Effect of a novel tyrosine kinase inhibitor nintedanib on bFGF and VEGF concentrations in a rabbit retinal vein occlusion model

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Abstract

● **AIM:** To evaluate whether a novel tyrosine kinase inhibitor nintedanib could inhibit basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) simultaneously for retinal vascular disease *in vivo*.

● **METHODS:** After a laser induced rabbit retinal vein occlusion (RVO) model was made, 0.5 mg of nintedanib was injected intravitreally in the left eye on the third day while the right eye was as a control. Intracameral samples were taken on the day before laser treatment and days 1, 3, 7, 14, 21, and 28 after treatment. Enzyme-linked immunosorbent assay (ELISA) was used to test the bFGF and VEGF-A concentrations in the aqueous humor.

● **RESULTS:** Both bFGF and VEGF-A rose significantly on the third day after laser treatment in both eyes. In the control eye the bFGF concentration peaked on the 14th day while the VEGF-A concentration dropped rapidly soon after the third day. After nintadanib injection in the study eye, both bFGF and VEGF-A showed a significant reduction on the 4th day (7th day after laser treatment) when compared to the control eye, and kept on low level in the following several weeks.

● **CONCLUSION:** Intravitreal injection of nintedanib can inhibit the expression of bFGF and VEGF in the process of RVO model to a certain extent, which is expected to become a new method for the treatment of retinal vascular diseases or fibrotic diseases.

● **KEYWORDS:** retinal vein occlusion; nintedanib; tyrosine kinase inhibitor; basic fibroblast growth factor; vascular endothelial growth factor; rabbit model

INTRODUCTION

Retinal and choroidal vascular diseases have become one of the leading disorders with irreversible blindness. Neovascularization (NV) is the common pathological pathway of those diseases such as retinal vein occlusion (RVO), diabetic retinopathy (DR), and choroidal NV, *etc*[1]. Up to date, the mainstream treatment for those disorders is anti-vascular drug administration, such as bevacizumab, ranibizumab and aflibercept, *etc*[2-4]. However, in addition to the pronounced anti-vascular effect, the ensuing fibrosis may also bring about many severe problems, like retinal pigment epithelium tear, tractional retinal detachment and so on[5-10]. The normal angiogenesis process requires a fine balance between the pro-vascular and anti-vascular biomolecules, including angiopoietin 2, vascular endothelial growth factor (VEGF), tumor necrosis factor α (TNFα), platelet derived growth factor (PDGF), and basic fibroblast growth factor (bFGF), *etc*[11]. Cytokines like angiopoietin 2 and VEGF could promote endothelium proliferation, while TNFα could facilitate lumenization. bFGF and PDGF could promote pericyte proliferation and differentiation, maturation of the new-formed vessels[12-14]. However, overexpression of bFGF and PDGF could also contribute to tissue fibrosis[12,15-16]. Contraction of the fibrous tissue would result in such complications mentioned above. Anti-VEGF treatment could upregulate bFGF and promote tissue fibrosis[6,16-18], so inhibition of both NV and fibrosis simultaneously through anti-bFGF and -VEGF may provide a promising choice for the ocular vasculopathy or fibrosis. Nintedanib is a tyrosine kinase inhibitor that specifically target on platelet derived growth factor receptor α and β (PDGFR α, β), fibroblast growth factor receptor 1, 2, and 3 (FGFR 1, 2, 3), and vascular endothelial growth factor receptor 1, 2, and

3 (VEGFR 1, 2, 3)[19-22]. Oral administration of nintedanib has been approved by Food and Drug Administration (FDA) for idiopathic pulmonary fibrosis in clinic. Experimental cellular researches also showed that nintedanib could inhibit migration and differentiation of fibrocyte and downregulate bFGF and VEGF level[23]. Recently topically used nintedanib was proved to inhibit corneal NV in rabbit models[24]. Patients with RVO usually showed higher bFGF and VEGF concentration in aqueous humors, which were parallel with the disease activity[8,25-29]. Therefore, we postulate that intravitreal injection of nintedanib would inhibit bFGF and VEGF expression elevated by RVO. In this study, we aim to evaluate those growth factors changes after intravitreal use of nintedanib in rabbit RVO models.

MATERIALS AND METHODS

Ethical Approval　Animal experiments were approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University & Laboratory Animal Centre of Wenzhou Medical University (Wydw2019-0388).

Retinal Vein Occlusion Model　Conventional gray rabbit (Huashu Biotechnology, Hangzhou, China) about 2.5-3.0 kg was chosen in this study, regardless of sex. Xylazine hydrochloride (LuMianNing, Huamu, Jilin Province, China) was intramuscularly injected for general anesthesia (10 mg/kg). The rabbits' pupils were dilated with compound tropicamide eye drops (Jinyao, Univision, China) three times with 5min intervals. After intravenous injection of indocyanine green (ICGA; Ruidu, Dandong Yichuang, China) in a concentration of 5 mg/mL (0.5 mg/kg), 810 nm solid infrared laser (Supra 810, Quantel Medical, France) was introduced to the two retinal veins about two papillary discs diameter apart from the optic papillary margin in both eyes, using a headpiece instrument (Keeler Vantage Plus, Keeler, UK). The total laser burning area were about two papillary discs in diameter along the retinal veins. Delivered laser power was set at 300-400 mW and the duration of each ablation was 0.5s. Direct sign of RVO was venous constriction or complete occlusion at the burning area and torturous, engorged, or dilated vein shaping in distal area, some of which might show superficial retinal hemorrhage (Figure 1). Cases with Bruch membrane rupture, vitreous hemorrhage, retinal detachment or vitreoretinal fibrovascular proliferation were excluded. The vitreous cavity of the left eye was injected with nintedanib suspension on the third day after laser treatment (2 mm from the limbus), and the right eye was left as the control eye. Rabbit's fundus photos were taken through a portable fundus camera (Smartscope EY4, Optomed, Finland).

Nintedanib Preparation and Injection　Nintedanib suspension was prepared from oral capsules (150 mg, nintedanib esilate soft capsule, Ofev, Boehringer Ingelheim
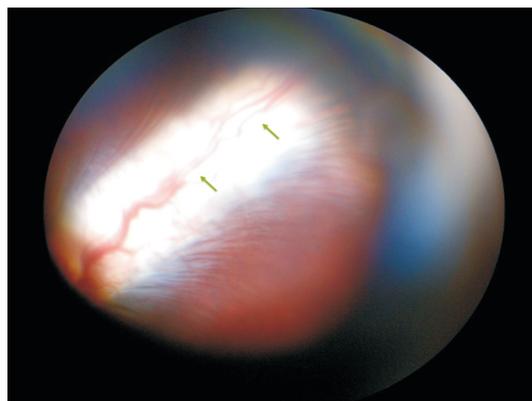

Figure 1 Rabbit retinal vein occlusion model　Rabbit retinal vein occlusion model was induced by 810 nm solid infrared laser that was enhanced by indocyanine green dye. The arrows indicated the occluded retinal vein immediately after laser delivery.
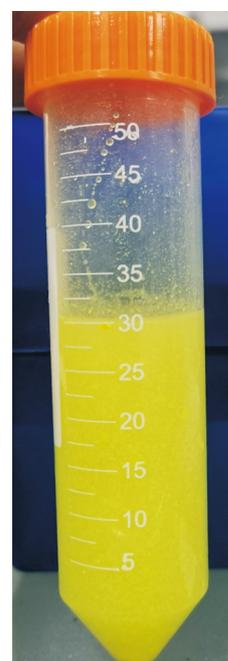

Figure 2 Nintedanib preparation for intravitreal injection　Nintedanib suspension was prepared from oral capsules. A capsule of 150 mg nintedanib was dissolved in 30 mL saline solution with a concentration of 5 mg/mL. A dose of 0.1 mL (0.5mg) nintedanib was used for intravitreal injection.

International GmbH, Germany). A 1-mL syringe was used to extract the drug from capsule and dissolved in saline solution with a concentration of 5 mg/mL, then 0.1 mL (0.5 mg) was injected into the rabbit's left vitreous cavity. During preparation, we assumed that rabbits had a similar pharmacodynamical process with human beings. The vitreous-applied dosage of nintedanib (200 ng/mL) was calculated from the empirical vitreous/peak serum concentration ratio (5:1) in clinical practice. The effective serum concentration of nintedanib was 40 ng/mL[30], while the mean volume of rabbit's vitreous cavity was 2.5 mL[31], so the total injection dose of nintedanib in each eye was set at 0.5 mg accordingly (Figure 2).
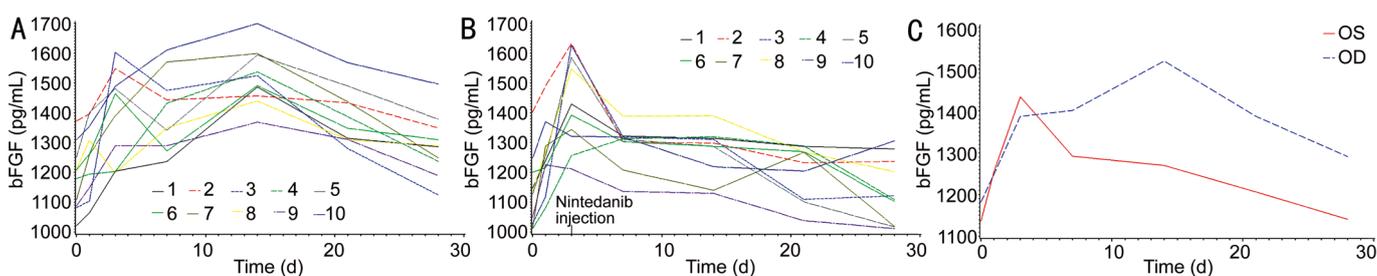
Figure 3 Trend analysis of basic fibroblast growth factor (bFGF) in the two eyes of rabbit models A: bFGF concentration trend on the sampling days in the control eyes (OD); B: bFGF concentration trend in the study eyes (OS), in which nintedanib was injected on the day 3; C: Comparison between the study eyes and the contralateral eyes.
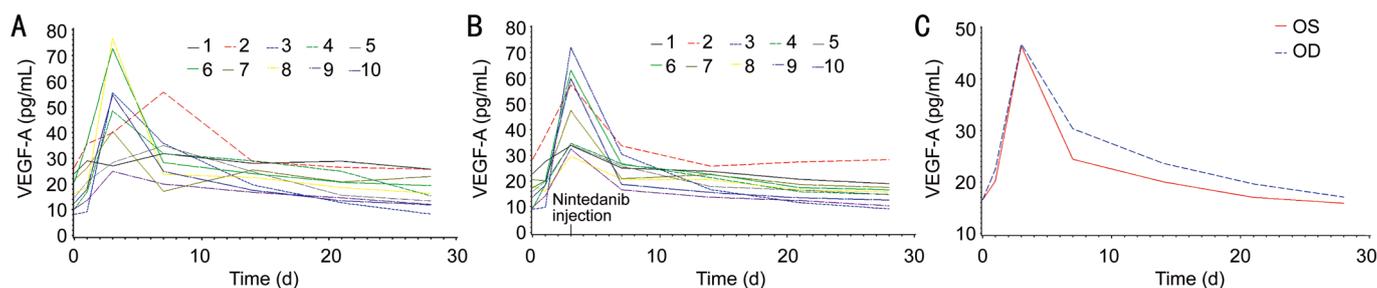

Figure 4 Trend analysis of vascular endothelial growth factor-A (VEGF-A) in the two eyes of rabbit models A: VEGF-A concentration trend on the sampling days in the control eyes (OD); B: VEGF-A concentration trend in the study eyes (OS), in which nintedanib was injected on the 3rd day; C: Comparison between the study eyes and the contralateral eyes.

Aqueous Humor Analysis of bFGF and VEGF Aqueous humor samples were taken through a 27 gauge 1-mL syringe after topical anesthesia (proparacaine hydrochloride eye drops, Alcaine, s.a. Alcon-Couvreur n.v., Belgium). The 0.1 mL of undiluted aqueous samples were taken on the day before laser treatment (baseline), and the day 1, 3, 7, 14, 21, and 28 after laser treatment. On the day 3, the left eye sample was collected before nintedanib injection. All samples were immediately stored in a sterile tube and transferred into a -80°C refrigerator for further investigation less than three months. bFGF and VEGF were tested by enzyme-linked immunosorbent assay (ELISA) using rabbit immunoassays (rabbit bFGF ELISA kit and rabbit VEGF ELISA kit, 96t, Fine test, Wuhan, China) according to the manufacturer's instruction, with a minimum detectable concentration of 15.625 pg/mL for bFGF and 1.562 pg/mL for VEGF-A.

Statistical Analysis All measures were recorded as mean±standard deviation (SD). A repeated analysis of variance method was performed to compare the sequential measures on the planned sampling days with the baseline. Paired t-test was used to analyze the difference between the nintedanib injected eye (OS) and the control eye (OD). P value of 0.05 was considered as the significant threshold. All statistical analysis was conducted by a commercial statistical software SAS 9.2 (SAS Institution Inc., Cary, NC, USA).

RESULTS

Thirteen rabbits were treated with laser coagulation, but three of which were excluded. One died on the day 7 due to suspected pulmonary fungal infection. One had vitreous hemorrhage during laser delivery and the other one had intracameral hemorrhage during intracameral sampling. No case of retinal detachment or vitreoretinal fibrovascular proliferation was observed during experiments. Total number of lasers spots delivered in the study eye were 53.80±10.25, and 54.40±10.63 in the control eye (P=0.8992). The bFGF concentration was 1136.45±123.67 pg/mL at baseline in the study eye and 1182.57±109.93 pg/mL in the control eye (P=0.1102), while the VEGF-A concentration was 16.49±6.35 pg/mL and 16.52±6.39 pg/mL respectively (P=0.9482).

After laser treatment in the control eye, the bFGF concentration rose significantly on the day 3, and peaked on the day 14. Then, it dropped gradually but remained higher than the baseline level on the day 28 (Figure 3A). However, after intravitreal injection of nintadanib on the day 3 in the study eye, the bFGF showed a significant reduction on the day 7, and keep decreasing in the following several weeks (Figure 3B). Then it returned to the baseline level on the day 28. In the terms of comparison between two eyes of cases, the study eye showed a significant decrease of bFGF at each testing point after intravitreal injection of nintadanib (Figure 3C; Table 1).

By contrast, the VEGF-A concentration peaked significantly on the day 3, but dropped rapidly soon after, and gradually returned to the baseline level from the day 7 to the day 28 (Figure 4A). When compared to the baseline, VEGF-A showed a significant increase on the days 1, 3, 7, and 14 (Table 2). After

Table 1 Comparison of intracameral bFGF concentration between two eyes of rabbit models pg/mL

bFGF	OD	OS	d	t	P
D0	1182.57±109.93	1136.45±123.67	-46.12±82.32	-1.77	0.1102
D1	1248.76±118.98[a]	1254.60±116.09[a]	5.83±96.99	0.19	0.8529
D3	1386.83±152.57[a]	1434.32±154.78[a]	47.49±153.56	0.98	0.3536
D7	1401.66±126.00[a]	1291.79±69.89[a]	-109.87±144.66	-2.40	0.0398
D14	1519.19±93.82[a]	1269.56±82.23[a]	-249.63±132.54	-5.96	0.0002
D21	1387.36±92.99[a]	1206.49±90.78	-180.88±127.30	-4.49	0.0015
D28	1290.75±103.48[a]	1140.32±109.85	-150.43±107.80	-4.41	0.0017

bFGF: Basic fibroblast growth factor; OD: The control eye in which only retinal vein occlusion was induced; OS: The study eye in which nintedanib was injected on the day 3 after retinal vein occlusion was induced; d: Concentration difference between two the eyes (C_{OS}-C_{OD}); D0-D28: The sampling days. Paired t-test was used to analyze the difference between two eyes. [a]Indicated significant increasement of bFGF concentration on sampling days when compared with the baseline (D0) through a repeated analysis of variance analysis.

Table 2 Comparison of intracameral VEGF-A concentration between two eyes of rabbit models pg/mL

VEGF-A	OD	OS	d	t	P
D0	16.52±6.39	16.49±6.35	-0.04±1.66	-0.07	0.9482
D1	22.44±9.11[a]	20.20±7.59[a]	-2.24±5.48	-1.29	0.2280
D3	46.83±18.24[a]	46.42±15.47[a]	-0.41±19.08	-0.07	0.9474
D7	30.38±10.85[a]	24.46±5.31[a]	-5.92±6.65	-2.82	0.0201
D14	23.64±4.47[a]	20.01±3.92[a]	-3.63±2.09	-5.47	0.0004
D21	19.65±5.69	17.03±4.62	-2.62±3.48	-2.38	0.0412
D28	17.10±6.10	15.86±5.34	-1.24±2.96	-1.33	0.2172

VEGF: Vascular endothelial growth factor; OD: The control eye in which only retinal vein occlusion was induced; OS: The study eye in which nintedanib was injected on the day 3 after retinal vein occlusion was induced; d: Concentration difference between the two eyes (C_{OS}-C_{OD}); D0-D28: The sampling days. Paired t-test was used to analyze the difference between two eyes. [a]Indicated significant increasement of bFGF concentration on sampling days when compared with the baseline (D0) through repeated analysis of variance analysis.

intravitreal injection of nintedanib on the day 3 in the study eye, the VEGF-A showed a significant reduction on the day 7, and remained reducing in the following days (Figure 4B). Though compared to the baseline VEGF-A of the study eye also showed significantly higher on the days 1, 3, 7, and 14, it showed a significant reduction when compared to the contralateral eye, except on the days 21 and 28 (Figure 4C; Table 2).

DISCUSSION
bFGF is an important cytokine in RVO pathogenesis, which is a heparin-binding growth factor, one of the nine members of the fibroblast growth factor (FGF) family[14]. bFGF can regulate cell growth and differentiation and promote retinal pigment epithelium (RPE) cells, fibroblasts and vascular endothelial cell mitosis and massive hyperplasia[32]. It can also promote leukocytes (like monocyte, T cell and neutrophil) recruitment to the inflammation sites through enhancing expressing of adhesion molecules in vascular endothelium[33]. After anti-VEGF therapy in a wide range of retinal or choroidal vasculopathy, fibrosis might ensue and cause severe

complications, like tractional retinal detachment, RPE tear, etc[6]. bFGF had been proved involving in those courses. In our study, we found bFGF concentration rose significantly on the day 3 of RVO induction in rabbit model, and peaked on the day 14, and we proved on animal level that nintedanib could inhibit bFGF expression effectively for several weeks. Nintedanib is a tyrosine kinase inhibitor that is specific for PDGFR, FGFR, and VEGFR. Treatment of oral nintedanib for one year has showed well control of clinical features of patients with idiopathic pulmonary fibrosis. Therefore, we tried to adopt nintedanib into ocular vascular or fibrotic diseases experimentally. So far, several researches demonstrated anti-fibrotic methods in retinal fibrosis so such as inhibiting Notch or TGF-β signaling pathway, a novel platelet activating factor receptor inhibitor, and connective tissue growth factor (CTGF) inhibitor, etc[34]. However, most of them focused on a single pathway of fibrosis, hereby we provide another potential choice through inhibition of both bFGF and VEGF levels.
The role of VEGF in pathogenesis in RVO is quite well established. RVO patients are characterized with increased

VEGF in their eyes, and the VEGF concentration in aqueous humor are correlated with severity of macular edema, NV of the iris, and vascular permeability[35-36]. In rabbits, few studies have been carried out to describe the VEGF changing curvature after RVO induction. Herein, we showed the VEGF concentration rose significantly on the day 3 after laser-induced RVO, but also dropped quickly after then. It was unsimilar with Neo *et al*'s[37] study, in which the VEGF concentration peaked on the day 7, and almost dropped back to the baseline in a couple of weeks. The discrepancy maybe resulted from the rabbit model inducing procedure. Rabbit RVO was mostly induced by argon green laser and enhanced by Bengal rose dye. But in this study, we induced RVO with infrared 810 nm laser and enhanced it with ICGA. The laser power of argon green laser was assumptively most absorbed by the red blood cells in the venous lumen and caused occlusion of the vein, but infrared laser was majorly absorbed by RPE cells. Though ICGA was used to enhance the intravenous absorption, extravascular damage (like RPE cells, neurocytes) of infrared laser was severer than that of argon green laser, which might result in the different secretion levels of VEGF. By the way, in our study, after vitreous injection of nintedanib in the left eye on the 3rd day, aqueous concentration of VEGF decreased significantly on the 7th day when compared to the control eye, and kept on a lower level until the 28th day. Interestingly, it was observed that after RVO induction, the concentration of VEGF peaked ahead of bFGF, but both could be effectively inhibited by nintedanib in several days after intravitreal administration.

The intravitreous dosage calculation of nintedanib applied in this study was a rough method. We just empirically deduced the dosage from some frequently used examples (like ceftazidime) in clinical practice. Furthermore, commercially used nintedanib was prepared in capsules, so it was not so accurate to prepare the nintedanib suspension here, even though we irrigated the capsular cavity repeatedly to minimize the dosage bias. However, to some degree, we still considered it as a potential approach to deal with retinal vasculopathy or fibrosis in the future.

As we mentioned above, another limit in this study was that we adopt 810 nm infrared thermal laser for induction of RVO model. When compared to the photodynamic method of RVO model preparation, thermal laser method had not only a more severe damage on the extravascular structures, and but also a shorter revascularization period. Usually, about two weeks after RVO induction, the occluded retinal vein might reopen again and showed a relatively normal fundus, so it was not suitable for further intervention for RVO researches, in case longer venous occlusion duration was required.

In conclusion, intravitreal use of nintedanib *in vivo* could to some degree inhibit bFGF and VEGF levels simultaneously in RVO development, which might be a promising approach for retinal vasculopathy or fibrotic diseases.

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