• Basic Research •

Vascular endothelial growth factor-165b protects the blood-retinal barrier from damage after acute high intraocular pressure in rats

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Abstract

● **AIM:** To elucidate the role of vascular endothelial growth factor-165b (VEGF-165b) in blood-retinal barrier (BRB) injury in the rat acute glaucoma model.

● **METHODS:** In this study, the rat acute high intraocular pressure (HIOP) model was established before and after intravitreous injection of anti-VEGF-165b antibody. The expression of VEGF-165b and zonula occludens-1 (ZO-1) in rat retina was detected by double immunofluorescence staining and Western blotting, and the breakdown of BRB was detected by Evans blue (EB) dye.

● **RESULTS:** The intact retina of rats expressed VEGF-165b and ZO-1 protein, which were mainly located in the retinal ganglion cell layer and the inner nuclear layer and were both co-expressed with vascular endothelial cell markers CD31. After acute HIOP, the expression of VEGF-165b was up-regulated; the expression of ZO-1 was down-regulated at 12h and then recovered at 3d; EB leakage increased, peaking at 12h. After intravitreous injection of anti-VEGF-165b antibody, the expression of VEGF-165b protein was no significantly changed; and the down-regulation of the expression of ZO-1 was more obvious; EB leakage became more serious, peaking at 3d. EB analysis also showed that EB leakage in the peripheral retina was greater than that in the central retina.

● **CONCLUSION:** The endogenous VEGF-165b protein may protect the BRB from acute HIOP by regulating the expression of ZO-1. The differential destruction of BRB after acute HIOP may be related to the selective loss of retinal ganglion cells.

● **KEYWORDS:** vascular endothelial growth factor-165b; blood-retinal barrier; high intraocular pressure; Evans blue; zonula occludens-1

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INTRODUCTION

Glaucoma is the second-leading irreversible blinding eye disease in the world. In recent years, many studies have focused on retinal ganglion cell (RGC) death[1]. The survival of retinal nerve cells is closely related to the microenvironment around them. The blood-retinal barrier (BRB) is essential in maintaining the retinal homeostasis of the microenvironment[2]. Structurally, the BRB is composed of two distinct barriers: the outer blood-retinal barrier (oBRB), consisting of retinal pigment epithelium that regulates transport between the choriocapillaris and the retina, and the inner blood-retinal barrier (iBRB), which regulates transport across retinal capillaries[3]. The local microenvironment of RGCs is mainly protected by iBRB[3]. The iBRB is not an absolute barrier because substances from the blood can cross it by two distinct mechanisms, caveola-mediated transport (transcellular) and paracellular transport[3]. Paracellular transport is strictly dependent on tight junctions (TJs), such as claudins and zonula occludens (ZO); adherens junctions (AJs), such as vascular endothelial cadherin (VE-cadherin); and gap junctions (GJs),

such as connexin 43 (Cx43). Studies have shown that ZO-1 is a marker of BRB integrity, and the loss or reduction of ZO-1 is related to an increase in barrier permeability[3-4].

Many diseases can cause retinal ischemia-hypoxia[5], such as diabetic retinopathy, age-related macular degeneration, retinal detachment, and partial acute glaucoma. The damage to BRB under hypoxia-ischemia conditions is one of the causes of the degeneration of retinal nerve cells[5]. The acute high intraocular pressure (HIOP) animal model is a kind of acute experimental glaucoma model[6]. Previous studies have found that BRB breakdown occurs after acute HIOP in rats[7], and hypoxia-inducible factor-1 alpha (HIF-1α) plays an important role in this process[8-9]. Elevated intraocular pressure can induce up-regulation of HIF-1α protein and its target gene, *vegf-α*[9], but another study found up-regulation of only vascular endothelial growth factor (VEGF)-165b, one subtype of VEGF-A in the same model[10]. VEGF-A is the principal angiogenic factor that increases microvascular permeability during physiological and pathological angiogenesis[11]. VEGF-A is generated by alternative splicing of 8 exons to produce two subtypes, proangiogenic VEGF-165a and anti-angiogenic VEGF-165b[11]. It has been reported that VEGF-165b was cytoprotective and antiangiogenic in the retina[11-12]. However, the role of VEGF-165b in iBRB breakdown after acute HIOP has not yet been clarified. In this study, the rat acute HIOP model was established before and after intravitreous injection of anti-VEGF-165b antibody. Immunohistochemistry, Western blotting and Evans blue (EB) staining were performed to elucidate the role of VEGF-165b in BRB injury in the rat acute glaucoma model.

MATERIALS AND METHODS

Ethical Approval Animal scarification and tissue collection were approved by the Animal Ethics Committee of Hainan Medical University and were performed according to the guidelines of the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985). The SD rats we used in this study strictly followed the ARVO statement for the use of animals in ophthalmic and vision research.

Animals Care and Grouping Adult male SD rats weighing 250-300 g (Changsha Tianqin Biotechnology Co. Ltd., Standard: GB14924.3-2010 Nutritional Composition of Mixed Feed for Laboratory Animals. Changsha, Hunan Province, China) were housed in a standard rat cage with unlimited access to water and food with a 12-hour continuous light supply every 24h. Rats were randomly divided into three groups: a control group, a 12h group and a 3d group. Each group was composed of 6 rats. Previous studies[7] found that BRB injury was more obvious in the early stage (within 24h) and gradually recovered in the late stage (1-7d). Therefore, in this study, the 12h time point was chosen to represent the early stage of injury, and the 3d time point was chosen to represent the late stage of injury. The control group did not have their intraocular pressure raised by inserting needles into the anterior chamber. The rats were sacrificed after 12h and 3d survival following the acute HIOP.

Establishment of Acute High Intraocular Pressure Models The animal model of acute HIOP was established as previously described[7-8,13]. Briefly, animals were anesthetized by intraperitoneal injection of 2% pentobarbital solution (40 mg/kg). A sterile disposable intravenous infusion needle connected to an instillation instrument filled with normal saline was inserted into the anterior chamber. The intraocular pressure of the two eyes was gradually elevated to 120 mm Hg and maintained for 60min, then slowly lowered to the normal level. A drop of levofloxacin eye drops was administered to the conjunctival sac for infection prevention. Rats with cataract or eye inflammation were excluded as unsuccessful models.

VEGF-165b Antibody Administration Previous studies have reported that endogenous retinal VEGF can be antagonized by intravitreal injection of a VEGF inhibitor[14] or antibody[15]. Therefore, Park HY and his team's method of administration[15] were used in this study. Briefly, 3 μL of VEGF-165b antibody (5 mg/mL) was intravitreally injected into the eyes of rats of all three groups. After the rats were anesthetized by intraperitoneal injection of 2% pentobarbital solution (40 mg/kg) and pupils were dilated by compound tropicamide eye drops (Yong guang Pharmaceutical Co., Ltd., Hebei Province, China), a 32-gauge needle connected to Hamilton syringe (Hamilton, 701N, Switzerland) was angled towards the optic nerve, inserted 1.5 mm deep into the vitreous at 2 mm posterior to the superotemporal limbus, and then we injected a single bolus of 3 μL solution under an operating microscope. Levofloxacin eye drops were topically administered. No cataract, vitreous hemorrhage or endophthalmitis related to intravitreal injection were observed after injection.

Retinal Tissue Preparation The retinal tissue preparation was performed as previously described[16]. Briefly, the rats were anesthetized by intraperitoneal injection of 2% pentobarbital solution (40 mg/kg) and sacrificed with excessive dose (80 mg/kg). For immunofluorescence staining, rats were transcardially perfused with normal saline followed by paraformaldehyde (PF) solution. After perfusion, the eyeballs were dissected out. The eyecups were gradually dehydrated and subsequently embedded in Tissue-Tek optimal cutting temperature medium. Using a cryomacrotome (Thermo Electron Corporation, Cheshire, UK), 10-μm-thick cryosections were obtained, and stored at 4°C.

For Western blotting analysis, the rats were anesthetized, the eyeballs were quickly dissected out over ice, the lens and vitreous body were removed, the retina was scraped and placed into a 1.5 mL centrifuge tube, frozen with liquid nitrogen, and preserved at -80°C.

Table 1 The antibodies used in this study

Product	Host	Company	Catalogue number	Dilution
Anti-VEGF 165b antibody[MRVL56/1]	Mouse	Abcam	ab14994	2 μg/mL for WB; 5 μg/mL for IF
Anti-CD31 antibody[EPR17260-263]	Rabbit	Abcam	ab222783	1:100 IF
Anti-CD31 antibody[P2B1]	Mouse	Abcam	Ab24590	2 μg/mL for IF
Anti-NeuN antibody	Rabbit	Abcam	ab104225	1:500 IF
Anti-ZO-1 antibody	Rabbit	Abcam	ab96587	1:1000 WB; 1:500 IF
Alexa Fluor 488 anti-rabbit IgG(H+L)	Donkey	Jackson	711-545-152	1:200 IF
Alexa Fluor® 594 anti-mouseIgG(H+L)	Donkey	Jackson	715-585-150	1:200 IF
Alexa Fluor® 488 anti-mouse IgG(H+L)	Donkey	Jackson	715-545-150	1:200 IF
Alexa Fluor® 594 anti-rabbit IgG(H+L)	Donkey	Jackson	711-585-152	1:200 IF
Anti-β-actin loading control	Rabbit	Abcam	Ab1801	1:2000 WB
HRP-conjugated anti-mouse IgG(H+L)	Goat	Abcam	Ab205719	1:10000 WB
HRP-conjugated anti-rabbit IgG(H+L)	Goat	Abcam	Ab205718	1:10000 WB

WB: Western blotting; IF: Immunofluorescence.

For EB test, the rats were anesthetized 2h before execution. After that, 3% EB (45 mg/kg, E2129, Sigma-Aldrich, CA, USA) was injected into the great saphenous vein within 2min, the eyes and toes of the animals turned blue rapidly, indicating that the dye was evenly distributed. The rats were sacrificed and transcardially perfused with PF. After that, the eyeball was dissected out immediately, the retina was scraped with a glass curved needle, and the whole retina was mounted onto the slide in darkness for photograph with confocal microscope. For EB quantitative detection, the eyeball was dissected out immediately, and the retina was taken out. With the optic nerve papilla as the center, the retina was cut into four quadrants, laid flat on the glass plate, and cut at the halfway point from the optic nerve papilla; the outer part of the retina is the peripheral part retina, and the inner part is the central retina. Retinas were dried at room temperature and weighed accurately, then put into 1.5 mL centrifuge tubes for later use.

Acquisition of Retinal Fluorescence Images on the Whole Mounted Retinal Slices After sealing with anti-fade mounting medium (E675011, Sangon Biotech, China) under dark conditions, EB red fluorescent spots in the retina were observed by confocal microscopy (Olympus FV1000, Japan) at an excitation wavelength of 654 nm, and the central and peripheral parts of the retina were photographed. The relative fluorescence intensity of each group was analysed by Image J software (National Institutes of Health, Maryland, USA) and normalized to the control group.

Quantitative Detection of Blood-Retinal Barrier Breakdown with EB The EB quantitative method was performed as previously reported[7, 17] with modification. Briefly, 1 μL of EB (2%) was diluted 1000-fold in 1 mL formamide (F9037, Sigma-Aldrich, CA, USA) to a concentration of 20 ng/mL, then semi diluted 7 times in turn, and a total of 8 standard tubes, including a formamide blank tube, were used to prepare a standard curve of EB in formamide. Each 1.5 mL centrifuge tube with dry retina was incubated with 160 μL formamide in a constant-temperature chamber at 60℃ for 24h. Then the extract was centrifuged at 4℃ and 16 100 g for 30min. The supernatant was taken (150 μL) and divided into three sample tubes (50 μL/tube). The optical density of each standard tube and sample tube was measured using a bio-spectrophotometer (Eppendorf, Germany) at 620 nm. The concentration of dye was calculated according to the standard curve of EB in formamide. EB (ng) content was standardized to retinal dry weight (mg), expressed as ng/mg.

Immunofluorescence Staining Retina sections were blocked with 5% donkey serum and incubated with the primary antibodies. Subsequently, in a dark chamber, the sections were incubated with the secondary antibodies. Dual labeling of VEGF-165b/CD31 (vascular endothelial cell marker), VEGF-165b/NeuN (retinal ganglion cell marker) and ZO-1/CD31 were performed in sections from the control animal respectively. The antibodies used in this study are shown in Table 1. Retinal sections were observed by confocal microscopy (Olympus FV1000, Japan).

Western Blotting Assay Rat retinas were homogenized and subjected to protein quantification with a Pierce BCA reagent kit (Thermo Fisher Scientific, 23227). Then, 50 μg of the protein lysate was loaded onto a 10% SDS-PAGE gel for electrophoresis and subsequently transferred to a nitrocellulose (NC, PALL 66485) membrane. After blocking, the membrane was incubated with the primary antibody (Table 1). Thereafter, the membrane was washed three times and incubated with an HRP-conjugated secondary antibody. Membranes developed

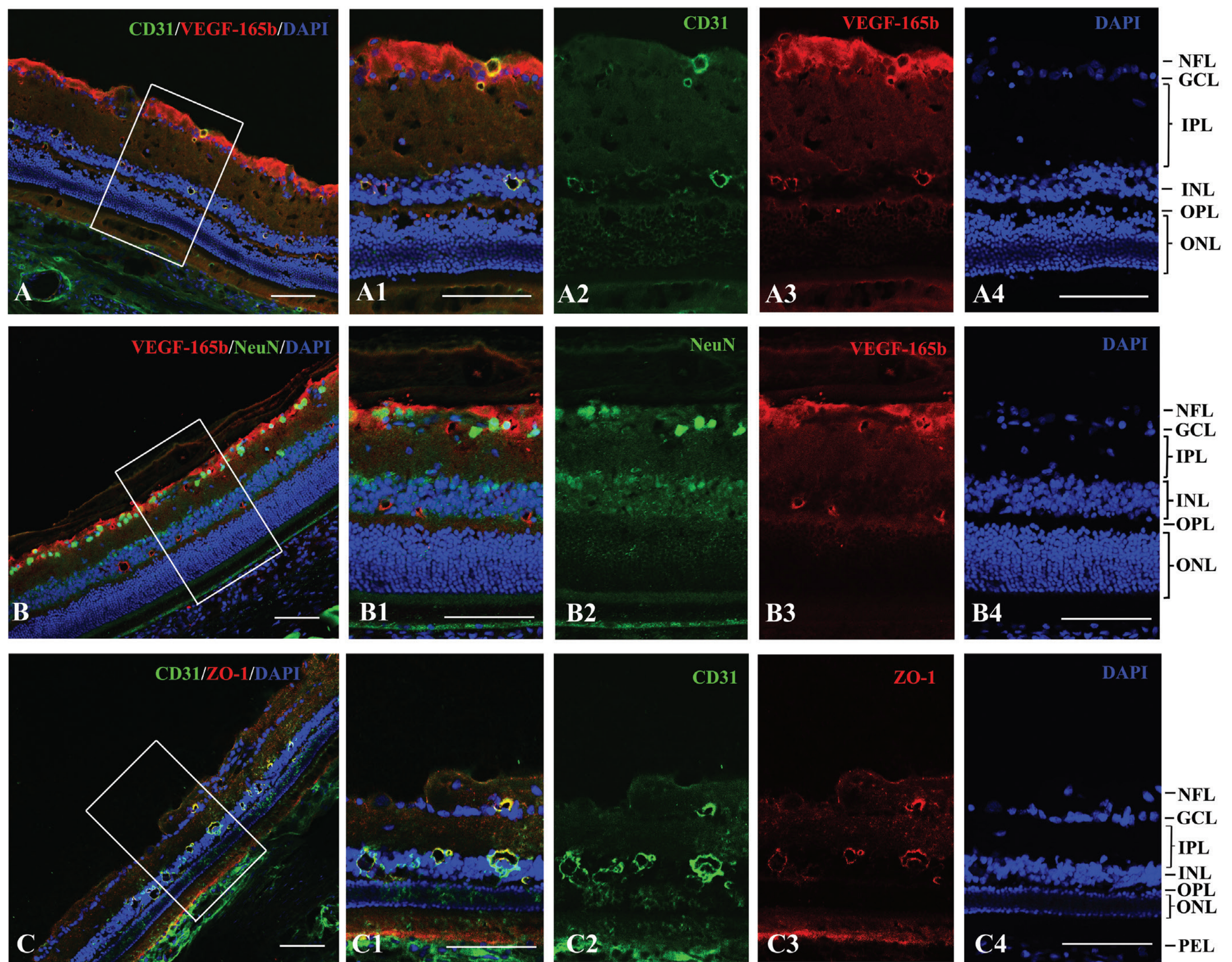

Figure 1 The localization of VEGF-165b in control retina as detected by immunofluorescence staining A: Double immunofluorescence staining was used to detect the expression of VEGF-165b (red) and CD31 (green) in retina. A1 is an enlarged picture of the box in image A, and it is also the merged graph of A2-A4 images. Vascular endothelial cells were labeled with CD31 (green), and the nucleus was labeled with DAPI (blue). B: Double immunofluorescence staining of VEGF-165b and NeuN in retina. B1 is an enlarged picture of the box in image B, and it is also the merged graph of B2-B4 images. Retinal ganglion cells were labeled with NeuN (green), and the nucleus was labeled with DAPI (blue). C: Double immunofluorescence staining of ZO-1 (red) and CD31 (green) in retina. C1 is an enlarged picture of the box in image C, and it is also the merged graph of C2-C4 images. Vascular endothelial cells were labeled with CD31 (green), and the nucleus was labeled with DAPI (blue). NFL: Nerve fiber layer; GCL: Ganglion cell layer; IPL: Inner retinal layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; PEL: Pigment epithelium layer. Bar=50 μm.

by incubation with β-actin were used as controls. Protein was visualized using the Pierce ECL reagent kit (Thermo Fisher Scientific, 32132). Quantitative analysis of proteins was carried out on the protein bands with Image J (National Institutes of Health) and Microsoft Excel (Microsoft Corp., USA). The amounts of VEGF-165b and ZO-1 protein were normalized to β-actin.

Statistical Analysis The obtained values are expressed as means and standard errors. Statistical analysis was performed with SPSS18.0 software (Statistical Product and Service Solutions18.0, Al Monk, New York, USA). Paired *t*-tests were used for comparisons between paired data. All the other data were analyzed using one-way ANOVA. n=6, P<0.05 was considered statistically significant.

RESULTS

Localization of Endogenous VEGF-165b and ZO-1 Protein Expression Detected by Double Immunofluorescence Staining Double immunofluorescence staining of VEGF-165b/CD31, VEGF-165b/NeuN, and ZO-1/CD31 in rat retinal sections showed that VEGF-165b protein was mainly expressed on the RGC layer, the nerve fiber layer and the inner nuclear layer and was co-expressed with CD31 protein, but only co-expressed with NeuN protein partly (Figure 1). The results also showed that ZO-1 protein was mainly expressed

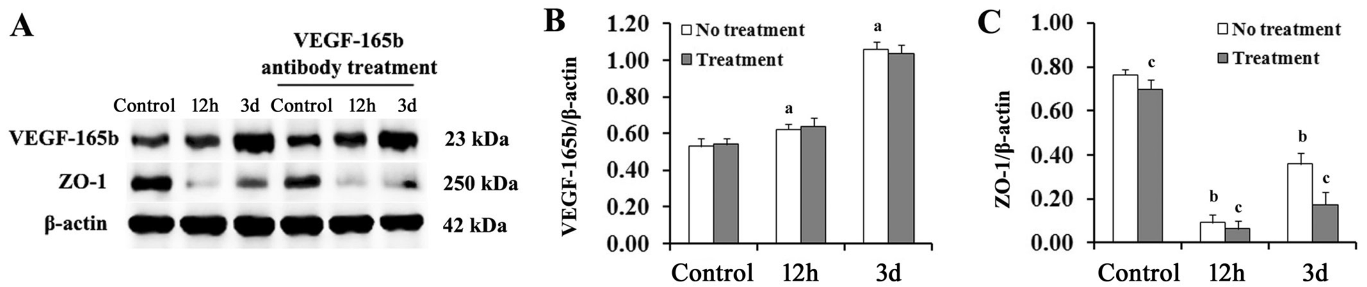

Figure 2 Changes in VEGF-165b and ZO-1 in retina analysed by Western blotting A: VEGF-165b and ZO-1 expression in retina before and after anti-VEGF-165b antibody treatment; B: The changes of VEGF-165b normalized to β-actin. *n*=6. [a]Compared to control, *P*<0.05; C: The changes of ZO-1 normalized to β-actin. *n*=6. [b]Compared to control, *P*<0.01; [c]Compared to the untreated groups, *P*<0.001.

on the RGC layer, the inner nuclear layer and the pigment epithelium layer and was co-expressed with CD31 protein (Figure 1).

Changes of VEGF-165b and ZO-1 in Rat Retina with Acute High Intraocular Pressure Detected by Western Blotting Before vitreous injection of anti-VEGF-165b antibody, the expression of VEGF-165b/β-actin in the control group, 12h group and 3d group was 0.530±0.042, 0.621±0.032, and 1.062±0.038, respectively. Compared with the control group, the expression of VEGF-165b protein in the 12h group and the 3d group was upregulated (*n*=6, *P*<0.05). The expression of ZO-1/β-actin in the control group, 12h group and 3d group was 0.764±0.025, 0.091±0.037, and 0.360±0.048, respectively. Compared with the control group, the expression of ZO-1 protein in the 12h group decreased significantly (*n*=6, *P*<0.01); the expression of ZO-1 protein in the 3d group recovered gradually but was still lower than that in the control group (*n*=6, *P*<0.01). After vitreous injection of anti-VEGF-165b antibody, the expression of VEGF-165b/β-actin in the control group, 12h group and 3d group was 0.541±0.034, 0.640±0.045, and 1.037±0.047, respectively. Compared with the corresponding groups of nontreatment, the expression of VEGF-165b protein was no significant changed (*n*=6, *P*>0.05). ZO-1/β-actin in the control group, 12h group and 3d group was 0.699±0.044, 0.063±0.035, and 0.174±0.057, respectively. Compared with the same group before treatment, the expression of ZO-1 protein was significantly downregulated (*n*=6, *P*<0.001; Figure 2).

Blood-Retinal Barrier Leakage in Rats Detected by EB Staining After EB was injected into the great saphenous vein of normal rats, the image taken by confocal microscopy of whole mounted retinal slices showed that the main trunk and branches of retinal blood vessels were clear, and there were no red EB leakage spots outside the vessels (Figure 3A2). No vascular distribution or red EB leakage spots were observed in the retina after PF perfusion (Figure 3A3). We used this method to detect the leakage of BRB before and after treatment in rats of each group. The results showed that before vitreous injection of anti-VEGF-165b antibody, no red EB leakage spots were found in the central or peripheral retina of the control group, and red EB leakage spots could be seen in the central and peripheral retina of the 12h and 3d groups. The EB relative fluorescence intensity of the peripheral retina in the 12h group and the 3d group was stronger than that in the central retina (*n*=6, *P*<0.05), and that in the 12h group and 3d group was stronger than that in the control group (*n*=6, *P*<0.001; Figure 3B1-B3, 3C1-C3). After vitreous injection of anti-VEGF-165b antibody, there were no red EB leakage spots in the central or peripheral retina of the control group. Many red EB leakage spots could be seen in the central and peripheral retina of the 12h group. Compared with the 12h group before treatment, the relative fluorescence intensity was stronger (*n*=6, *P*<0.01; Figure 3D). There were many red EB leakage plaques in the central and peripheral part of the retina in the 3d group and more and larger red EB leakage plaques in the peripheral part. Compared with the 3d group before treatment, the relative fluorescence intensity of EB in the 3d group was stronger (*n*=6, *P*<0.01; Figures 3B4-B6, 3C4-C6, 3D).

EB leakage of BRB was measured quantitatively before and after treatment. The results showed that before vitreous injection of anti-VEGF-165b antibody, the EB leakage in the central retina of the control group, 12h group and 3d group was 10.85±4.13, 67.44±7.12, and 27.38±4.46 ng/mg, respectively; the EB leakage in the peripheral retina of the control group, 12h group and 3d group was 12.29±2.89, 71.23±5.28, and 35.89±6.67 ng/mg, respectively. Compared with the control group, EB leakage increased significantly in the 12h group and the 3d group (*n*=6, *P*<0.001). After vitreous injection of anti-VEGF-165b antibody, the EB leakage in the central retina of the control group, 12h group and 3d group was 11.88±2.92, 83.76±4.63, and 67.55±8.21 ng/mg, respectively; the EB leakage in the peripheral retina of the control group, 12h group and 3d group was 13.52±2.35, 91.33±3.57, and 123.46±4.91 ng/mg, respectively. Compared with the corresponding group before injection, the EB leakage of the 12h and 3d groups increased significantly (*n*=6, *P*<0.01); the EB leakage of the peripheral

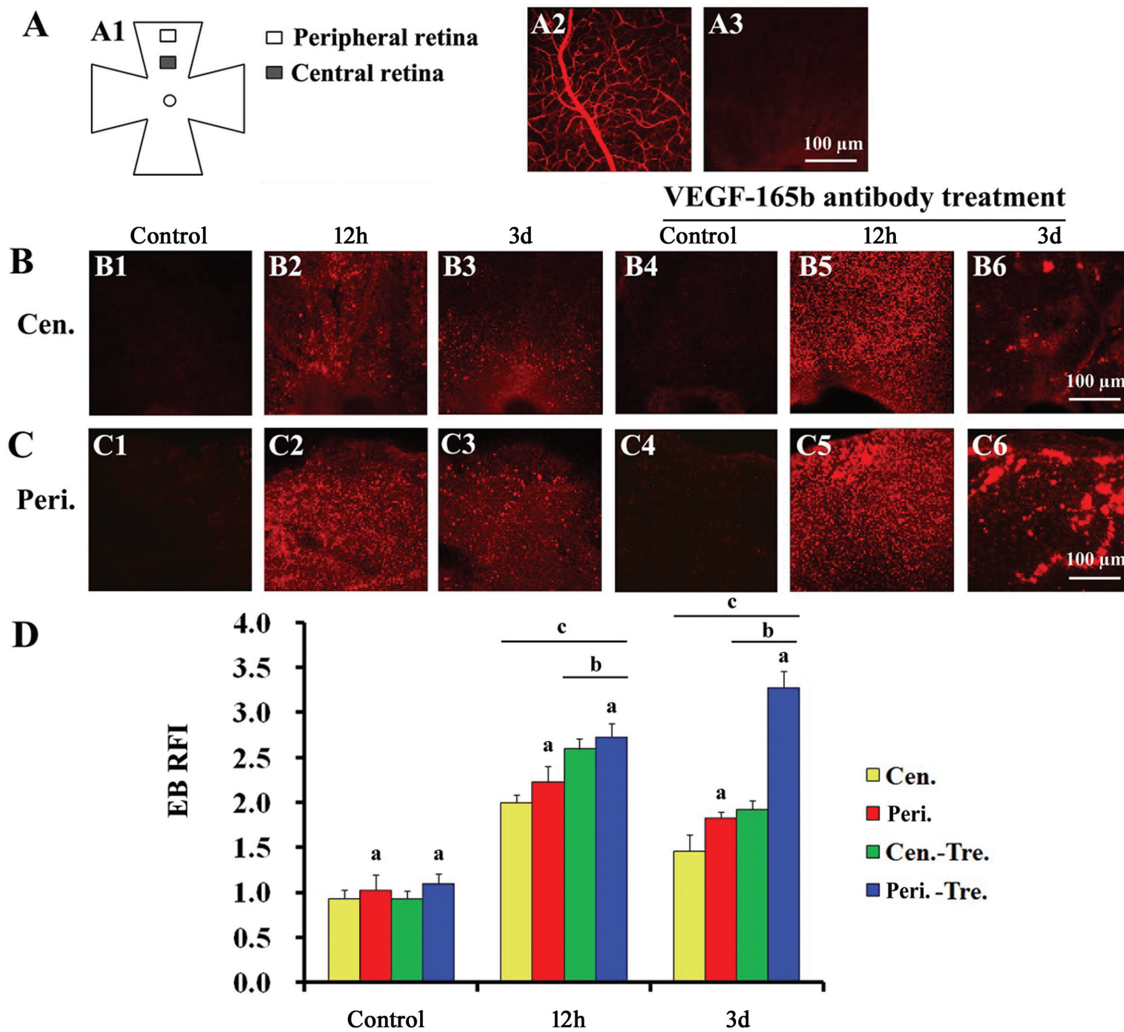

Figure 3 Detection of blood-retinal barrier leakage by EB dye in whole-mount retina A1: Diagram of whole-mount retina. The white box shows the central retina, and the black box shows the peripheral retina. A2: EB shows normal retinal vessels. A3: EB shows normal retinal vessels after perfusion. B: EB leakage in central retina before and after treatment. B1-B3 are the central retina of the control, 12h and 3d groups, respectively. B4, B5, and B6 are the central retina with treatment in the control, 12h and 3d groups, respectively. C: EB leakage in the peripheral retina before and after treatment. C1, C2 and C3 are the peripheral retina of the control, 12h and 3d groups, respectively. C4, C5, and C6 are the peripheral retina with treatment in the control, 12h, and 3d groups, respectively. D: Analysis of the relative fluorescence intensity of EB in retina of each group before and after intravenous injection of anti-VEGF-165b antibody. *n*=6, [c]Compared with the corresponding parts of the control group, *P*<0.001; [b]Compared with the corresponding parts of the same group before treatment, *P*<0.01; [a]Compared with the central retina of the same group, *P*<0.05. Cen.: Central retina; Peri.: Peripheral retina; Tre.: Retinal with treatment; RFI: Relative fluorescence intensity. Bar=100 μm.

retina in the 12h and 3d groups was greater than that in the central retina before and after intravitreous injection (*n*=6, *P*<0.05; Figure 4).

DISCUSSION

The retina of rats expressed the protein of VEGF-165b, which was mainly localized in the retinal vascular endothelial cells but only localized in some RGCs. The tight junction protein ZO-1 was also expressed in vascular endothelial cells of RGC layer and inner nuclear layer. After acute HIOP, the expression of VEGF-165b was up-regulated, the expression of ZO-1 was down-regulated and the EB leakage increased. After treatment, there was no change in the expression of VEGF-165b protein, the down-regulation of ZO-1 was more obvious, and the EB leakage became more serious. EB assays also showed that EB leakage in the peripheral retina was greater than that in the central retina.

VEGF-A was one of the downstream molecules of HIF-1α[9], and it has two isoforms: pro-angiogenic VEGF-(xxx)a and anti-angiogenic VEGF-(xxx)b. The VEGF-(xxx)b protein was expressed in rat experimental glaucoma model but the VEGF-(xxx)a protein was not expressed[10]. So, in this study, the role of VEGF-165b rather than HIF-1α was investigated in BRB destruction of rats with acute HIOP. Because in our previous study[8], we observed the role of HIF-1α in the BRB injury after acute HIOP in rats, and found that the up-regulation of HIF-1α promoted the BRB break down.

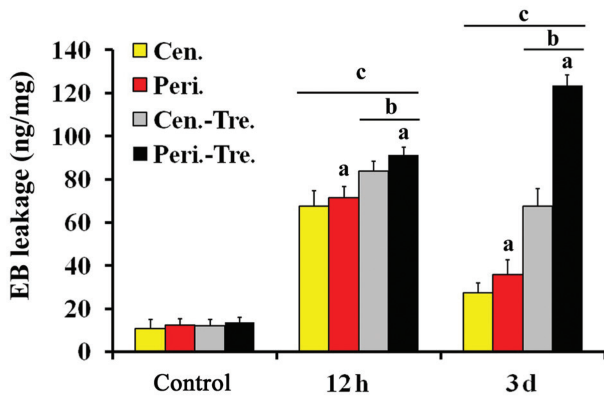

Figure 4 Quantitative detection of BRB leakage in rats by EB The longitudinal axis indicates EB leakage (ng EB/mg retina); the transverse axis indicates the experimental groups. *n*=6, [c]Compared with the corresponding parts of the control group, $P<0.001$; [b]Compared with the corresponding parts of the same group before treatment, $P<0.01$; [a]Compared with the central retina of the same group, $P<0.05$. Cen.: Central retina; Peri.: Peripheral retina; Cen.-Tre.: Central retina with treatment; Peri.-Tre.: Peripheral retina with treatment.

The expression of VEGF-165b protein was up-regulated in the retina of rats with acute HIOP, which was consistent with Ergorul *et al*[18] and his team's report. The expression of VEGF in diabetic retinopathy[19-20] and senile macular degeneration[21] is positively correlated with the destruction of BRB; it may be that a subtype of VEGF (VEGF-164a) that promotes angiogenesis plays a major role. However, recent studies[12,22-23] have shown that VEGF-165b has a protective role against retinal cell damage and BRB breakdown in the retina of an oxygen-induced retinopathy mouse model or in the diabetic retina. Our results showed that BRB breakdown in rats caused by acute HIOP was not alleviated after treatment but was more serious, as seen by the localization of endogenous VEGF-165b protein in retinal vascular endothelial cells, the down-regulation of ZO-1 and increase of EB leakage, suggesting that the endogenous VEGF-165b protein may maintain the homeostasis of the local microenvironment around the retinal nerve cells by protecting BRB from breakdown, thus protecting the retinal nerve cells from injury.

The inner retinal vascular system mainly consists of shallow and deep capillary networks. The shallow capillary networks are mainly distributed in the nerve fiber layer and ganglion cell layer, while the deep capillary networks are distributed around the inner nuclear layer[24]. The blood supply of the inner retina comes from the shallow capillary network and the deep capillary network, whose microenvironment is mainly regulated by iBRB[3]. BRB damage is associated with the destruction of tight junction proteins between capillary endothelial cells[25]. Studies have shown[3-4] that the loss or reduction of ZO-1 is related to an increase of BRB permeability. Our immunofluorescence staining showed that tight junction protein ZO-1 was mainly expressed in vascular endothelial cells (co-expressed with CD31) of RGC layer and inner nuclear layer, and it was also expressed in pigment epithelial cell layer. The expression of ZO-1 in retinal pigment epithelial cells may be related to the maintenance of the function of the outer BRB. Therefore, the changes of ZO-1 in the retina after acute HIOP can reflect the breakdown of BRB. We found that the decrease in ZO-1 protein after acute HIOP was negatively correlated with the leakage of BRB, which was consistent with previous studies[3-4]. After treatment, the decrease in ZO-1 protein was more obvious, indicating that BRB integrity was damaged more seriously after endogenous VEGF-165b was antagonized, suggesting that endogenous VEGF-165b has a protective effect on retinal endothelial cells, which is consistent with the results of Magnussen *et al*[12] and his team. Our results showed that endogenous VEGF protein and ZO-1 protein were localized in vascular endothelial cells (both co-expressed with CD31). These results also suggest that VEGF-165b may protect the BRB from acute HIOP by regulating the expression of ZO-1.

Our EB method for quantitative detection of BRB breakdown is not exactly the same as that reported by Xu *et al*[17]. After EB was injected into the caudal vein of normal rats, confocal images were taken from whole-retinal mounted slices. Retinal blood vessels and their branches were clearly visible. No red EB spots were found in the extravascular retinal space (Figure 3A2). After perfusion, no red fluorescent spots were found in the retina (Figure 3A3). If red EB spots or plaques appear in the retina after perfusion, it is the EB that leaks into the retinal tissue space, indicating that BRB has been damaged or broken down. The results observed by this method and the analysis of the relative fluorescence intensity of EB (Figure 3D) are basically consistent with the results of EB quantitative detection (Figure 4). This method is simple and effective; the difference in relative fluorescence intensity can also reflect the degree of BRB breakdown. After treatment, in addition to the red spots of EB in the retina, red plaques of EB were observed in the peripheral retina of the 12h group and the retina of the 3d group. The most red EB plaques were observed in the peripheral retina of the 3d group. One possible reason is that more EB leaked into the retinal space. After losing the protective effect of VEGF-165b, the more serious breakdown of BRB, retinal edema, and tissue gap loosening were more conducive to EB aggregation into plaques. The EB quantitative results (Figure 4) showed that most EB leakage was found in the peripheral retina of the 3d group, which also confirmed this hypothesis. The inner retina is highly sensitive to hypoxic stress[26]. Kaur *et al*[27] and his team found that iBRB is very sensitive to hypoxic-ischemic injury under the conditions

of ischemia-hypoxia, which shows that the response of the inner retina and iBRB to hypoxia-ischemia is isotropic. We observed that EB leakage in the peripheral retina was more severe than that in the central retina after acute HIOP before and after treatment, showing that the breakdown of BRB had a regional difference. Tong *et al*[28] and his team reported that there were site differences in retinal blood supply after acute HIOP, which was associated with selective retinal ganglion cell death, but this only partially explained the difference in retinal ganglion cell vulnerability. Therefore, we speculate that the characteristics of BRB injury may be related to the selective loss of ganglion cells under acute HIOP.

In summary, we found that the expression of retinal VEGF-165b was continuously up-regulated after acute HIOP, and the damage to BRB was severe in the early stage and recovered in the later stage. After inhibiting the endogenous VEGF-165b protein, the expression of VEGF-165b protein was not affected, but the expression of ZO-1 was down-regulated more obviously in the later stage, and the damage to BRB was more serious, indicating that anti-VEGF-165b treatment can make vascular injury more severe in acute HIOP model, it suggests that VEGF-165b has a protective role against BRB injury induced by ischemia-reperfusion; it may protect retinal nerve cells from ischemia-hypoxia injury by regulating the expression of ZO-1. The differential destruction of BRB after acute HIOP may be related to the selective loss of RGCs. We have to admit that there are some deficiencies in this experimental design. Bates *et al*[29] considered that the study of VEGF-165b isoform detection must have a positive control. Controls are always essential when studying protein isoforms that are highly similar to all other isoforms from the same protein. This study still lacks *in vitro* experimental evidence, such as coculture of retinal endothelial cells, pericytes and astrocytes[30], to study the protective effect of VEGF-165b on BRB. The specific molecular mechanism through which vascular endothelial cells maintain BRB integrity *via* VEGF-165b remains to be further studied.

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