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EXPERIMENTAL RESEARCH

Effect and mechanism of Buyang Huanwu decoction on retinal inflammation in streptozotocin-induced diabetic rats

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[Abstract] **Objective** To observe the protective effect of Buyang Huanwu decoction (BYHWD) against retinal inflammation in diabetic rats. **Methods** Healthy male Wistar rats were randomly divided into normal and diabetic model groups. Diabetic rats were given a one-time intraperitoneal injection of streptozotocin (STZ) to establish a diabetic phenotype and were randomly assigned to the negative control (saline), positive control (calcium dobesilate), and BYHWD treatment groups. After 8 weeks of treatment, fluorescein-labeled dextran and Evans blue dye were injected into the tail vein to observe vascular permeability and vascular leakage in the retina, respectively. Perfusion of fluorescein-labeled concanavalin was used to detect adhesion in rat retinal leukocytes. Western blotting was used to quantify the expression levels of the inflammatory factors TNF-α, NF-κBp65, and *p*-NF-κBp65 in retinal tissue. **Results** Compared with nondiabetic rats, saline-treated diabetic rats showed significantly increased retinal vascular leakage, leukocyte adhesion, and protein levels of TNF-α and *p*-NF-κBp65 (P<0.05). These effects were significantly reduced in the positive control and BYHWD groups (P<0.05). **Conclusion** Buyang Huanwu decoction can ameliorate retinal inflammation in STZ-induced diabetic rats. Its mechanism of action may be related to the inhibition of TNF-α expression and NF-κB pathway activation.

[Key words] Diabetes; Rats; Retinitis; Buyang Huanwu decoction; Mechanism

1 Introduction

Diabetic retinopathy (DR) is a common and serious complication of diabetes and is a leading cause of vision loss in the working age population. Among patients with diabetes, the incidence of DR is more than 40% five years after disease onset. This incidence gradually increases over time and can exceed 90% beyond 20 years in the course of the disease[1-2].

DR results from microvascular injury, with inflammation occurring in the early stages of DR. Early DR can be difficult to detect due to the lack of visual symptoms and signs of ocular pathological changes in patients. The pathological hallmarks of DR include basement membrane thickening, leukocyte adhesion, increased vascular permeability, blood-retinal barrier (BRB) damage, and capillary pericyte loss. These changes can cause retinal capillary leakage, eventually leading to macular edema and blindness. Recent studies have shown that increased activity of inflammatory

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markers, such as vascular endothelial growth factor (VEGF), intercellular adhesion molecule 1, tumor necrosis factor-α (TNF-α), and nuclear factor-κB (NF-κB), are associated with BRB damage and increased vascular permeability[3-5]. Therefore, intervention at the early stage of diabetes by inhibiting the overexpression of inflammatory markers in the retina can potentially be an effective approach in preventing the development of DR.

Presently, the drugs for DR generally have limitations or several complications. Ranibizumab and bevacizumab may cause intraocular inflammation and rhegmatogenous retinal detachment[6], while intravitreal injection may lead to intraocular hemorrhage, intraocular infection, and retinal detachment[7-9]. Traditional Chinese medicine (TCM) has been recognized for its holistic treatment, excellent safety, and fewer side effects. A viable approach for drug development is to identify a Chinese herbal compound from TCM that can effectively treat DR. Buyang Huanwu decoction (BYHWD) can be used to treat DR clinically but evidence for its mechanism of action is supported by only a few animal studies. Previous studies have confirmed that (Jiawei) BYHWD can increase retinal blood flow, reduce retinal vascular leakage, and inhibit VEGF overexpression in rat diabetes models[10-12]. However, its effectiveness in improving retinal inflammation and its mechanism of action remain unclear. This study investigated the effects and mechanism of BYHWD on retinal inflammation in streptozotocin-induced diabetic rats.

2 Materials and methods

2.1 Experimental animals

Eighty SPF male Wistar rats, with a body mass of 200–220 g, were purchased from Shanghai Slake Experimental Animal Co., Ltd. (License number SCXK 2017-0005). After slit lamp examination, animals without fundal abnormalities were selected. Rats were fed adaptively *libitum* and habituated in an animal facility for three days. Environmental conditions were room temperature of 18–22 ℃, relative humidity of 40%–70%, and a 12-hour light: 12-hour dark cycle.

2.2 Main instruments and reagents

Inverted fluorescence microscope (Nikon, Japan); Microplate reader (Molecular Devices, USA); Cryogenic centrifuge (Thermo, USA); Glucometer (Sannuo Biosensors Co., Ltd., China); XTL-165 Stereomicroscope (Phoenix Optics Group Co., Ltd., China); Lyophilizer (TAITEC, Japan); Electrophoresis apparatus and Membrane rotator (Bio-rad, USA). FITC-Con A (Vector lab, USA); FITC-dextram (Sigma, USA); NF-κBp65 (Cell Signaling Technology, USA); *p*-NF-κBp65 (Cell Signaling Technology, USA); β-actin (Cell Signaling Technology, USA); TNF-α (Abcam, Britain).

2.3 Drugs

Buyang Huanwu Decoction: *Astragalus membranaceus* (Huangqi, 120 g, Lot No.170908, Gansu), *Angelica sinensis* (Danggui, 6 g, Lot No.180201, Gansu), Radix paeoniae rubrathe root of common peony (Chishao, 5 g, Lot No.180202, Anhui), Earthworm (Dilong, 3 g, Lot No.180102, Guangdong), *Ligusticum wallichii* (Chuanxiong, 3 g, Lot No.180201, Sichuan), *Carthamus tinctorious* (Honghua, 3 g, Lot No.170401, Xinjiang), and Peach kernel (Taoren, 3 g, Lot No.180302, Hebei). Identified by Li Wei, Chief Pharmacist of Pharmacy Department of Xiangtan Central Hospital. Components were extracted by boiling and the decoction was concentrated and stored at a low temperature; Calcium dobesilate (Ningxia Kangya Pharmaceutical Co., Ltd., China).

2.4 Grouping and administration

The rats were divided into normal (20 rats) and diabetic model groups (60 rats). Rats were fasted for 10 h and weighed. Animals in the diabetic model group were intraperitoneally injected with streptozotocin (STZ, 55 mg/kg) once, while normal

rats received 0.9% sodium chloride solution of equal volume intraperitoneally. After 72 hours, blood was taken from the tail vein to measure the fasting blood glucose concentration. Blood glucose concentration was≥16.7 mmol/L. After 1 week of observation, induction of diabetes was deemed successful if the blood glucose remained stable. Diabetic rats were randomly assigned to receive the negative control, positive control, and BYHWD, with 20 rats in each group. The BYHWD group (DM+BYHWD) was given the decoction of BYHWD (12.87 g/kg). The dose used was obtained using the formula of equivalent dose ratio to convert the daily dose of clinical administration for gastric perfusion intervention in humans, taking into consideration the body surface area ratio between humans and animals. The positive control group (DM+CaD) was given calcium dobesilate (150 mg/kg) by gavage. Both the normal group (Con) and the negative control diabetic group (DM) were given 0.9% sodium chloride solution of equal volume by gavage. Treatments were given once a day in all groups for eight consecutive weeks before follow-up experiments were performed.

2.5 Observe the retinal vascular permeability of rats

According to the method described in the literature[3], after anesthesia of rats in each group, 250 μL FITC dextran (100 mg/mL) was injected through the tail vein, and the internal circulation was maintained for 10 min. The eyeball was removed and placed in 4% paraformaldehyde. After 15 min of fixation, the retina was separated. The retina was scattered on a colorless glass slide and covered with a glass slide. It was observed under a fluorescent microscope and photographed for preservation.

2.6 Detection of retinal vascular leakage in rats

According to the method described in the literature[3], the rats in each group were weighed and anesthetized, then injected Evans blue dye solution [Evans blue (EB), 30 mg/mL] into the tail vein at 45 mg/kg to maintain the internal circulation for 0.3 h. At 2 min, 15 min, 30 min, 1 h, and 2 h, blood was taken from the tail and stored in crushed ice. After 2 hours, EB was observed to fully diffuse into the skin as it turned blue. After opening the chest cavity, a perfusion needle was inserted into the left ventricle, the right atrial appendage was cut off, and normal saline was injected until the eyeball turned white. The eyeball was removed and the retina was separated. Samples were freeze-dried for 12 hours before weighing. After drying, 300 μL formamide was added, and the mixture was placed in a 70 ℃ thermostatic water bath for 18 h. Samples were centrifuged at 12 000 rpm and 4 ℃ for 15 min. A 100 μL aliquot of the supernatant was collected and placed in a microplate reader to measure the maximum and minimum absorbance values at 620 and 740 nm, respectively. This procedure was repeated three times and the average EB content was calculated. The final result was expressed as EB (μg) /Retina dry weight (g).

2.7 Detection of leukocyte adhesion in rat retina

According to the method described in the literature[3], the rats in each group were weighed and anesthetized. The experimenter then opened the chest cavity, inserted a perfusion needle into the left ventricle, cut off the right atrial appendage, and perfused with PBS buffer (250 mL/kg), and then perfused with 4% paraformaldehyde solution. After the head and neck of the rats were hardened, the rats were perfused with FITC-Con A at a ratio of 5 mg/kg, and then perfused with 1% BSA solution for 2 min. The eyeballs were removed and placed in 4% paraformaldehyde and the retina was separated after 15 min of fixation. The retina was scattered on a colorless glass slide and observed under the fluorescence microscope to count the number of leukocyte adhesion.

2.8 Determination of TNF-α, NF-κBp65 and *p*-NF-κBp65 protein expression in rat retina by western blotting method

The expression levels of TNF-α, NF-κBp65, and *p*-NF-κBp65 protein in rat retina were determined according to the method described in the literature[13]. β-actin was used as an internal reference.

2.9 Statistical analysis

SPSS 16.0 software was used for statistical analysis. Data were expressed as $\bar{x} \pm s$. One-way ANOVA and *t*-test were used for comparison between groups. $P<0.05$ was considered statistically significant.

3 Results

3.1 Effect of BYHWD on retinal vascular leakage in rats

The retinal vascular permeability of rats was qualitatively observed by using FITC dextran, as shown in Fig. 1A–1D. The retinal vessels in the DM group had obvious cotton wool-like exudations (as shown by the arrow), which were absent in the other three groups. The amount of retinal vascular leakage was quantified by EB leakage test, as shown in Fig. 1E. Compared with the normal control group, the level of EB leakage was significantly increased in the DM group ($P<0.001$). Compared with the DM group, retinal vascular leakage in the DM+BYHWD group was significantly reduced ($P<0.001$), with an effect similar to that of the positive control group. These results are consistent with the trend observed in the FITC dextran experiment.

3.2 Effect of BYHWD on the adhesion of leukocytes in retinal vessels of rats

As shown in Fig. 2, there were small, oval, green, bright spots in the retinal vascular

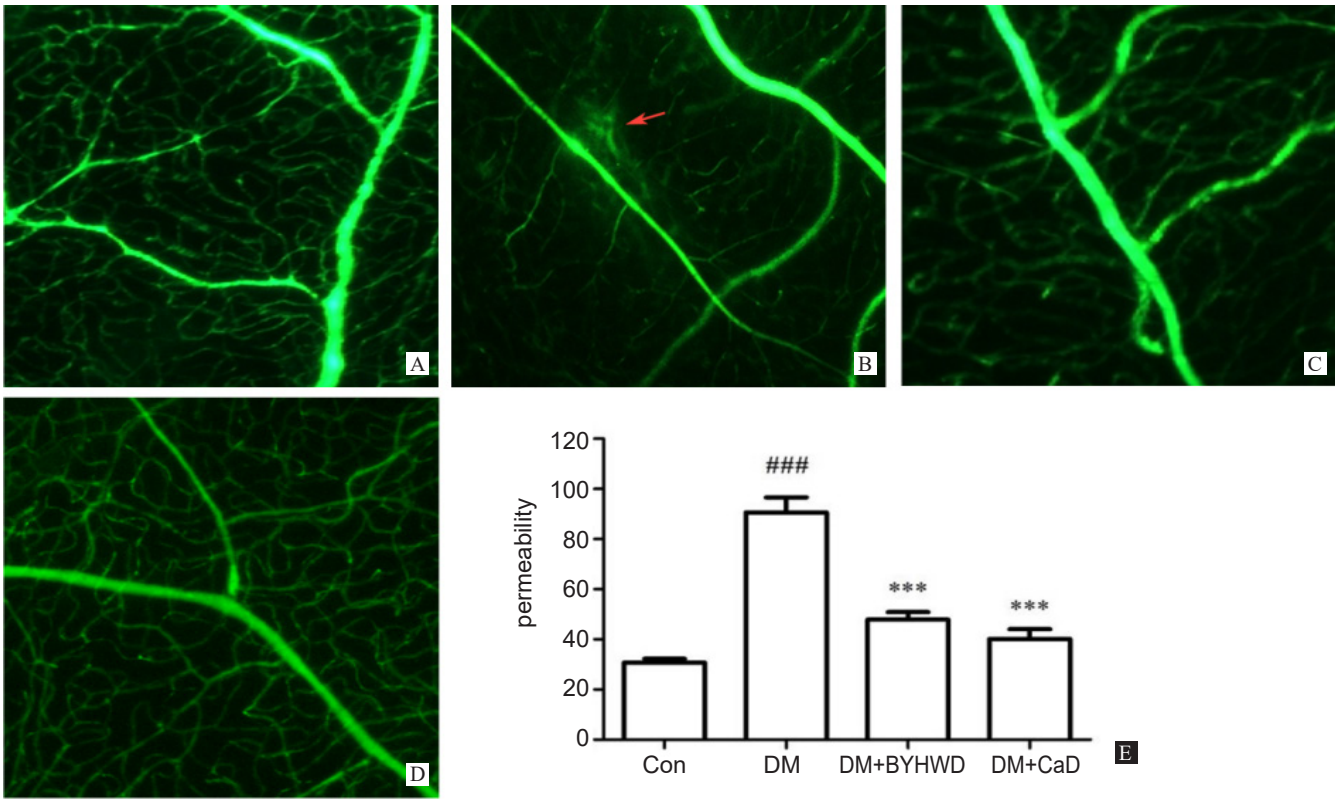

Fig. 1 Effects of BYHWD on retinal vascular leakage in diabetic rats (40×, *n*=8).
A. Con, normal control group; B. DM, saline-treated diabetic model group; C. DM+BYHWD, BYHWD group; D. DM+CaD, positive control group. The arrow shows the exudation; E. Quantitative detection of retinal vascular leakage by Evans blue method. $^{###}P<0.001$, compared with the normal control group; $^{***}P<0.001$, compared with model group.

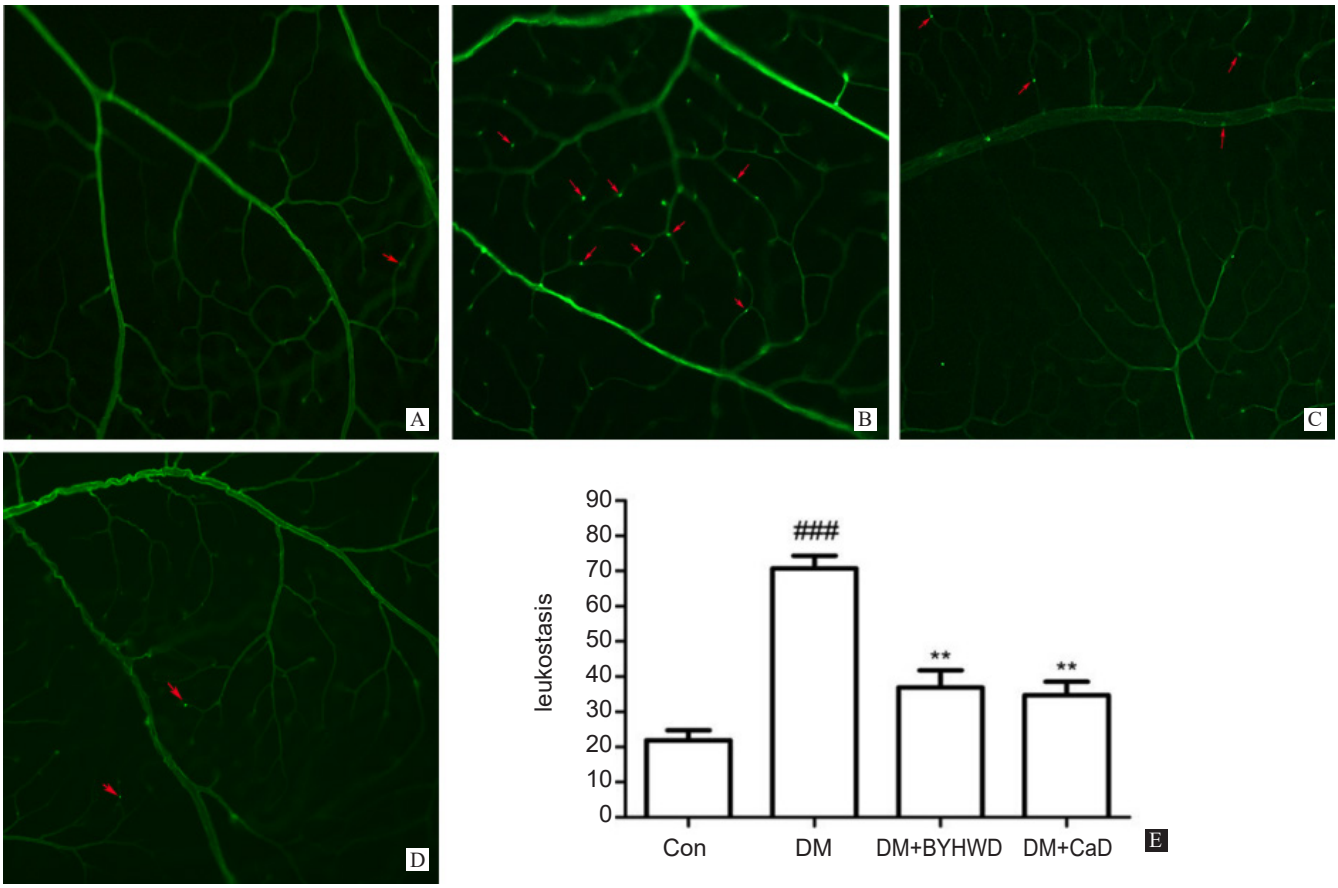

Fig. 2 Effects of BYHWD on diabetic retinal leukostasis in diabetic rats (100×, *n*=6).
A. Con, normal control group; B. DM, diabetic model group; C. DM+BYHWD, BYHWD group; D. DM+CaD, positive control group. Arrows show leukocyte adhesion; E. Number of leukocytes attached to the retina of rats in each group. $^{###}P<0.001$, compared with the normal control group; $^{**}P<0.01$, compared with DM group.

endothelium, which are fluorescein-labeled white blood cells (indicated by the arrow). Compared with the normal control group, the number of adhered white blood cells in the DM groups was significantly increased ($P<0.001$). Compared with the DM group, leukocyte adhesion in the DM+BYHWD group was significantly reduced ($P<0.01$), similar to that in the positive control group.

3.3 Effect of BYHWD on TNF-*α*, NF-*κ*Bp65, and *p*-NF-*κ*Bp65 expression in rat retina

As shown in Fig. 3, there was no significant difference in the expression levels of NF-*κ*Bp65 protein in the retina of rats in each group ($P>0.05$). Compared with normal controls, the expression levels of TNF-*α* and *p*-NF-*κ*Bp65 in the DM group were significantly increased ($P<0.05$). Compared with the DM group, the expression levels of TNF-*α* and *p*-NF-*κ*Bp65 in the DM+BYHWD and DM+CaD groups were decreased significantly ($P<0.01$, $P<0.05$).

4 Discussion

BRB injury is one of the important pathological characteristics of early DR and is closely related to DR pathogenesis. It is mainly due to the decrease in the number of periretinal cells resulting from high glucose levels. This inhibits the contraction of capillaries and thickens the vascular basement membrane, thus affecting the tension in the capillaries. When the blood viscosity increases in diabetic patients, the blood flow velocity becomes slow and turbulent, which increases the risk of thrombus formation that can result in the blockage of capillaries. This leads to retinal capillary stenosis, ischemia, hypoxia, and ultimately, causes

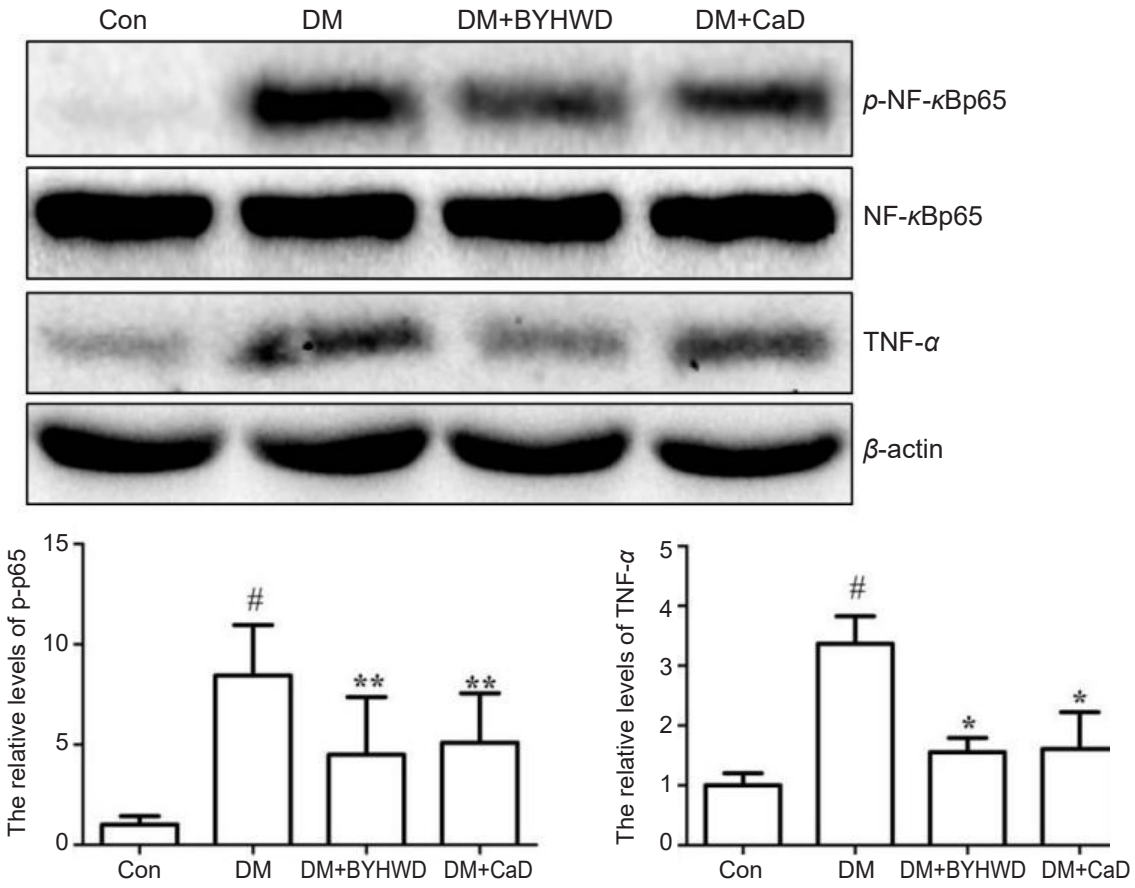

Fig. 3 Effects of BYHWD on the expression levels of TNF-*α*, NF-*κ*Bp65 and *p*-NF-*κ*Bp65 in the retina of diabetic rats (*n*=6). Con, normal control group; DM, diabetic model group; DM+BYHWD, BYHWD group; DM+CaD, positive control group. $^{\#}P<0.05$, compared with the normal control group; $^{**}P<0.01$, $^{*}P<0.05$, compared with DM group.

the destruction of the BRB structure. In addition, retinopathy can cause the activation of white blood cells, which is a hallmark event of the inflammatory response. From the perspective of hemodynamics, under the condition of retinal capillary ischemia and hypoxia, white blood cells will slowly gather and stagnate, and adhere to the endothelial lining of the blood vessels, thus, inducing inflammatory reaction that can damage the structure and function of endothelial cells and cause BRB dysfunction. This can lead to increased vascular permeability and vascular leakage[14-16]. TNF-*α* is an inflammatory cytokine that can be upregulated in the retina of DR patients. As the initiator of the inflammatory response, it participates in many processes, such as inflammatory response and immune response. Its increased expression will aggravate leukocyte adhesion and BRB destruction. Moreover, as a transcription factor, NF-*κ*B participates in a variety of physiological and pathological processes, including inflammation. It usually exists as a dimer. The p65∶p50 pair has been studied more widely. NF-*κ*Bp65 is inactive under normal conditions, but it will induce its transcription when stimulated by bacteria or viruses, thus enhancing the release of inflammatory factors such as TNF-*α*[3,17,18]. Therefore, vascular leakage, leukocyte adhesion, TNF-*α*, and NF-*κ*Bp65 play important roles in the occurrence and development of DR.

DR in TCM belongs to the category of "Long time Xiaoke leads to blood vessel" and "eyeful firefly". Its basic pathogenesis is based on yin deficiency, marked by dryness and heat, blood stasis in collaterals, blood not following meridians, repeated bleeding leading to blindness[19], and usually requires symptomatic treatment. BYHWD

comes from "*YilinGaicuo*," which has the functions of tonifying Qi, activating blood, and unblocking collaterals, and is mainly used for the syndrome of Qi deficiency and blood stasis in apoplexy. This prescription reuses *Astragalus membranaceus* to tonifying middle-Jiao and Qi. It aims to promote blood circulation when Qi is strong, remove blood stasis, and unblock collaterals when blood circulation is strong. At the same time, it promotes water and detumescence, which can accelerate the absorption of exudates from the fundus of the eye, and it is the monarch medicine. *Angelica sinensis* leaves its body and takes its tail to promote blood circulation and remove blood stasis without hurting blood, and it is the ministerial drug. "*Xuere Lun*" said "if the old blood is not removed, the new blood will not be generated and impede circulation of Qi and blood," therefore, Radix paeoniae rubrathe root of common peony and *Ligusticum wallichii* are used to promote blood circulation and remove blood stasis, which means that old blood can be removed and new blood can be generated, so that Qi and blood can run smoothly. Among them, *Ligusticum wallichii* is pungent and warm in nature and can be dispersed. With its upward power, it can lead medicine to the top and act on the eye; Peach kernel and Carthamus tinctorious can promote blood circulation and remove blood stasis better. Earthworm can dredge meridians and activate collaterals. The above drugs can be used as an adjuvant. When all medicines are used together, they can cure both the symptoms and root causes. Moreover, the Qi will be increased, the blood stasis will be eliminated, and the collaterals will be unblocked, and all symptoms will heal[20-21].

By observing the effect of BYHWD on retinal vascular leakage in diabetes rats, it was found that the retina of diabetic rats had cotton wool-like exudation, indicating that the structural integrity of BRB in rats was damaged. In contrast, the retina of the other three groups did not show obvious exudation. In order to determine the effect of DM+BYHWD, further analysis of the EB leakage test showed that the EB leakage of rats in the model group was significantly higher than that of rats in the normal control group. In comparison, the EB leakage of rats in the DM+BYHWD group was significantly lower than that of the model group, and the effect was close to that of the positive control group, which was consistent with the trend of the leukocyte adhesion test. In the western blotting experiment, BYHWD can reverse the increased expression of TNF-α and *p*-NF-κBp65 in the retina of diabetic rats, and the effect is slightly better than that of the positive control group. The above results indicate that diabetes can cause increased retinal vascular leakage and leukocyte adhesion, as well as increased levels of TNF-α and *p*-NF-κBp65 expression. At the same time, BYHWD can control the level of inflammatory factor TNF-α by inhibiting the expression of *p*-NF-κBp65, thus improving the inflammatory performance of retinal vascular leakage and leukocyte adhesion in diabetes rats.

In summary, this study shows that BYHWD reduces STZ-induced retinal inflammation in rats, and its mechanism of action may be related to the inhibition of TNF-α expression and NF-κB pathway activation. However, the precise mechanism of the anti-inflammatory effect of BYHWD in the retina has yet to be elucidated. Future research can explore this question to provide an experimental basis for the clinical use of BYHWD in treating diabetic retinal inflammation.

5 Conflicts of interest

These authors have no conflict of interest to declare.

6 Acknowledgments

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