

Role of vascular endothelial growth factor-165b in the breakdown of the blood-retinal barrier after acute high intraocular pressure in rats

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Research article

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Abstract

Background: The blood-retinal barrier (BRB) is essential in maintaining the retinal homeostasis of the microenvironment, previous studies have found that BRB breakdown occurs after acute high intraocular pressure (HIOP) in rats, elevated intraocular pressure can induce upregulation of vascular endothelial growth factor-165b (VEGF-165b) protein in the retina, but the role of VEGF-A165b in BRB breakdown after acute HIOP is still undetermined.

Methods: In this study, the rat acute HIOP model was established before and after intravitreal injection of anti-VEGF-165b antibody. The expression of VEGF-165b and ZO-1 in rat retina was detected by immunohistochemistry or western blotting, and the breakdown of BRB was detected by Evans blue (EB) dye.

Results: The normal retina of rats expressed VEGF-165b protein, which was mainly located in the retinal ganglion cell (RGC) layer and the inner nuclear layer and was coexpressed with tight junction protein ZO-1. After acute HIOP, the expression of VEGF-165b was upregulated ($P < 0.01$); The expression of ZO-1 was downregulated ($P < 0.01$) at 12 h and then recovered at 3 d; EB leakage increased, peaking at 12 h ($P < 0.01$). After intravitreal injection of anti-VEGF-165b antibody, the expression of VEGF-165b protein was significantly downregulated ($P < 0.01$); and the downregulation of the expression of ZO-1 was more obvious ($P < 0.01$); EB leakage became more serious, peaking at 3 d ($P < 0.01$). EB analysis also showed that EB leakage in the peripheral retina was greater than that in the central retina ($P < 0.01$).

Conclusions: 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 RGCs.

Background

Glaucoma is the second-leading irreversible blinding eye disease in the world. In recent years, many studies have focused on retinal ganglion cell 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 BRB (oBRB), consisting of retinal pigment epithelium that regulates transport between the choriocapillaris and the retina, and the inner BRB (iBRB), which regulates transport across retinal capillaries [3]. The local microenvironment of retinal ganglion cells (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), and the maintenance of physiological retinal cell structure requires complicated cell-to-cell interactions. These interactions occur at special contact sites called cell junctions, which include 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, 6], 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 [7]. Previous studies have found that BRB breakdown occurs after acute HIOP in rats [8], and hypoxia-inducible factor-1 alpha (HIF-1 α) plays an important role in this process [9, 10]. HIF-1 α is an upstream transcription factor of the expression of VEGF [11]. Elevated intraocular pressure can induce upregulation of HIF-1 α protein and its target gene, *vegf-a* [10], but another study found upregulation of only VEGF-A165b, one subtype of VEGF-A in the same model [12]. VEGF-A is the principal angiogenic factor that increases microvascular permeability during physiological and pathological angiogenesis [13, 14]. VEGF is generated by alternative splicing of 8 exons to produce two subtypes, proangiogenic VEGF-165a, formed by proximal splicing in exon 8 (exon 8a), and anti-angiogenic VEGF-165b, generated by distal splicing in exon 8 (exon 8b) [13]. It has been reported that VEGF-165b was cytoprotective and antiangiogenic in the retina [15]. However, the role of VEGF-A165b in iBRB breakdown after acute HIOP has not yet been clarified. In this study, the rat acute HIOP model was established before and after intravitreal injection of anti-VEGF-165b antibody. The expression of VEGF-165b and ZO-1 in rat retina was detected by immunohistochemistry or western blotting, and the breakdown of BRB was detected by Evans blue (EB) staining. Data collection and analysis were performed to elucidate the role of VEGF-165b in BRB injury in the rat acute glaucoma model.

Methods

1. Animals

Adult male SD rats (250-300 g body weight) were purchased from the experimental animal department, Hunan Agricultural University (Changsha, Hunan, China) and housed in a standard rat cage with unlimited access to water and food with a 12-hour continuous light supply every 24 hours. Rats were randomly divided into a normal group and an experimental group. The experimental group consisted of a control group, a 12 h group and a 3 d group. Previous studies[8] found that BRB injury was more obvious in the early stage (within 24 hours) and gradually recovered in the late stage (1-7 days). Therefore, in this study, the 12 h time point was chosen to represent the early stage of injury, and the 3 d time point was chosen to represent the late stage of injury. Normal rats were not given any treatment. The control group did not have their intraocular pressure raised by inserting needles into the anterior chamber. The rats in the 12 h group and the 3 d group survived 12 hours and 3 days after establishing the acute HIOP model.

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).

2. Establishment of acute HIOP models

The animal model of acute HIOP was established as previously described [8, 9, 16, 17]. 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 mmHg and maintained for 60 min, then slowly lowered to the normal level. A drop of levofloxacin eye drops was administered to the conjunctival sac for infection prevention. For the sham surgery group as a control, the needle was inserted into the anterior chamber without elevating the pressure. Rats with cataract or eye inflammation were excluded as unsuccessful models. Each group was composed of 6 rats.

3. VEGF-A165b antibody administration

Previous studies have reported that endogenous retinal VEGF can be antagonized by intravitreal injection of a VEGF inhibitor [18] or antibody [19]. Therefore, Park HY and his team's method of administration [19] were used in this study. Briefly, 3 μ l of VEGF-A165b 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, a 32-gauge microinjector needle (Hamilton, Reno, NV, USA) 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 μ l solution into the vitreous under an operating microscope. Injections were given slowly over 30 s to allow diffusion of the liquid, and then levofloxacin eye drops were topically administered on the eyes three times per day after intravitreal injection. No cataract, vitreous hemorrhage or endophthalmitis related to intravitreal injection were observed after injection.

4. Retinal tissue preparation

The retinal tissue preparation was performed as previously described [20]. Briefly, the rats were sacrificed with excessive 2% pentobarbital solution intravitreal injection (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), 15- μ m-thick cryosections were obtained, and stored at 4°C.

The preparation of retinal tissue for western blotting is summarized as follows: after anesthetizing rats with excessive 2% pentobarbital solution (80 mg/kg), the eyeballs were quickly dissected out over ice, the lens and vitreous body were removed, the retina was scraped and placed into the EP tube, and it was frozen with liquid nitrogen. Then the retina was kept at - 80 °C.

The rats were anesthetized by intraperitoneal injection of 2% pentobarbital solution (40 mg/kg) 2 hours before execution. After that, 3% Evans blue (45 mg/kg, E2129, Sigma-Aldrich, CA, USA) was injected into

the great saphenous vein within 2 minutes, the eyes and toes of the animals turned blue rapidly, indicating that the dye was evenly distributed. The rats were anesthetized by intraperitoneal injection of 2% pentobarbital solution (80 mg/kg) and transcardially perfused with normal saline followed by PF. After perfusion, the following operations were performed: (1) 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. (2) 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 EP tubes for later use.

5. 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. Fluorescence images of EB in the control group, 12 h group and 3 d group were taken. The relative fluorescence intensity of each group was analyzed by ImageJ software (National Institutes of Health, Maryland, USA) and normalized to the fluorescence images of the control group. The obtained values are expressed as means and standard errors.

6. Quantitative detection of BRB breakdown with Evans blue

The EB quantitative method was performed as previously reported [8, 21] with modification. Briefly, 1 μ l of EB (2%) was diluted 1000-fold in 1 ml formamide (F9037, Sigma-Aldrich, CA, USA) to a concentration of 20 ng/ml, then semidiluted 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 EP tube with dry retina was incubated with 160 μ l formamide in a constant-temperature chamber at 60 °C for 24 hours. Then the extract was centrifuged at 4 °C and 12 000 rpm for 30 minutes. The supernatant was taken (150 μ l) and divided into three sample tubes (50 μ l/tube). The optical density of each standard tube and sample tube was measured using a biospectrophotometer (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. The obtained values are expressed as means and standard errors.

7. Immunofluorescence staining

Retina sections were incubated with 5% donkey serum for 1 hour at room temperature before being incubated with the primary antibodies in the refrigerator overnight. Subsequently, in a dark chamber, the sections were incubated with the secondary antibodies for 2 hours at room temperature. Sections incubated with 2% donkey serum without a primary antibody were used as a negative control.

VEGF-165b was used for single labeling of retina sections, and dual labeling of VEGF-165b and ZO-1 was performed in sections from the control animal. The antibodies used in this study are shown in Table 1. Retinal sections were observed by fluorescence microscopy (Olympus BX50, Japan), and fluorescence images under the 40-fold objective were taken for analysis. Fluorescence images of VEGF-165b in the control group, 12 h group and 3 d group were taken. The relative fluorescence intensity of each group was analyzed by ImageJ software (National Institutes of Health) and normalized to the fluorescence images of the negative control. The obtained values are expressed as means and standard errors.

8. Western blotting

Rat retinas were homogenized with homogenization buffer (20 mM Tris, pH 8, 137 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The supernatant was removed and subjected to protein quantification with a Pierce BCA reagent kit (Thermo Fisher Scientific, 23227). Then, 50 μ g 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 with a 5% milk-PBS solution, 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 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 ImageJ (National Institutes of Health) and Microsoft Excel (Microsoft Corp.). The amounts of VEGF-165b and ZO-1 protein were normalized to β -actin. The obtained values are expressed as means and standard errors.

9. Statistical analysis

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. $P < 0.05$ was considered statistically significant.

Results

1. Dynamic changes in VEGF-165b protein level in rat retina with acute HIOP before and after treatment detected by immunofluorescence staining and western blotting.

Immunofluorescence staining of VEGF-165b showed that it was mainly expressed in the ganglion cell layer and the inner nuclear layer of the retina. The expression of VEGF-165b in the normal retina was similar to that in the control group (image not shown). The relative fluorescence intensity of VEGF-165b in the 12 h group and the 3 d group was stronger than that in the control group ($P < 0.01$) (Figure 1A). The result is consistent with that of western blotting (Figure 2A). Before vitreous injection of anti-VEGF-165b antibody, the expression of VEGF-165b/ β -actin in the control group, 12 h group and 3 d group was 0.743 ± 0.023 , 1.039 ± 0.035 , and 1.974 ± 0.039 , respectively. Compared with the control group, the expression of VEGF-165b protein in the 12 h group and the 3 d group was upregulated ($P < 0.01$). After vitreous

injection of anti-VEGF-165b antibody, the expression of VEGF-165b/ β -actin in the control group, 12 h group and 3 d group was 0.348 ± 0.031 , 0.433 ± 0.026 , and 0.305 ± 0.031 , respectively. Compared with nontreatment, the expression of VEGF-165b protein was significantly downregulated ($P < 0.01$).

2. Localization of ZO-1 in rat retina by double immunofluorescence staining and detection of the dynamic changes in ZO-1 expression before and after treatment by western blotting

Double immunofluorescence staining of VEGF-165b and ZO-1 in rat retinal sections showed that ZO-1 protein was mainly expressed on the retinal ganglion cell layer and both sides of the inner nuclear layer and was coexpressed with VEGF-165b protein (Figure 1B). Western blotting showed that before vitreous injection of anti-VEGF-165b antibody, the expression of ZO-1/ β -actin in the control group, 12 h group and 3 d group was 0.708 ± 0.025 , 0.414 ± 0.032 , and 0.582 ± 0.034 , respectively. Compared with the control group, the expression of ZO-1 protein in the 12 h group decreased significantly ($P < 0.01$); the expression of ZO-1 protein in the 3 d group recovered gradually but was still lower than that in the control group ($P < 0.01$). After vitreous injection of anti-VEGF-165b antibody, ZO-1/ β -actin in the control group, 12 h group and 3 d group was 0.735 ± 0.021 , 0.406 ± 0.019 , and 0.145 ± 0.026 , respectively. Compared with the control group, the expression of ZO-1 protein in the 12 h group and the 3 d group was significantly downregulated ($P < 0.01$). Compared with the same group before treatment, the expression of ZO-1 protein in the 3 d group was significantly downregulated ($P < 0.01$).

3. Detection of blood-retinal barrier leakage in rats by Evans blue 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 3 A2). No vascular distribution or red EB leakage spots were observed in the retina after saline perfusion (Figure 3 A3). 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 12 h and 3 d groups. The EB relative fluorescence intensity of the peripheral retina in the 12 h group and the 3 d group was stronger than that in the central retina ($P < 0.01$), and that of the central and peripheral retina in the 12 h group was stronger than that in the 3 d group ($P < 0.01$) (Figure 3 B1-3, C1-3). 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 12 h group. Compared with the 12 h group before treatment, the relative fluorescence intensity was stronger ($P < 0.01$). There were many red EB leakage plaques in the central and peripheral part of the retina in the 3 d group and more and larger red EB leakage plaques in the peripheral part. Compared with the 3 d group before treatment, the relative fluorescence intensity of EB in the 3 d group was stronger ($P < 0.01$) (Figure 3B4-6, C4-6).

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, 12 h group and 3 d group was 10.85 ± 4.13 ng/mg, 67.44 ± 7.12 ng/mg, and 27.38 ± 4.46 ng/mg, respectively; the EB leakage in the peripheral retina of the control group, 12 h group and 3 d group was 12.29 ± 2.89 ng/mg, 71.23 ± 5.28 ng/mg, and 35.89 ± 6.67 ng/mg, respectively. Compared with the control group, Evans blue leakage increased significantly in the 12 h group and the 3 d group ($P < 0.01$). After vitreous injection of anti-VEGF-165b antibody, the EB leakage in the central retina of the control group, 12 h group and 3 d group was 11.88 ± 2.92 ng/mg, 83.76 ± 4.63 ng/mg, and 67.55 ± 8.21 ng/mg, respectively; the EB leakage in the peripheral retina of the control group, 12 h group and 3 d group was 13.52 ± 2.35 ng/mg, 91.33 ± 3.57 ng/mg, and 123.46 ± 4.91 ng/mg, respectively. Compared with the corresponding group before injection, the EB leakage of the 12 h and 3 d groups increased significantly ($P < 0.01$); the EB leakage of the peripheral retina in the 12 h and 3 d groups was greater than that in the central retina before and after intravitreal injection ($P < 0.01$) (Figure 4).

Discussion

The normal retina of rats expressed the protein of VEGF-165b, which was mainly located in the ganglion cell layer and the inner nuclear layer of the retina. After acute HIOP, the expression of VEGF-165b was upregulated. After intravitreal injection of anti-VEGF-165b antibody, the expression of VEGF-165b protein was significantly downregulated. ZO-1 protein was mainly expressed on the retinal ganglion cell layer and both sides of the inner nuclear layer and was coexpressed with VEGF-165b protein. The expression of ZO-1 was downregulated at 12 h after acute HIOP and then recovered at 3 d, and the downregulation was more obvious after intravitreal injection. EB leakage increased after acute HIOP, peaking at 12 h. After treatment, EB leakage did not decrease but became more serious in the 3 d group. EB tracing on the whole mounted retina and EB quantification also showed that EB leakage in the peripheral retina was greater than that in the peripheral retina.

The expression of VEGF-165b protein was upregulated in the retina of rats with acute HIOP, which was consistent with Ergorul C and his team's report [12]. The expression of VEGF in diabetic retinopathy [22, 23] and senile macular degeneration [24] is positively correlated with the destruction of BRB; it may be that a subtype of VEGF (VEGF-164) that promotes angiogenesis plays a major role. However, recent studies [15, 25, 26] have shown that VEGF-165b has a protective role against retinal cell damage and BRB breakdown. Magnussen AL and its team [15] found that VEGF-A165b is cytoprotective and antiangiogenic in the retina of an oxygen-induced retinopathy mouse model. Beazley-Long N [25] and his team demonstrated in vivo and in vitro that VEGF-A165b has neuroprotective effects, including retinal ganglion cells. Ved N and his team [26] also confirmed that VEGF-165b ameliorates outer-retinal barrier and vascular dysfunction in the diabetic retina. Our results showed that BRB breakdown in rats caused by acute HIOP was not alleviated after intravitreal injection of anti-VEGF-165b antibody to antagonize endogenous VEGF-165b protein but was more serious, as seen by the downregulation of ZO-1 and increase of EB leakage, suggesting that the expression of endogenous VEGF-165b protein protected the maintenance of the integrity of BRB. VEGF-165b 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 [27, 28]. The blood supply of the inner retina comes from the shallow capillary network and the deep capillary network composed of branches of the central retinal artery, whose microenvironment is mainly regulated by iBRB[3]. ZO-1 is one of the tight junction proteins between the adjacent capillary endothelial cells of BRB[3]. Immunofluorescence showed that ZO-1 was mainly expressed in the retinal ganglion cell layer and both sides of the inner nuclear layer, indicating that ZO-1 was distributed in the superficial retinal capillary network and the deep retinal capillary network. BRB damage is associated with the destruction of tight junction proteins between capillary endothelial cells [29]. Studies have shown [3, 4] that the tight junction protein ZO-1 is a marker of BRB integrity, as loss or reduction of ZO-1 is related to an increase of barrier permeability. Therefore, the changes in ZO-1 in the retina of 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 intravitreal injection of anti-VEGF-165b antibody, 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 AL and his team [15]. 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.[21]. 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 3 A2). After perfusion with 0.9% saline and 4% PF, no red fluorescent spots were found in the retina (Figure 3 A3). 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 intravitreal injection of anti-VEGF-165b antibody, in addition to the red spots of EB in the retina, red plaques of EB were observed in the peripheral retina of the 12 h group and the retina of the 3 d group. The most red EB plaques were observed in the peripheral retina of the 3 d group. One possible reason is that more Evans blue 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 3 d group, which also confirmed this hypothesis. The inner retina is highly sensitive to hypoxic stress [30]. Kaur C and his team [31] 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 JB and his team [17] 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.

Conclusions

We found that the expression of retinal VEGF-165b was continuously upregulated 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 and ZO-1 was downregulated more obviously, and the damage to BRB was more serious, suggesting 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 retinal ganglion cells.

This study still lacks in vitro experimental evidence, such as coculture of retinal endothelial cells, pericytes and astrocytes, to study the protective effect of VEGF-165b on BRB. The specific molecular mechanism through which vascular endothelial cells (VECs) maintain BRB integrity via VEGF-165b remains to be further studied.

Abbreviations

blood-retinal barrier (BRB); high intraocular pressure (HIOP); Evans blue (EB); retinal ganglion cell (RGC); vascular endothelial growth factor-165b (VEGF-165b); outer BRB (oBRB); inner BRB (iBRB); tight junctions (TJs); zonula occludens (ZO); adherens junctions (AJs); vascular endothelial cadherin (VE-cadherin); gap junctions (GJs); connexin 43 (Cx43); hypoxia-inducible factor-1 alpha (HIF-1 α); paraformaldehyde (PF); Statistical Product and Service Solutions18.0 (SPSS18.0); vascular endothelial cells (VECs); ganglion cell layer (GCL); inner retinal layer (IPL); inner nuclear layer (INL); outer nuclear layer (ONL); Relative fluorescence intensity (RFI); Western blotting (WB); Immunofluorescence (IF); central retina(Cen.); peripheral retina (Peri.); central retina with treatment (Cen.-Tre.); peripheral retina with treatment (Peri.-Tre.).

Declarations

- Ethics approval and consent to participate

Not applicable

- Consent to publish

Not applicable

- Availability of data and materials

Raw data can be obtained from corresponding authors.

- Competing Interests

There is no conflict of interest to be disclosed by the author.

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- Author Contributions

QP Z and J S designed the experiments; J S, QP Z, ZH L, XN Y, M L, WX L, HL S and L W performed the experiments; QP Z provided the experimental reagents and access to the data analysis software; QP Z, XN Y and J S carried out data analysis and wrote the manuscript.

The manuscript has been read and approved for publication by all authors.

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Table

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
Anti-ZO-1 antibody	Rabbit	abcam	ab96587	5 µg/ml for IF 1:1000 for WB
Alexa Fluor 488 anti-rabbit IgG (H+L)	donkey	Jackson	711-545-152	1:200 for IF 1:200 IF
Alexa Fluor® 488 anti-Mouse IgG(H+L)	donkey	Jackson	715-545-150	1:200 IF
Alexa Fluor® 594 anti-Mouse IgG(H+L)	donkey	Jackson	715-545-150	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

Figures

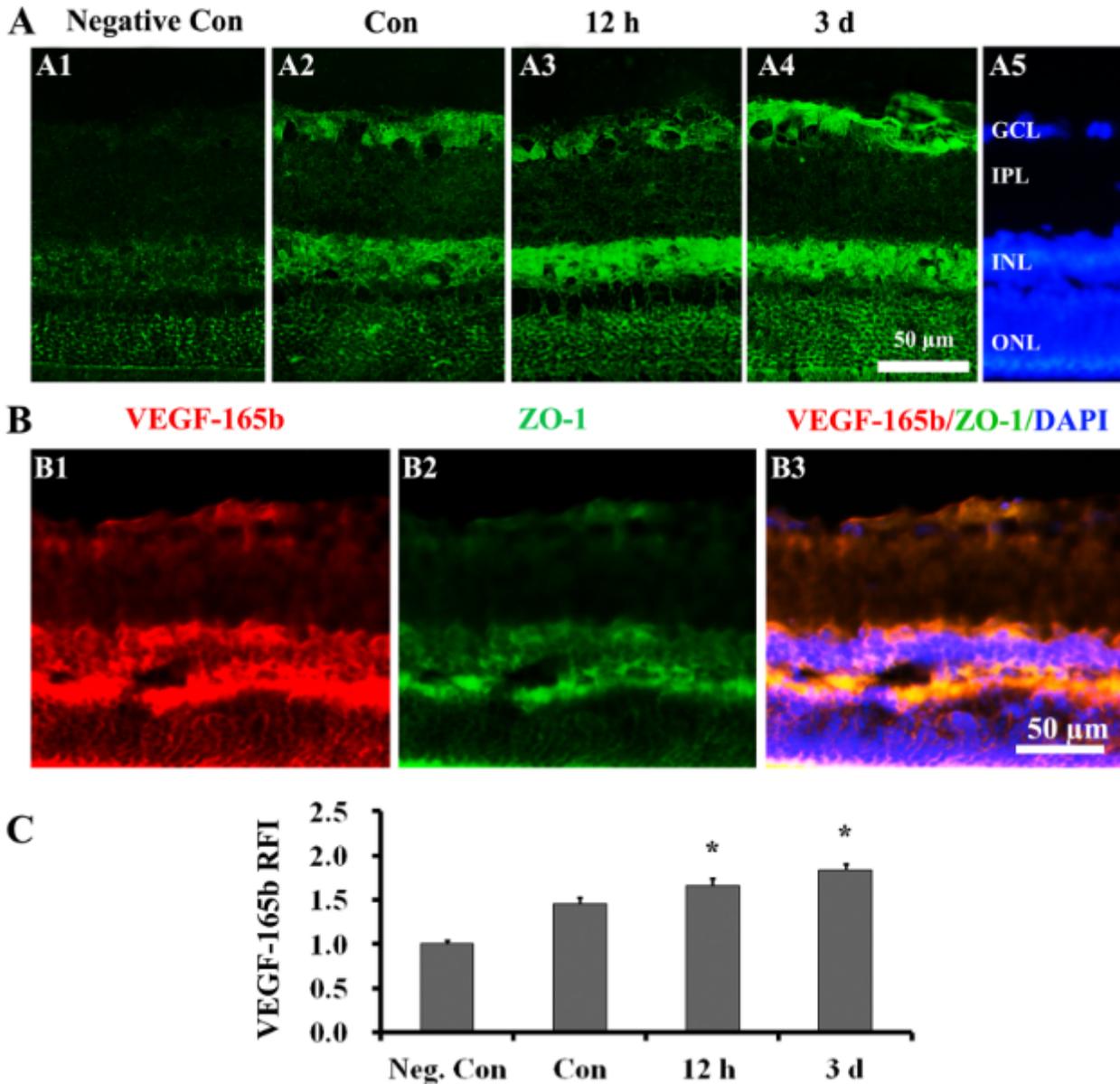


Figure 1

The expression of VEGF-165b and ZO-1 in retina as detected by immunofluorescence staining (A) Immunofluorescence staining was used to detect the expression of VEGF-165b (green) in retina. A1 is a negative control image, and A2-4 are images taken from the control group, 12 h group and 3 d group, respectively. A5 shows retinal DAPI nuclear staining (blue). (B) Double immunofluorescence staining of VEGF-165b and ZO-1 in normal retina. B1 is an immunofluorescence image of VEGF-165b (red), B2 is an image of ZO-1 (green), B3 is a merged image of VEGF-165b, ZO-1 and DAPI (blue). (C) Relative fluorescence intensity analysis of retinal VEGF-165b protein expression, normalized to the negative control. *, Compared with the control, $P < 0.05$. GCL, ganglion cell layer; IPL, inner retinal layer; INL, inner nuclear layer; ONL, outer nuclear layer. Bar = 50 μm . RFI, relative fluorescence intensity.

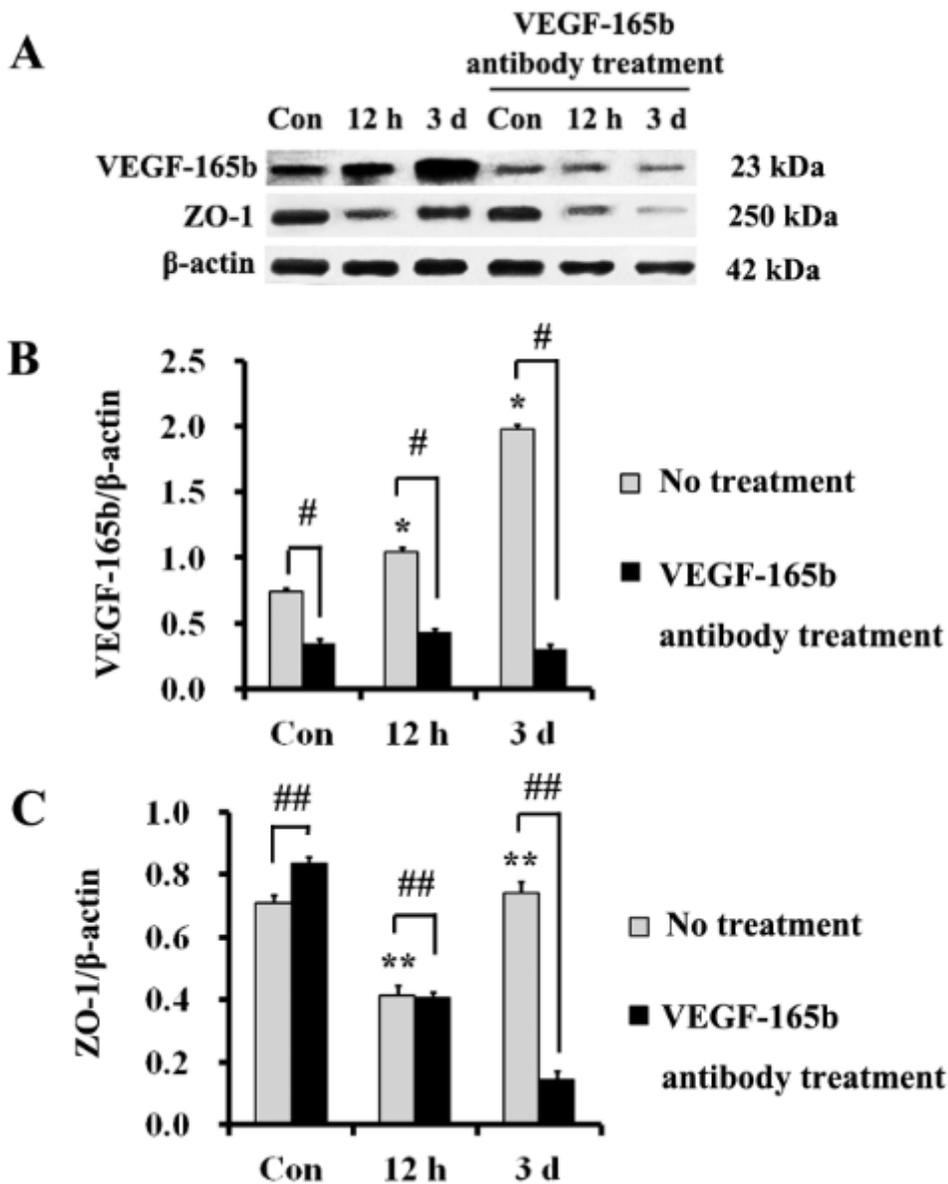


Figure 2

Changes in VEGF-165b and ZO-1 in retina analyzed by western blotting (A) Western blot to detect VEGF-165b and ZO-1 expression in retina before and after anti-VEGF-165b antibody treatment. (B) Western blot to analyze the changes in VEGF-165b, normalized to β -actin. *, compared to control, $P < 0.01$; #, compared to the untreated groups, $P < 0.01$. (C) Western blot to analyze the changes in ZO-1, normalized to β -actin. **, Compared to control, $P < 0.01$; ##, compared to the nontreated groups, $P < 0.01$.

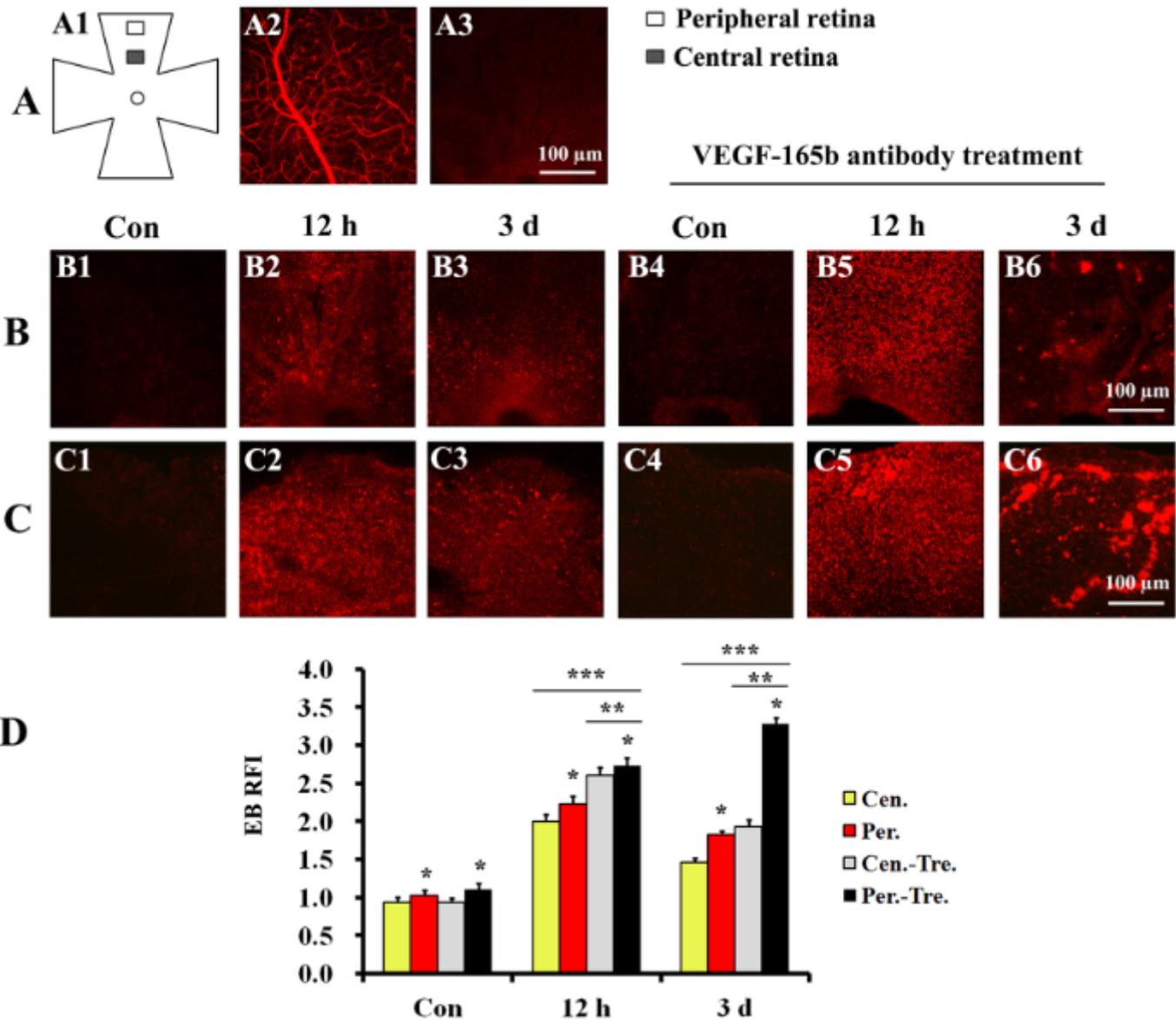


Figure 3

Detection of blood-retinal barrier leakage by Evans blue 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) Evans blue shows normal retinal vessels. (A3) Evans blue shows normal retinal vessels after 0.9% saline infusion. (B) Evans blue detection of EB leakage in central retina before and after treatment. B1-3 are the central retina of the control, 12 h and 3 d groups, respectively. B4-6 are the central retina with treatment in the control, 12 h and 3 d groups, respectively. (C) Evans blue detection of EB leakage in the peripheral retina before and after treatment. C1-3 are the peripheral retina of the control, 12 h and 3 d groups, respectively. C4-6 are the peripheral retina with treatment in the control, 12 h and 3 d 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. ***, Compared with the corresponding parts of the control group, $P < 0.01$; **, compared with the corresponding parts of the same group before treatment, P

< 0.01; *, compared with the central retina of the same group, P < 0.01. Cen., central retina; Peri., peripheral retina; RFI, relative fluorescence intensity. Bar = 100 µm.

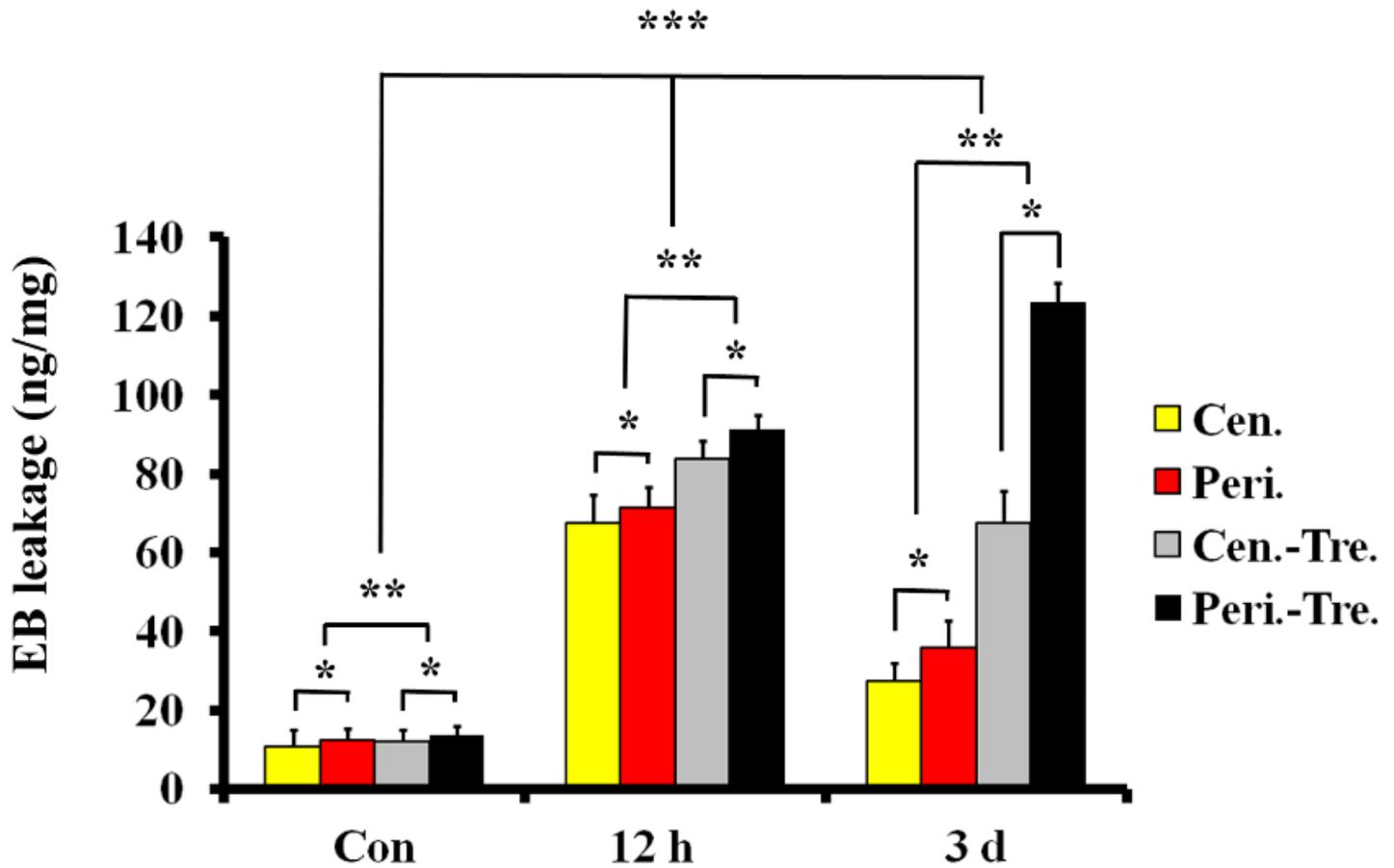


Figure 4

Quantitative detection of BRB leakage in rats by Evans blue The longitudinal axis indicates EB leakage (ng EB/mg retina); the transverse axis indicates the experimental groups. ***, Compared with the corresponding parts of the control group, P < 0.01; **, compared with the corresponding parts of the same group before treatment, P < 0.01 in the 12 h group and 3 d group, P > 0.05 in the control group; *, compared with the central retina of the same group, P < 0.01 in the 12 h group and 3 d group, P > 0.05 in the control group. Cen., central retina; Peri., peripheral retina; Cen.-Tre., central retina with treatment; Peri.-Tre., peripheral retina with treatment.

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