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Original Article

Optic Nerve Blood Flow in Neonatal Rats with Retinopathy of Prematurity-Like Abnormal Vascular Growth Induced by Vascular Endothelial Growth Factor Receptor Inhibitors

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ABSTRACT

Introduction: Retinopathy of prematurity (ROP) is a leading cause of childhood blindness. Understanding the underlying mechanisms and vascular changes in ROP is essential for improving diagnostic and therapeutic strategies. Therefore, this study aimed to determine the relationship between ROP-like fundus changes and optic nerve blood flow in rats.

Methods: Overall, 62 Sprague-Dawley rats were used. We induced ROP-like fundus changes in 29 rats by administering KRN633, a vascular endothelial growth factor (VEGF) receptor inhibitor, on postnatal days (P) 7 and 8. Subsequently, 33 age-matched control rats were treated with a vehicle (0.5% methylcellulose). We measured the optic nerve blood flow using laser speckle flowgraphy. Additionally, we determined arteriolar tortuosity, arteriolar diameter, and avascular and neovascular areas in the retinas using retinal flat-mount preparations. These were used as vascular parameters for evaluating fundus changes.

Results: The rats with ROP-like fundus on P21 had significantly higher mean blur rate (MBR) than the control rats, and MBR positively correlated with arteriolar tortuosity. The increase in MBR between P14 and P21 was correlated with arteriolar tortuosity, avascular area, and neovascular area. Multiple regression analysis revealed that arteriolar tortuosity had the strongest association with changes in optic nerve blood flow.

Conclusions: The rats with ROP-like fundus had significantly increased optic nerve blood flow than the controls, and this increase was associated with the severity of fundus lesions. The elevated optic nerve blood flow may be a novel indicator for predicting arteriolar tortuosity.

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KEYWORDS: abnormal retinal vascular growth, laser speckle flowgraphy, optic nerve blood flow, retinopathy of prematurity, VEGF receptor inhibitor

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Introduction

Retinopathy of prematurity (ROP) is a major cause of childhood blindness.[1] Recent advancements in neonatal care have improved the survival rate of newborns with severe illness.[2, 3] Therefore, establishing appropriate medical examinations and treatment options is crucial to prevent blindness in infants with ROP.

Sufficient optic nerve blood flow is important for retinal tissue development and retinal function maintenance. Our previous study demonstrated that optic papillary blood flow increased with growth.[4] However, laser speckle flowgraphy (LSFG) revealed a decreased optic papillary blood flow following treatment for severe ROP using retinal photocoagulation.[5] Therefore, an increased optic nerve blood flow may predict ROP severity.

Clinical studies involving patients with ROP are essential to understand the hemodynamics and pathophysiology of ROP fundus. However, the diverse pathological backgrounds of ROP and the limited number of patients pose challenges. In infants, accurate measurement of optic nerve blood flow requires advanced techniques. The fundus findings also change with eye manipulation, such as intraocular pressure. Moreover, continuous blood flow measurement until the condition becomes severe is challenging in actual clinical practice. These factors further complicate human ROP clinical studies.

Currently, the oxygen-induced retinopathy (OIR) model is the most common ROP animal model.[6, 7] Previous studies have revealed higher optic nerve blood flow in rats with OIR than in normal rats.[8] However, no study has investigated optic nerve blood flow in ROP models other than the OIR model. Additionally, abnormal retinal vascular growth and patterns have been observed after the short-term administration of vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibitors in neonatal mice and rats.[9, 10] The retinas of neonatal rats treated with VEGF receptor inhibitors displayed morphological features of the ROP fundus, including increased vascular density and tortuous arteries. However, whether the hemodynamic and vascular functions are altered has not been extensively addressed. Therefore, this study aimed to determine the relationship between optic nerve papillary blood flow and retinal lesions in an ROP-like abnormal vascular growth induced in the retina by treating neonatal rats with a VEGF receptor inhibitor.

Methods

Animals

All animal procedures followed the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research and the Regulations for the Care and Use of Laboratory Animals at Kitasato University, adopted by the Institutional Animal Care and Use Committee of Kitasato University (20-16), Tokyo, Japan.

Pregnant Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Tokyo, Japan) and maintained on a standard diet (Oriental Yeast, Tokyo, Japan) with *ad libitum* access to water. We inspected the rats daily to determine the date of offspring birth, which was defined as postnatal day (P)0. The animal housing facility had a lighting schedule from 8:00 a.m. to 8:00 p.m.

ROP animal model

We randomly divided the neonatal rats into control (n = 33) and ROP-like fundus (n = 29) groups. The sample size was approximately 30 cases based on previous studies.[8] We induced ROP-like fundus changes in the rats by injecting the VEGF receptor inhibitor, KRN633 (10 mg/kg), subcutaneously on P7 and P8.[10] We administered KRN633 (synthesized by Dr. Toru Nagamitsu at the Kitasato University School of Pharmaceutical Sciences, Department of Organic Synthesis) as a suspension in 0.5% methylcellulose in water. However, we subcutaneously injected the control group with the vehicle (0.5% methylcellulose) on P7 and P8.[10]

LSFG measurements

We used the LSFG-Micro System (Softcare, Fukuoka, Japan), designed for small animals, to measure the fundus blood flow.[11] The LSFG measures the relative velocity of moving red blood cells in the retina and choroid by calculating the mean blur rate (MBR) of the red blood cells.[12, 13] Previous studies have demonstrated that KRN633-treated rats (ROP-like fundus groups) exhibited abnormalities in capillary density and arteriolar tortuosity on P14, which increased further on P21.[10] Therefore, the LSFG measurements were conducted on P14 and P21 (Fig. 1A), and all assessments were performed between 2:00 p.m. and 5:00 p.m.

The animals were anesthetized via an intraperitoneal injection of a mixture containing butorphanol tartrate (2.5 mg/kg; Meiji Seika Pharma, Tokyo, Japan), medetomidine hydrochloride (0.375 mg/kg; Meiji Seika Pharma, Tokyo, Japan), and midazolam (2 mg/kg; Teva Takeda Pharma,

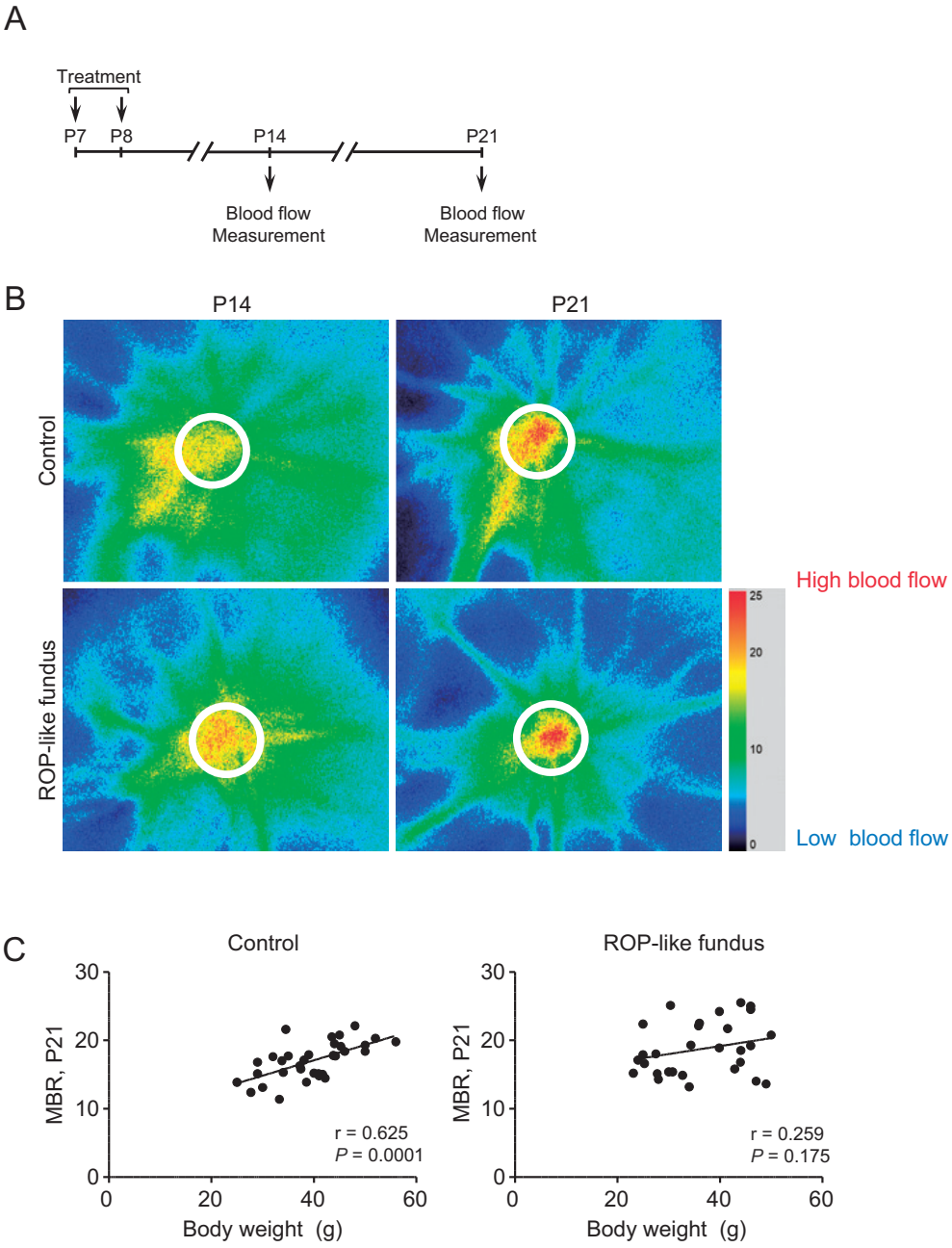

Fig. 1 Changes in ocular blood flow in control and retinopathy of prematurity ROP-like fundus groups. A, A schematic representation of the study protocol. Rats were treated subcutaneously with KRN633 (10 mg/kg) or vehicle (0.5% methylcellulose) on postnatal days (P)7 and P8. Laser speckle flowgraphy (LSFG) measurements were performed on P14 and P21. B, Representative LSFG images obtained from P14 and P21 rats treated with KRN633 (ROP-like fundus) or the vehicle (control) on P7 and P8. Red indicates high blood flow, whereas blue indicates low blood flow. A 50×50-pixel (50 pixels=0.25 mm) rubber band is positioned on the optic nerve disc. C, Correlation between body weights and mean blur rate (MBR). A significant positive correlation between body weights and MBR was observed in the control group, whereas no significant correlation was observed in the ROP group.

Nagoya, Japan). We placed a piece of viscoelastic material (hydroxyethyl cellulose) and a cover glass on the right cornea of each rat to measure the optic nerve blood flow. The cover glass was square with a weight that maintained the eyeball's shape. At the time of measurement, viscoelastic material was applied to the ocular surface, and the cover

glass was placed over it enough not to deform the eyeball. We focused on the retinal vessels centered on the optic nerve head (ONH). Vitreous blood vessels from the optic nerve papilla to the lens were also observed; however, we measured blood flow over the optic nerve papilla by focusing on the retinal surface information in this study. We performed the measurements thrice within a 5-minute interval. We analyzed the MBR by fixing a rubber band on a circle with a diameter of 50 pixels (0.25 mm) centered on the ONH center (Fig. 1B) using the LSFG Analyzer software program (version 3.1.14.0, Softcare Co.). The recorded MBR values at each time point represented the averages of three successive measurements.

Retinal tissue processing

After measuring the blood flow on P21, the animals were deeply anesthetized with sodium pentobarbital (Nacalai, Kyoto, Japan) and underwent transaortic perfusion with 1% paraformaldehyde in phosphate-buffered saline (PBS). Following the perfusion, we enucleated the eyes and stored them in a fixative at 4℃ for 24 hours. Subsequently, we removed the corneas, lenses, and sclera and incubated the retinas in 5% normal hamster serum in PBS containing 0.3% Triton-X (PBS-T; Nacalai, Kyoto, Japan) for 0.5-1.0 hours at room temperature. We used an anti-platelet endothelial cell adhesion molecule-1 antibody (1:500; R&D Systems, Minneapolis, MN, USA), an endothelial cell marker, and a fluorescein isothiocyanate-conjugated anti-alpha-smooth muscle actin (anti-αSMA) antibody (1:500; Sigma-Aldrich, MO, USA) as primary antibodies to visualize the vascular network and arteries/arterioles, respectively. We could easily identify arteries and arterioles because αSMA is highly expressed in arterial pericytes. Furthermore, we used Cy3-conjugated donkey antibody against goat immunoglobulins (1:400 dilution; Jackson ImmunoResearch, West Grove, PA, USA) as a secondary antibody. The retinas were rinsed in PBS-T, and retinal flat mounts were prepared using a fluorescence mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA).

We utilized the index of arteriolar tortuosity and arteriolar diameter, including the avascular and neovascular areas, as parameters to evaluate fundus changes. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to obtain the measured values. Fig. 2B shows the methods for measuring these parameters. We calculated the tortuosity index by dividing the measured vascular length of the artery from the central part of the optic disc to its first branch (b) by the linear distance (a) between its endpoints. Measurements were obtained for all the retinal arteries observed on the retinal flat mounts. We divided the sum of the measurements by the number of vessels measured, and the average value represented the degree of arteriolar tortuosity in the retina. The avascular area was determined as the size of the avascular peripheral retina relative to the entire retinal surface area. Similarly, we calculated the neovascular area as the area of the neovascular region relative to the vascular area.[10] The diameters of all the arteries near the optic disc were measured, and their average values were calculated to determine the arteriolar diameter. We performed all measurements thrice, and the average values were used for analysis. The ImageJ analysis was performed in a blinded manner to the experimental conditions.

Statistical analysis

The values are presented as mean ± standard deviation. We utilized GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA, USA) and JMP software version 12.1 (SAS, Cary, NC, USA) for the statistical analysis. Body weight (BW), MBR, changes in MBR, arteriolar tortuosity, arteriolar diameter, avascular area, and neovascular area on P14 and P21 were compared between the control and ROP-like fundus groups using the unpaired *t*-test. We evaluated the correlations between MBR at P21 and vascular parameters, including changes in MBR from P14 to P21 and vascular parameters using Pearson's correlation analysis. Multiple regression analysis was performed in rats with ROP-like fundus using MBR as the dependent variable and BW, degree of arteriolar tortuosity, avascular area, and neovascular area as the independent variables. Statistical significance was set at $p < 0.05$. Multiple regression analysis was performed using the forced-entry method.

Results

Optic nerve blood flow in ROP-like fundus rats

Fig. 1B shows representative LSFG images from P14 and P21 rats in the control and ROP-like fundus groups. In both groups, optic nerve blood flow increased with increasing age. However, the P21 rats with ROP-like fundus displayed greater optic nerve blood flow than the age-matched controls. The MBR was higher in P14 rats with ROP-like fundus than in P14 control rats, although the changes were insignificant (Table 1). In contrast, the MBR of P21 rats with ROP-like fundus was significantly higher

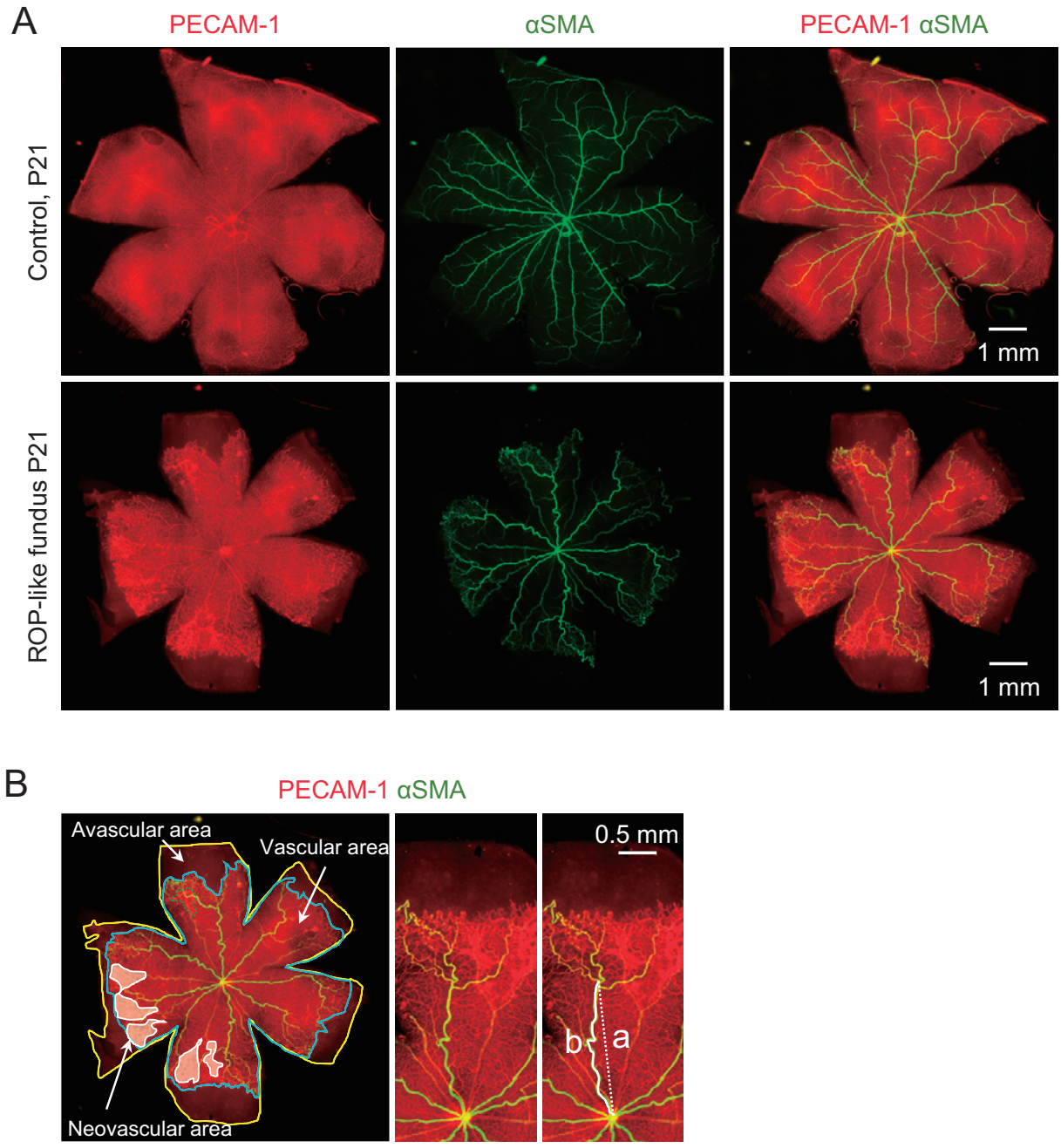

Fig. 2 Retinal vascular abnormalities in P21 rats with ROP-like fundus. A, Representative retinal whole mounts stained with platelet endothelial cell adhesion molecule-1 and alpha-smooth muscle actin (αSMA) obtained from P14 and P21 rats treated with KRN633 (ROP-like fundus) or the vehicle (control). B, The methods for assessing retinal vascular abnormalities. The tortuosity index of an artery was calculated by dividing its measured vascular length from the central part of the optic disc to its first branch (b) by the linear distance between the optic disc and the first branch of the retinal artery (a). The size of the avascular peripheral retina relative to that of the entire retinal surface area was determined as a measure of avascular area. The area of the neovascular region relative to the vascular area was calculated as a measure of the neovascular area.

than that of age-matched control rats.

BW comparison between control and ROP-like fundus groups

The BWs varied widely between the ROP-like fundus group and the control group (Fig. 1C). However, no significant differences in BWs were observed between both groups (Table 1). A significant positive correlation was observed between MBR and BWs in the control group (r =

Table 1 Body weight, heart rate, MBR, and changes in MBR (%) in control and ROP-like fundus groups

	Group	P14	P21
Body weight (g)			
	Control	28.5 ± 5.8	39.6 ± 7.5
	ROP-like fundus	27.1 ± 2.7	36.2 ± 8.6
	p-value	0.226	0.102
Heart rate (min^{-1})			
	Control	-	386.8 ± 38.6
	ROP-like fundus	-	367.7 ± 33.1
	p-value	-	0.043
MBR			
	Control	15.3 ± 2.7	17.0 ± 2.7
	ROP-like fundus	16.4 ± 3.7	18.7 ± 3.89
	p-value	0.18	0.045
Change in MBR (%)			
	Control	-	13.3 ± 19.3
	ROP-like fundus	-	18.9 ± 31.1
	p-value	-	0.398

MBR: mean blur rate, ROP: retinopathy of prematurity
p-value: unpaired *t*-test

0.625, $p = 0.0001$), whereas no significant correlation was observed in the ROP-like fundus group (r = 0.259, $p = 0.175$) (Fig. 1C).

Retinal vascular phenotypes in ROP-like fundus rats

Consistent with observations in previous studies,[10] the assessment of the retinal vasculature in P21 rats of the ROP-like fundus group revealed tortuous arteries and avascular and neovascular areas, similar to the vascular phenotypes observed in patients with ROP (Fig. 2A). Fig. 2B details the methods used to assess the retinal vasculature, and Table 2 summarizes the vascular parameters in each group. The ROP-like fundus group had a remarkably higher degree of arteriolar tortuosity and increased arteriolar diameter than the control group. The rats with ROP-like fundus exhibited avascular and neovascularized areas, which were not observed in the control rats.

Correlation between vascular parameters and optic nerve blood flow in ROP-like fundus rats

We examined the correlation between each vascular parameter and the MBR using values obtained from P21 rats in the ROP-like fundus group. The arteriolar tortuosity positively correlated with the MBR (r = 0.546, $p = 0.002$) (Fig. 3A). However, no significant correlation was observed between the arteriolar diameter (r = 0.091, $p = 0.640$)(Fig. 3B), avascular area (r = 0.273, $p = 0.152$) (Fig. 3C), or neovascular area (r = 0.355, $p = 0.059$)(Fig. 3D) and the MBR. The multiple regression analysis revealed that arteriolar tortuosity was the most significant contributor to MBR in P21 rats (Table 3).

Relationship between changes in MBR and vascular parameters in ROP-like fundus rats

We examined whether the changes in MBR from P14 to P21 were correlated with vascular parameters. We observed no significant difference in the degree of MBR change from P14 to P21 between control and ROP-like fundus groups (Control, 13.3 ± 19.3, vs. ROP-like fundus, 18.9 ± 31.1, $p = 0.39$). However, positive correlations were identified between changes in the MBR, arteriolar tortuosity (r = 0.484, $p = 0.008$), the avascular area (r = 0.410, $p = 0.027$), and neovascular area (r = 0.397, $p = 0.033$) in the ROP-like fundus group. The results of the multiple regression analyses revealed that no factor contributed to the changes in the MBR (Table 4).

Discussion

This study demonstrated that optic nerve blood flow increased in rats with ROP-like fundus induced by the short-term administration of the VEGF receptor inhibitor KRN 633. Furthermore, we observed that arteriolar tortuosity was strongly correlated with MBR, and changes in MBR in the ROP-like fundus rats were positively correlated with arteriolar tortuosity, avascular areas, and neovascular areas. Arteriolar tortuosity was the most significant

Table 2 Comparison of each retinopathy parameter between the control and ROP-like fundus groups

	Group	P14	P21
Arteriolar tortuosity			
	Control	-	1.03 ± 0.01
	ROP-like fundus	-	1.14 ± 0.07
	p-value	-	＜0.001
Arteriolar diameter (μm)			
	Control	-	33.93 ± 3.7
	ROP-like fundus	-	38.71 ± 5.1
	p-value	-	＜0.0001
Avascular area (%)			
	Control	-	0
	ROP-like fundus	-	14.7 ± 9.8
	p-value	-	＜0.001
Neovascular area (%)			
	Control	-	0
	ROP-like fundus	-	4.78 ± 3.5
	p-value	-	＜0.001

ROP: retinopathy of prematurity
p-value: unpaired *t*-test

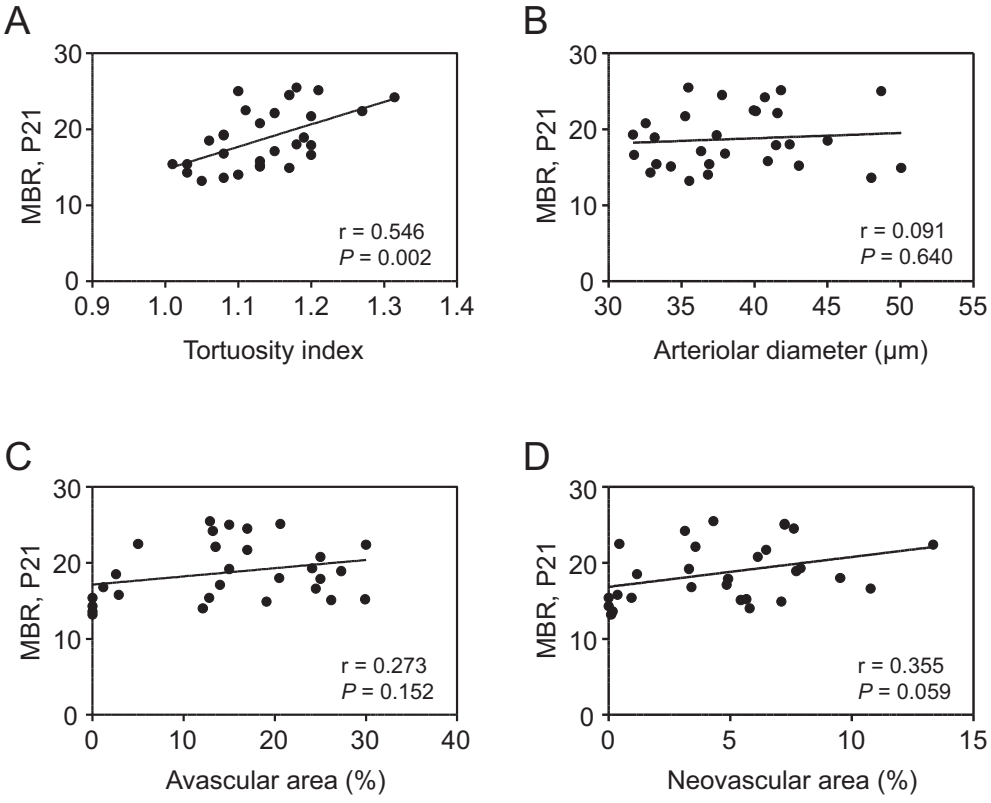

Fig. 3 Relationship between each vascular parameter and MBR in P21 rats of ROP-like fundus group. A, arteriolar tortuosity; B, arteriolar diameter; C, avascular area; D, neovascular area. A positive correlation was observed between arteriolar tortuosity and the MBR, whereas no correlation was observed with arteriolar diameter, avascular area, or neovascular area.

Table 3 Multiple regression analysis between MBR and body weight and vascular parameters in P21 rats in ROP-like fundus group

	β	*p*-value	VIF
Body weight	0.165	0.039	1.154
Arteriolar tortuosity	29.262	0.014	1.719
Avascular area	0.114	0.726	3.529
Neovascular area	− 0.002	0.984	3.536

VIF: variance inflation factor, MBR: mean blur rate, ROP: retinopathy of prematurity, P21: prenatal day 21

Table 4 Multiple regression analysis between changes in MBR and body weight and vascular parameters in P21 rats in ROP-like fundus group

	β	*p*-value	VIF
Body weight	− 0.006	0.374	1.154
Arteriolar tortuosity	1.610	0.114	1.719
Avascular area	0.004	0.891	3.529
Neovascular area	0.003	0.784	3.536

VIF: variance inflation factor, MBR: mean blur rate, ROP: retinopathy of prematurity, P21: prenatal day 21

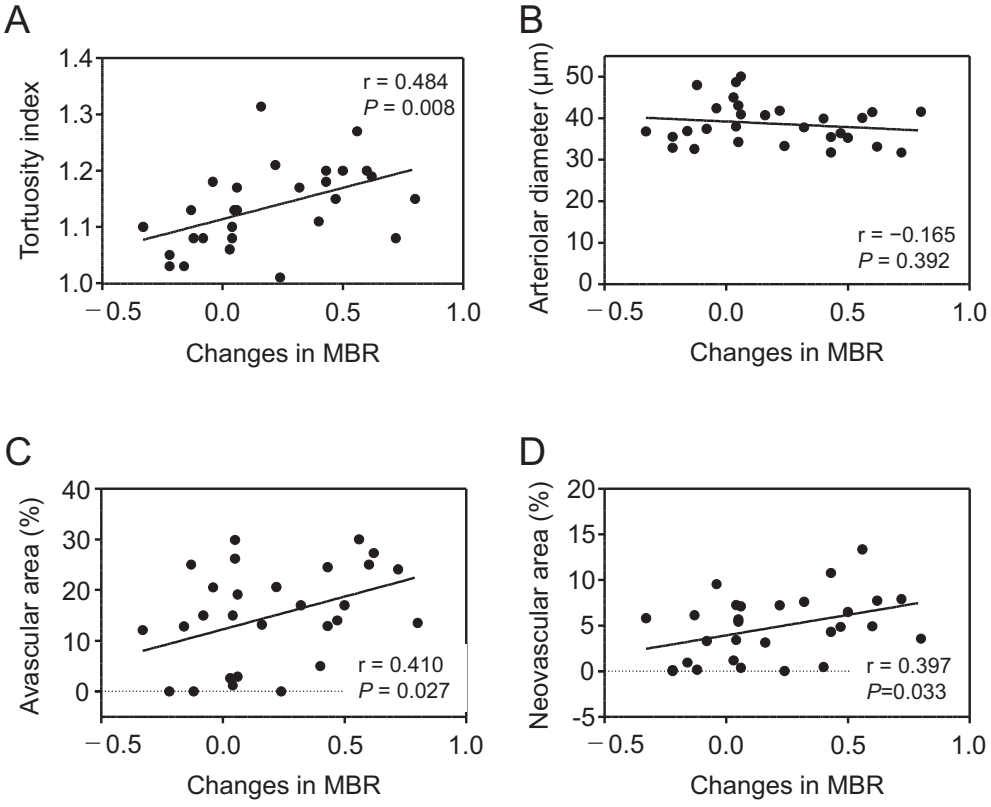

Fig. 4 Relationship between the changes in MBR and each vascular parameter in ROP-like fundus group. The changes in MBR from P14 to P21 (%) were calculated, and the correlations between the changes in MBR and each vascular parameter were determined.
Positive correlations were observed between the changes in MBR and arteriolar tortuosity (A), avascular area (C), and neovascular area (D).

contributor to MBR values.

Animal experiments are useful for ROP because human ROP cases are few, and pathological studies regarding ROP are difficult. The OIR is currently the most used model in ROP animal experiments.

Rodent models of OIR are widely used in ROP studies owing to their resemblance in pathologic changes with human ROP under hyperoxic conditions. However, OIR models are difficult to produce, and only a few facilities can produce them. The model used in this study is useful because it is relatively easier to fabricate than the OIR model, which is usually used for animal experiments. Previous studies have reported increased ocular blood flow on P18 in OIR rats when retinopathy is most severe.[8] These findings suggest that ROP causes peripheral ischemia. However, in cases of severe ROP, blood flow in the posterior pole, particularly in the optic nerve, increases. Unlike OIR models, short-term treatment with VEGF inhibitors

can induce fundus changes similar to human ROP without hyperoxia exposure.[10] Furthermore, we discovered that rats with ROP-like fundus treated with KRN633 on P7 and P8 had higher MBR values than the age-matched controls. Additionally, the increased blood flow to the optic nerve papillae in our study was consistent with the findings of previous studies in neonatal and OIR-induced ROP models.[5, 8] Similarly, ocular arterial blood flow increases with ROP severity in human neonates with ROP.[14, 15] In addition, optic nerve blood flow decreases after retinal photocoagulation or intravitreal injection of bevacizumab (Avastin®) for ROP.[5, 16] The similarities in optic nerve blood flow alterations observed in human ROP and rodent ROP models induced by exposure to hyperoxia or VEGF inhibitors suggest that these experimental models are valuable for investigating ROP pathology. The VEGF inhibitor-induced models of retinal vascular abnormalities do not require facilities to keep animals under hyperoxic conditions, making them useful for investigating ocular blood flow changes under pathological conditions.

Patel et al. reported that diabetic retinopathy, which causes retinal ischemic changes similar to ROP, exhibits retinal vessel hyperperfusion to compensate for retinal ischemia.[17] Previous studies have indicated increased optic nerve blood flow in the early stages of proliferative diabetic retinopathy.[18] Excessive blood inflow may cause vascular remodeling, including changes in the vascular smooth muscle phenotypes,[19] potentially contributing to the retinal arteriolar tortuosity formation. Further pathological clarification of the relationship between arteriolar tortuosity and blood flow is required; however, increased blood flow is associated with arteriolar tortuosity. Evaluating the presence or absence of arteriolar tortuosity is a subjective process; however, determining the factors for appropriate treatment is important. Therefore, based on our findings, we propose that increased MBR values may serve as objective parameters for determining the presence or absence of vascular meandering.

The increased blood flow in the optic nerve papillary region and arteriolar tortuosity in the ROP-like fundus may be associated with increased ocular VEGF levels. VEGF increases retinal blood flow in rats,[20] and VEGF neutralization decreases vascular tortuosity in OIR rats.[21] Clinical studies revealed that anti-VEGF therapy reduces ocular blood flow in neonates with ROP.[16] These findings suggest that increased intraocular VEGF associated with ROP promotes retinal blood vessel dilation, resulting in increased blood flow in the optic nerve papillary area. Nakano et al. demonstrated enhanced immunoreactivity of VEGF and VEGF receptor 2 in the retinas of P14 rats treated with KRN633 on P7 and P8.[10] Therefore, VEGF/VEGF receptor 2-signaling pathway enhancement may be involved in vascular abnormality progression and increased ocular blood flow in rats with ROP-like fundus.

This study had some limitations. First, the accurate interpretation of intraocular blood flow requires the knowledge of systemic blood pressure and intraocular pressure (IOP), which influence ocular perfusion pressure and blood flow. However, owing to the small sample size of the neonatal rats, accurate blood pressure and IOP measurements were impossible, resulting in the lack of information on these parameters. Second, LSFG provides relative values, making inter-individual comparisons challenging.[22] Dervenis et al. also reported that LSFG is a relative assessment in their summary of the methods used for assessing ocular blood flow.[23] However, the issue of relative values can be eliminated when evaluating changes in blood flow within the same individual. Finally, in this study, we induced an ROP-like fundus through systemic administration of a VEGF receptor tyrosine kinase inhibitor. Such systemic administration of antiangiogenic compounds can affect the development and structure of blood vessels in several organs, including the eye. Furthermore, the overall body size and growth of neonatal rats may influence optic nerve blood flow. Our results showed no significant difference in BWs between the control and ROP-like fundus groups, and the inhibitory effect of KRN633 on the VEGF receptor signaling pathway was eliminated on P9 and later. However, the possibility that the systemic and potential off-target effects of the VEGF inhibitor may have influenced the observed phenomenon cannot be excluded. Also, we did not measure VEGF expression in this study. Although perfusion is necessary to accurately evaluate fundus lesions, we prioritized accurate fundus evaluation in this study because VEGF concentration cannot be measured after perfusion. VEGF concentration measurement is needed in the future.

In conclusion, rats with ROP-like fundus showed significantly increased blood flow in the optic nerve papillary region, consistent with previously reported findings in neonatal human ROP or OIR rat models. The positive correlation between increased blood flow and arteriolar tortuosity indicates that optic nerve blood flow could indicate arteriolar tortuosity. However, further studies must eluci-

date the relationship between optic nerve papillary blood flow and arteriolar tortuosity pathophysiology in neonatal rats.

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Authors' contribution: M.T., T.M., and T.N. designed the study; M.T., T.M., M.A., and T.I. performed the experiments and analyzed the data; A.M. and T.N. provided critical reagents; T.N. and Y.H. supervised the experiments; M.T., T.M., and T.N. wrote the manuscript.

Ethics statement: Full name and affiliation of ethics committee and approved number (Committee at Kitasato University 20-16)

Conflicts of interest: T.M. owns stocks of Matsumoto Kiyoshi HD.

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