and P53 signaling pathways, promote autophagy [23]. The mTOR signaling pathway is involved in the development of retinal diseases. Studies on cell survival have found that mTOR plays an important role in regulating the vitality of a variety of cells that are critical to the initiation and progression of DR, indicating that mTOR is a potential target in the treatment of DR [6]. The present study observed the mechanism of autophagy in relation to the protective effect of LBP, and the results showed that LBP treatment can significantly inhibit the abnormal activation of autophagy in ARPE-19 cells under high-glucose conditions, *i.e.*, increase the expression of autophagy inhibitory protein p-mTOR and reduce intracellular autophagic flux, thus suggesting that the mTOR signaling pathway is involved in RPE cell injury in DR and may be an important target in the treatment of DR using LBP. The results of the present study are similar to those observed in H_2O_2-induced ARPE-19 cell injury, where LBP can improve cell survival, promote cell proliferation, and reduce the abnormally high expression of autophagy proteins [24].

The rate of blindness and morbidity of DR are high, and it is difficult to treat this condition. DR seriously

threatens the health of all mankind, presents a heavy disease burden to society, and affects the mental health of the patients and their families. The present study provides an experimental basis for clarifying the role and mechanism of LBP in protecting RPE under high-glucose conditions and new ideas for using LBP as an intervention measure to treat DR in anticipation of improving its curative effect and the prognosis of DR when combined with existing prevention and treatment methods. LBP, as an extract of *Lycium barbarum* fruit, is abundant and inexpensive. The medicinal value of LBP for DR has been proven, and it has important social significance as it can reduce the economic burden of treatment on patients. However, the present study cannot provide direct evidence that LBP inhibits autophagy and plays a protective role. Therefore, further studies are needed to confirm these hypotheses. In addition, animal models of DR and clinical samples of patients may be used to verify the protective effect of LBP and its relationship with autophagy regulation.

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Disclosure statement

The authors declare no conflict of interest.

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