Original Article

Correlation of Vascular Endothelial Growth Factor Production with Photochemical Reaction-induced Retinal Edema

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Abstract

Background: Retinal edema is the major complication of retinal vein occlusion and diabetic retinopathy; it can damage visual function by influencing macular region. This study was to establish a rat retinal edema model and explore the related VEGF expression and observe the responses to anti-VEGF drugs in this model.

Methods: A rat retinal edema model was established by inducing photochemical reaction using a 532 nm laser after the intravenous injection of Erythrosin B. Immediately after the laser treatment, models were given intravitreal injections of Ranibizumab or Conbercept to inhibit VEGF expression, and the changes of retinal thickness were measured. Retinal edema was observed using fundus photography (FP), optical coherence tomography (OCT), and fluoresce in fundus angiography (FFA) at 0, 1, 2, 4, 7 and 14 days after intervention. The retinal VEGF expression was measured using enzyme-linked immunosorbent assay (ELISA) and western blotting at each time point. The rat retinal edema model was also used to verify the function of anti-VEGF polypeptide ZY1.

Results: Both retinal edema and vascular leakage were clearly observed at 1, 2 and 4 days after photochemical induction and the retinal thickness increased notably over the same period. The retinal VEGF expression peaked at day 1 and retina became thickening simultaneously. After the interventions, the VEGF expression of the Ranibizumab and Conbercept groups decreased at each time point compared to the edema group (26.90 ± 3.57 vs. 40.29 ± 6.68, $F = 31.269$ on day 1 and 22.36 ± 1.12 vs. 29.92 ± 0.93 $F = 163.789$ on day 2, both $P < 0.01$); the mean RT (278 ± 4 vs. 288 ± 3, $F = 134.190$ on day 1 and 274 ± 7 vs. 284 ± 6, $F = 64.367$ on day 2, both $P < 0.05$) and vascular leakage in these groups also decreased. The same results were observed in the ZY1 group, particularly at day 2 ($P < 0.05$).

Conclusions: This retinal edema model induced by a photochemical reaction is reliable and repeatable. Induced edema increases expression of VEGF. This model can be used to test new drugs.

Key words: Animal Model; Anti-vascular Endothelial Growth Factor; Erythrosin B; Photochemical Reaction; Retinal Edema

Introduction

Retinal edema is the major complication of retinal vein occlusion and diabetic retinopathy; it can damage visual function by influencing macular region.[1,2] High blood pressure and diabetes are very common diseases that cause high morbidity of retinal vein occlusion and diabetic retinopathy.[3,4] There is currently no simple retinal edema animal model, which is urgently required for the development of new drugs and treatments for this condition.

Erythrosin B (EB) is a dyestuff with photochemical properties. Similar to Rose Bengal, EB is a Type II photosensitive dye that, when excited by laser irradiation after entering the blood, can cause puncta injury in vascular endothelial barrier by producing singlet oxygen.[5] This damage can lead to retinal vasogenic edema and even cause thrombosis. Previous studies have shown that optic nerve and retinal disease models can be induced with Rose Bengal, which undergoes a photochemical action.[6,7] EB has been used to establish animal models of posterior ischemic optic neuropathy, neuropathic pain, and distal occlusion of the

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.4103/0366-6999.195463

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Received: 31-07-2016 **Edited by:** Li-Min Chen
How to cite this article: Shan L, Zheng M, Zhang Y, Qu Y, Niu T, Gu Q, Liu K, Xia X. Correlation of Vascular Endothelial Growth Factor Production with Photochemical Reaction-induced Retinal Edema. Chin Med J 2016;129:2944-50.

arteria cerebri media.[8-10] We have successfully constructed a retinal vein occlusion model using this feature of EB in an earlier study.[11] In all of the preceding models, edema occurs. However, we established a new retinal edema model with EB that avoids thrombosis by adjusting the dosage of EB, the intensity and timing of laser irradiation.

Vascular endothelial growth factor (VEGF) is a nutritional factor of retinal neuron cells and vascular endothelial cells; it is also a main factor in increasing vascular permeability. The concentrations of VEGF in the vitreous samples of patients with macula swelling caused by diabetic retinopathy or retinal vein occlusion were higher than the concentrations in normal controls.[12,13] In the retinal vascular system, VEGF plays an important role in the pathogenesis of retinal vascular permeability by affecting the integrity of tight junctions between the endothelial cells.[14] A study has reported that anti-VEGF treatment could reduce retinal edema in retinal diseases.[15] Ranibizumab and conbercept are typical drugs used in anti-VEGF treatments of ophthalmological conditions. In this experiment, we constructed a retinal edema model and used ranibizumab and conbercept intervention to verify the edema model and explore the expression of VEGF in retinal edema.

Placental growth factor (PLGF) is an important part of the VEGF family. PLGF has been found to increase vascular permeability, which is closely associated with the occurrence of edema. ZY1[16] is a 21-amino acid peptide with the sequence of CVSLLRCTGCCGDENLHCVPV from loop b3 to b4 of PLGF-1.[17] It may block the effects of VEGF-A, VEGF-B, and PLGF through VEGF receptor 1 (VEGFR1). On this basis, we used this retinal edema model to verify the function of this small molecular polypeptide ZY1 and to provide preclinical research results for future clinical transformation.

Methods

Animals

All the procedures involving animals adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Experimental Centre of Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine.

A total of 134 Sprague Dawley rats (180–200 g) were provided by the Shanghai Experimental Animal Centre, Chinese Academy of Sciences. Thirty rats were used in examinations of FP, FFA and OCT, 52 rats were used in ELISA tests and also 52 rats were used in Western blotting.

Establishment of the rat photochemical action-induced retinal edema model

EB (2%) was injected intravenously at a dose of 20 mg/kg under 4% chloral hydrate anesthesia. The power supply voltage of the laser was adjusted to 2 mW (output spot energy). The light beam used in the laser irradiation was kept vertical to the pupil for 8 min. Rats in the control group (n = 30) were injected with the same amount of placebo saline solution instead of EB; they also received 8 min laser irradiation. All the rats received laser irradiation only in the right eye; the left eye was not irradiated.

Intravitreal injection of anti-vascular endothelial growth factor drugs

After induction, intravitreal injections of anti-VEGF drugs were administered immediately in the ranibizumab group (n = 30; ranibizumab 3 μl, 10 mg/ml, Novartis Co.), conbercept group (n = 30; conbercept 3 μl, 10 mg/ml, Chengdu Kanghong Biotechnologies Co. Ltd.), and ZY1 group (n = 6; ZY1 polypeptide 3 μl, 2 mg/ml, China Peptides Co., Ltd.), and 3 μl of phosphate-buffered saline was administered to the control (n = 38) and edema groups (n = 30). The injections were applied slowly over 30 s to allow diffusion of the drugs and vehicles.

Fundus photography, fundus fluorescein angiography, and optical coherence tomography examinations

Fundus photography, fundus fluorescein angiography (FFA), and optical coherence tomography (OCT) were performed at 0, 1, 2, 4, 7, and 14 days after treatment to observe the progress of the retinal edema and any related retinal response. Fundus images were taken with a digital camera (Canon DS126231, Japan). Fluorescein sodium (10%, 0.2 ml) was intravenously injected, and penetration into the eyes was confirmed by the color of the conjunctiva turning to yellow, at which time the angiographs were recorded (Heidelberg HRT2, Germany). Retinal structure and retinal thickness were measured using OCT (Heidelberg HRT2, Germany).

Retinal thickness between the retinal pigment epithelium layer and the retinal nerve fiber layer were measured in four quadrants (superior, inferior, nasal, and temporal quadrants; 1 mm from the center of the optic nerve), and then the average was calculated.

ELISA

Retinal tissues were collected before induction and at 1, 2, and 4 days after induction. The stripped retinal tissues were homogenized on ice cubes. The VEGF levels in the retinal tissue were detected using the commercially available R-VEGF ELISA kit (Multisciences, Shanghai, China), following the manufacturer's instructions. The optical density was read at 450 nm using a microplate reader (Thermo Multiskan MK3) with a correction wavelength of 570 nm. The VEGF concentration of each sample was calculated from the standard curve.

Western blotting

Retinas were dissected, snap-frozen in dry ice, and homogenized before induction and at 1, 2, and 4 days after induction. Concentration of total protein in each sample was determined by bicinchoninic acid method. Total protein of 20 μg from each sample was electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membranes were washed in Tris-Tween buffered saline and probed with a rabbit polyclonal anti-VEGF (1:400) overnight at 4°C, then with anti-rabbit immunoglobulin (Ig) G (1:2500) for 1 h.

Then, enhanced chemiluminescence (ECL) detection was performed, and the membranes were exposed to X-ray film.

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analyses were performed with one-way analysis of variance (ANOVA), followed by Student's *t*-test. Differences with $P < 0.05$ were considered statistically significant. All statistical analyses were conducted using the SPSS version 19.0 software package (IBM, Chicago, IL, USA).

Results

Fundus photography

Compared to the control group, prominent retinal whitening and edema were observed in the edema group on day 1, with obvious retinal vasoconstriction and the disappearance of the optic disc pit, followed by venous dilatation and tortuosity on days 2 and 4. Retinal pallor also reduced at the same time. On days 7 and 14, retinal whitening reappeared [Figure 1a]. There was no significant change in retinal morphology in the control group at each time point.

In the ranibizumab, conbercept, and ZY1 groups, there was only mild vasoconstriction and retinal pallor on day 1. Especially in the ranibizumab group, less change was observed. Compared to the edema group, the vascular tortuosity in these three intervention groups was lighter, and the optic disc pit recovered its topography [Figure 1b-1d].

Fundus fluorescence angiography

There was widespread hyperfluorescence with leakage around the optic disc in the edema group on day 1 compared to the control group. On day 2, the lesions looked similar to those on day 1, except for more dilated and tortuous vessels on retina. On days 4 and 7, the retinal vessels began to return to normal, with only weak leakage remaining. These changes disappeared by day 14 [Figure 2a]. There was no fluorescence leakage in the control group at each time point.

Compared to the edema group, hyperfluorescence and leakage in the intervention groups during the first 2 days had a great improvement. In the conbercept group, the fluorescence leakage disappeared at day 4. While in the ranibizumab group, leakage could not be observed at day 7. Moreover, the vessels in both groups returned to normal. Fourteen days after induction, there was no leakage in the ZY1 group [Figure 2b-2d].

Optical coherence tomography and retinal thickness

Compared to the control group, the photodynamically treated eyes in edema group at day 1 [Figure 3a2] showed hyperreflectivity of the inner retinal layers around the disc and loss of a clear distinction between the layers of the retina, which became more pronounced by day 2 [Figure 3a3]. Shallow subretinal fluid was detected in a small number of

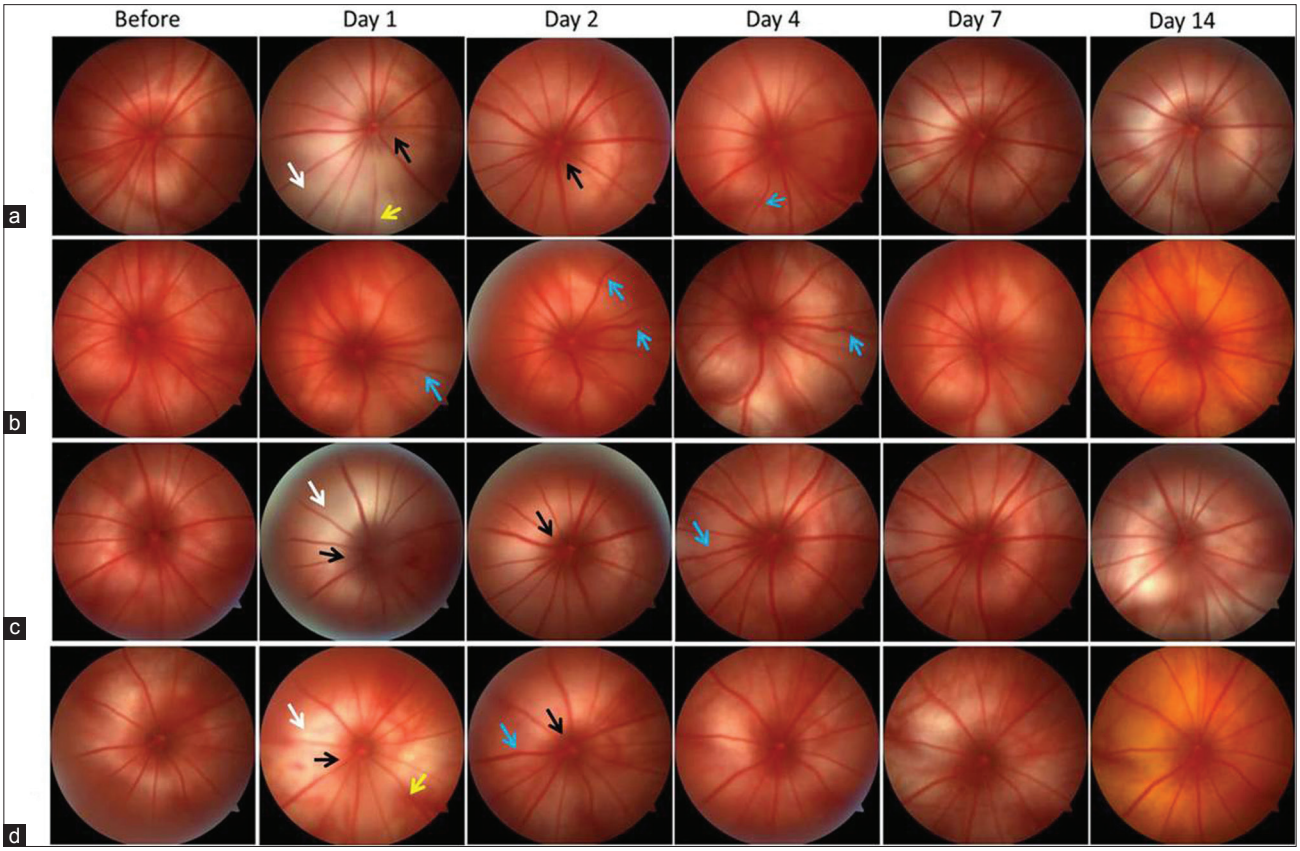

Figure 1: Retinal morphological changes through fundus photography at different time points in each group ($n = 6$). (a) Edema group; (b) Ranibizumab group; (c) Conbercept group; (d) ZY1 group. Black arrows: Disappearance of the optic disc pit; White arrows: Retinal whitening; Yellow arrows: Retinal vasoconstriction; Blue arrows: Vessel expansion and tortuosity.

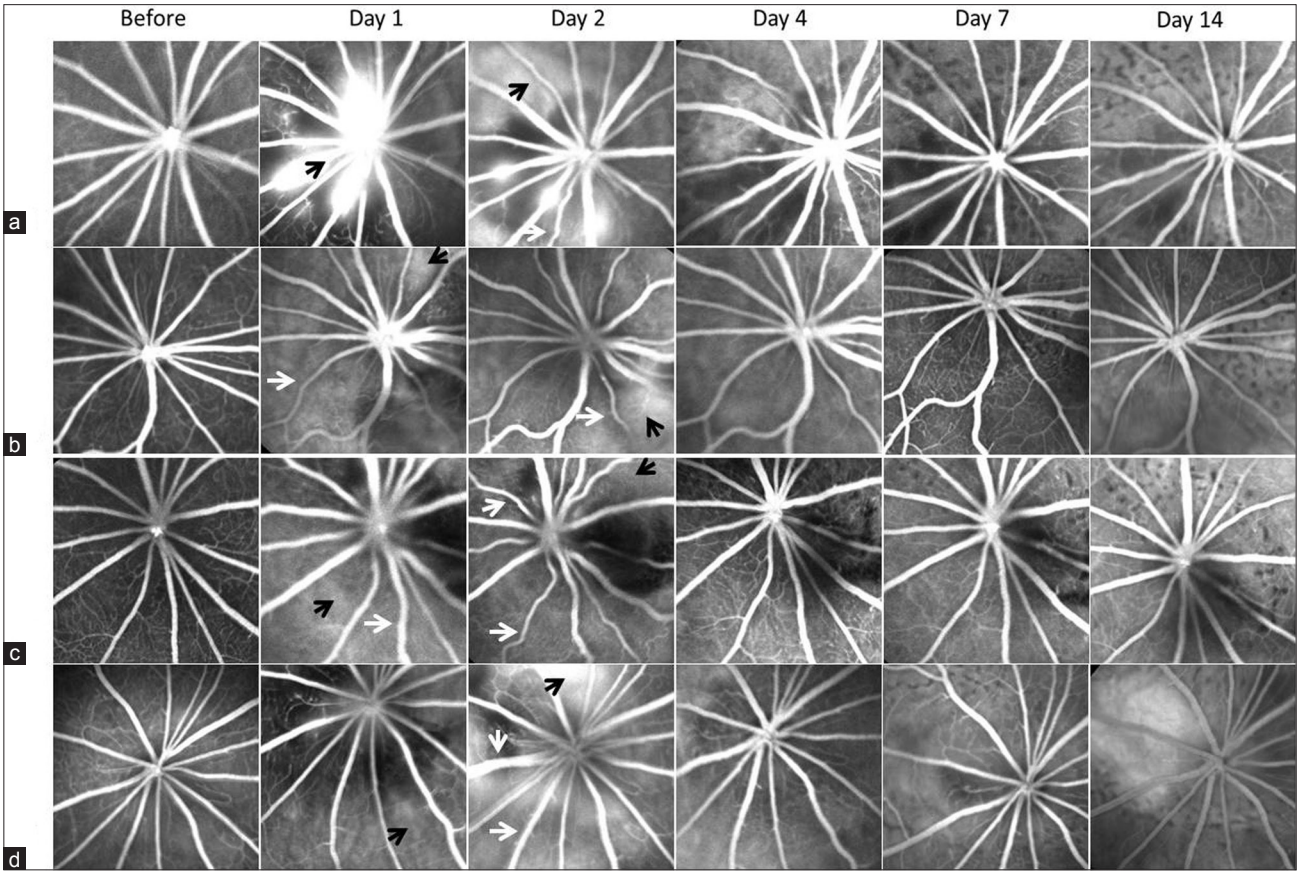

Figure 2: Vascular abnormalities through fluorescein fundus angiography at different time points in each group ($n = 6$). (a) Edema group; (b) Ranibizumab group; (c) Conbercept group; (d) ZY1 group. Black arrows: Fluorescence leakage; White arrows: Vessel expansion and tortuosity.

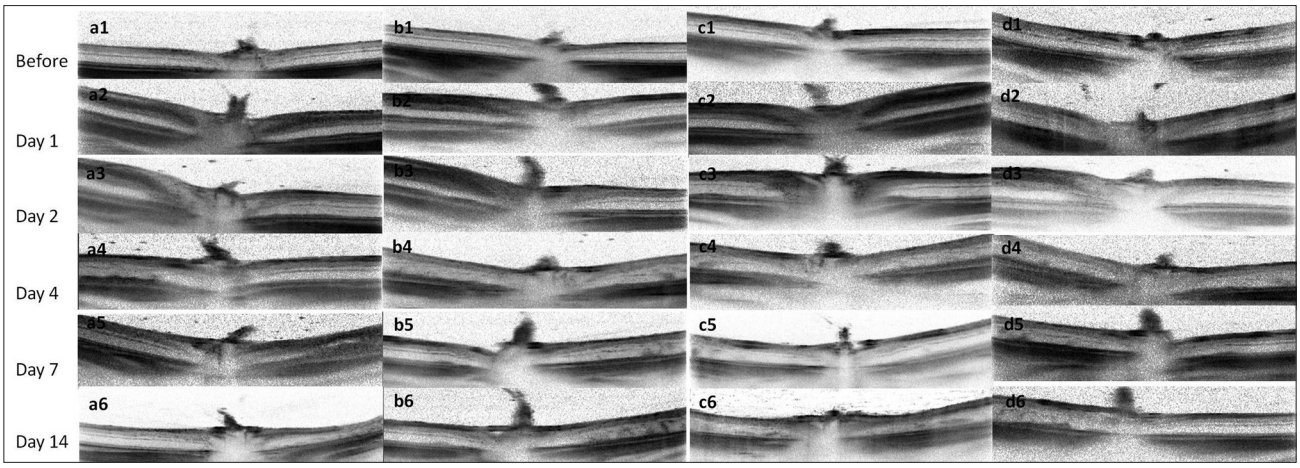

Figure 3: Retinal edema through optical coherence tomography at different time points in each group ($n = 6$). (a1-a6) Edema group; (b1-b6) Ranibizumab group; (c1-c6) Conbercept group; (d1-d6) ZY1 group. Pictures from top to bottom show changes of retinal layers, in order on day 0 (a1, b1, c1, d1), day 1 (a2, b2, c2, d2), day 2 (a3, b3, c3, d3), day 4 (a4, b4, c4, d4), day 7 (a5, b5, c5, d5), and day 14 (a6, b6, c6, d6).

treated eyes. Retinal swelling reduced on day 4 [Figure 3a4] and the edema was resolved by day 7 [Figure 3a5] and returned to normal by day 14 [Figure 3a6], although with some retinal atrophy. There were no obvious changes in the control group at any time point.

Retinal thickness increased quickly after photochemical induction, which was observed on day 1 postinduction and became thicken on day 2. However, retinal thickness decreased from day 4 without any intervention [Table 1]. There was no significant variation in the retinal thickness of the control group throughout the observation period ($P > 0.05$).

Figure 3b-3d showed the reduction of retinal thickness by anti-VEGF intervention in the retinal edema model. On day 2, there were significant differences in the retinal thicknesses of the edema group and the ranibizumab group (274 ± 7 μm, $F = 64.367$, $P < 0.001$), conbercept

group (284 ± 6 μm, $F = 64.367$, $P < 0.01$), and ZY1 group (290 ± 11 μm, $F = 64.367$, $P < 0.05$). The retinal thickness of the conbercept group (239 ± 6 μm, $F = 20.000$, $P < 0.05$) was thinner than that of the edema group on day 4. However, the retinal thickness of the ranibizumab group (212 ± 9 μm, $F = 13.192$, $P < 0.01$) was thicker than that of the edema group on day 14.

ELISA

The ELISA results [Table 2] showed that the concentration of VEGF in the rat retina of the edema group increased immediately after the laser treatment and decreased steadily starting at day 2. There was no significant variation in the concentration of VEGF in the control group throughout the observation period ($P > 0.05$).

Table 2 shows the reduction of the VEGF concentration due to anti-VEGF intervention in the retinal edema model. The VEGF expression in the ranibizumab group (26.90 ± 3.57 pg/ml, $F = 31.269$, $P < 0.05$) was lower than that in the edema group on day 1. On day 2, there were significant differences between the VEGF concentrations in the edema group and the ranibizumab group (22.36 ± 1.12 pg/ml, $F = 163.789$, $P < 0.001$) and conbercept group (29.92 ± 0.93 pg/ml, $F = 163.789$, $P < 0.001$).

Western blotting

No statistical differences in the VEGF protein levels in the control group were observed at any time point. Compared to the control group, the Western blotting analysis revealed that the protein level of VEGF in the edema group increased 1.40-fold (1 day), $F = 159.467$, $P < 0.001$; 1.31-fold (2 days), $F = 12.149$, $P < 0.01$; and 1.05-fold (4 days), $P > 0.05$ [Figure 4].

Compared to the edema group, the ranibizumab and conbercept groups had decreased VEGF protein levels [Figure 4]. However, there were no statistically significant differences between any of the groups by day 4.

Discussion

Retinal edema is commonly seen in a variety of eye diseases, and it could lead to vision impairment if the diseases were not treated properly. It is generally believed that the blood–retinal barrier (BRB) impairment is the major step leading to the development of retinal edema.[18] BRB can be compromised by ischemia and inflammation. Many factors, including pro-inflammatory cytokines, prostaglandins, and VEGF, are known to induce vascular leakage that leads to retinal edema.[19]

In this study, we subjected rat eyes to low-intensity green laser irradiation immediately after the rats had received injections of EB. As a result of this photochemical procedure, retinal pallor, vascular tortuosity, fluorescein leakage, an increase in retinal thickness, and the destruction of the layer structure were observed whereas FFA analyses did not reveal any evidence of vascular thrombosis.

In this rodent retinal edema model, retinal thickening was observed on the first day after induction and retinal layer become fuzzy in OCT. The degree of retinal edema reached its maximum level on the second day after model induction and maintained this level until the fourth day after the laser irradiation. Fluorescein leakage with retinal vascular dilatation and congestion around optic disc was observed in FFA, which was paralleled by changes in the OCT. Severe edema can interfere with tissue microcirculation, which leads to tissue ischemia and hypoxia, resulting in the death of retinal cells. On the second day, the retinal thickness in edema group was thicker than that in the ranibizumab group, which indicated that retinal cell has been protected by ranibizumab from death. Hence, on 14th day, the retinal thickness in ranibizumab group was thicker than that in the edema group.

VEGF is the main factor in the vascular permeability. In primate experiments, injection of VEGF into the vitreous cavity may lead to retinal vascular fluorescein leakage.[20] We found that the protein level of VEGF in rat retinas

Table 1: Comparison of the mean retinal thickness data for each group at each time point (μm, $n = 6$)

Time (day)	Control group	Edema group	Ranibizumab group	Conbercept group	ZY1 group	F	P
0	216 ± 2	215 ± 3	216 ± 2	216 ± 3	217 ± 3	0.151	0.958
1	215 ± 4	287 ± 7*	278 ± 4†	288 ± 3	286 ± 6	134.190	<0.001
2	216 ± 2	310 ± 10*	274 ± 7†	284 ± 6†	290 ± 11†	64.367	<0.001
4	216 ± 3	252 ± 8*	256 ± 6	239 ± 6†	247 ± 7	20.000	<0.001
7	217 ± 2	225 ± 8	230 ± 7	219 ± 5	225 ± 6	2.117	0.153
14	217 ± 3	191 ± 6*	212 ± 10†	199 ± 5	186 ± 6	13.192	0.001

All data were expressed as mean ± SD. *P<0.01 versus Control group, †P<0.05 versus Edema group. SD: Standard deviation.

Table 2: Comparison of the ELISA results for each group at each examined time point (pg/ml, $n = 4$)

Time (day)	Control group	Edema group	Ranibizumab group	Conbercept group	F	P
0	13.48 ± 1.28	13.48 ± 1.28	13.48 ± 1.28	13.48 ± 1.28	0	1.000
1	15.21 ± 1.41	44.43 ± 2.90*	26.90 ± 3.57†	40.29 ± 6.68	31.269	<0.001
2	14.21 ± 1.67	36.00 ± 1.27*	22.36 ± 1.12†	29.92 ± 0.93†	163.789	<0.001
4	14.23 ± 1.82	15.17 ± 2.63	15.77 ± 2.64	15.52 ± 2.05	0.325	0.807

All data were expressed as mean ± SD.*P<0.001 versus Control group, †P<0.01versus Edema group. SD: Standard deviation.

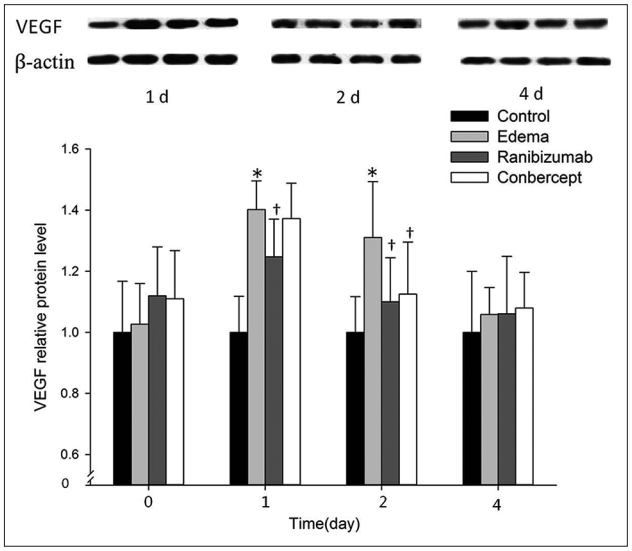

Figure 4: Changes of vascular endothelial growth factor protein expression level in each group at different time points. *$P < 0.01$ versus control group; †$P < 0.05$ versus edema group.

significantly increased on the first and second days after photochemical induction and began to decrease on the fourth day after induction until it reached the premodeling level. In this model, VEGF protein expression changes simultaneously with fluorescence leakage and retinal thickening.

Ranibizumab is a high-affinity recombinant Fab that neutralizes all of the isoforms of VEGF-A.[21] Conbercept is a recombinant fusion protein containing the second Ig domain of VEGFR1, the third and fourth Ig domains of VEGFR2, and the Fc region of human IgG.[22]

Some studies have found that the concentration of VEGF increases in retinal edema-related diseases and that anti-VEGF treatment can inhibit retinal edema.[15] We found that the expression level of retinal VEGF protein significantly decreased after anti-VEGF drug intervention, following with a decrease in retinal fluorescence leakage and retinal thickness after the intervention. These further proved the efficiency and usefulness of the rodent retinal edema model, which could be used for drug screening and the other potential edema treatment.

Polypeptide ZY1 is a peptide sequence screened from PLGF-1, including important binding sites for PLGF-1 and VEGFR-1.[23] Studies have confirmed that the polypeptide ZY1 can compete with PLGF and VEGF for VEGFR-1 receptor, and thus block the downstream signal transduction pathway and inhibit the formation of new blood vessels.[16] Some studies have shown that a high expression of PLGF in keratinocytes can induce a significant increase in vascular permeability.[24] Oura *et al*.[25] found that the lack of PLGF in PLGF gene-defective mice can reduce the proliferation of blood vessels during inflammation, and that a high expression of the PLGF gene in mouse skin can cause blood vessels to expand and edema to significantly increase. Miyamoto *et al*.[26] observed damage to rat retinal barriers and plasma extravasation after the intravitreous injection of PLGF-1, which resulted in subretinal fluid and retinal edema. In this study, we used polypeptide ZY1 to inhibit the function of PLGF and VEGF in this retinal edema model. From retinal vascular, thickness, color, and fluorescence leakage, ZY1 group made a quicker recovery than the edema group.

In summary, this study provides the first instance of the induction of a vasogenic edema model of rat retina by a photochemical reaction. This model involves retinal morphological changes that are similar to the alterations that occur in human retinal edema. In addition, by using a low-intensity laser, obvious retinal vascular leakage was observed, whereas no significant vascular occlusion was detected; thus, the aggravation of retinal edema by interference from various factors, such as ischemia, was avoided. The major improvement needed for this model is the shorter edema duration, while retinal edema in clinical case usually exists weeks or months. We are looking forward to improving this model to portray symptoms and progress of retinal edema completely. Anti-VEGF and polypeptide ZY1 provide further evident that this model is a new, reliable, and effective tool for research of vasogenic retinal edema.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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