SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR AFTER BEVACIZUMAB OR RANIBIZUMAB TREATMENT FOR RETINOPATHY OF PREMATURITY

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Purpose: To investigate vascular endothelial growth factor (VEGF) levels in the systemic circulation after intravitreal injections of bevacizumab (IVB) or ranibizumab (IVR) in patients with Type 1 retinopathy of prematurity (ROP).

Methods: Patients who had Type 1 ROP and received IVB or IVR were enrolled. Serum samples were collected before and up to 12 weeks after IVB or IVR treatment. The main outcome measurements were serum levels of VEGF up to 12 weeks after anti-VEGF treatment.

Results: In total, 10 patients with Type 1 ROP were enrolled in this study. All the eyes had complete resolution of abnormal neovascularization of ROP after IVB or IVR. In the direct comparison of IVB with IVR, serum VEGF was found to be suppressed more in patients with Type 1 ROP who received IVB treatment, compared with those who received IVR treatment ($P = 0.01$, $P = 0.03$, and $P = 0.03$, respectively, 2, 4, and 8 weeks after intravitreal injection).

Conclusion: Serum VEGF levels in patients with Type 1 ROP were suppressed for 2 months after treatment with IVB, and VEGF levels were less affected after IVR treatment. Further studies are warranted to investigate the long-term effects of VEGF changes in ROP patients.

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Retinopathy of prematurity (ROP) is a leading cause of childhood blindness. Previous studies have found that a primary pathologic growth factor mediating neovascularization and the development of ROP is vascular endothelial growth factor (VEGF).[1] In addition to the standard treatment for ROP using laser photocoagulation, the identification of angiogenesis regulators has enabled the development of novel therapeutic approaches involving the use of anti-VEGF medications to treat patients with Stage 3+ or Type 1 ROP.[2,3] Most of the blindness caused by ROP could therefore be prevented if the treatments are administered in a timely fashion.

Bevacizumab (Avastin; Genentech Inc, South San Francisco, CA) and ranibizumab (Lucentis; Genentech Inc) are 2 anti-VEGF agents with different molecular sizes, structures, and half-lives.[4,5] Intravitreal injections of bevacizumab (IVB) and ranibizumab (IVR) have demonstrated efficacy in the treatment of Type 1 ROP.[2,3,6–8]

However, Sato et al[9] found that IVB resulted in bevacizumab entering the systemic circulation, and serum VEGF was suppressed accordingly for up to 2 weeks after IVB; our previous study confirmed that bevacizumab was in the systemic circulation 1 day after IVB, and VEGF was suppressed for 2 months.[10] Vascular endothelial growth factor is considered to be an important neurodevelopmental growth factor in the early newborn period.[11] The safety of anti-VEGF medications in the treatment of ROP remains to be elucidated.[3,8]

Both serum and plasma samples have been used in clinical studies. However, which sample is more representative of the peripheral VEGF level remains uncertain. Plasma has been suggested to represent a more accurate assessment of circulating VEGF.[12] However, because citrated plasma VEGF levels are low and usually lie at the lower detection limit of currently available enzyme-linked immunosorbent assay (ELISA), serum assessments might have greater sensitivity.[13]

To understand better the systemic suppression of VEGF after treatment of ROP patients with anti-VEGF agents, we investigated the serum concentrations of VEGF in the systemic circulation before and up to 3 months after IVB or IVR for high-risk ROP patients. In this prospective study, serum VEGF changes were compared in patients treated with bevacizumab or ranibizumab. The assay only measured free VEGF and did not measure total VEGF.[10] Specifically, VEGF bound to bevacizumab or ranibizumab could not be detected.

Methods

Patients

This investigation was a prospective cohort study assessing the serum levels of VEGF in Type 1 ROP patients before and after IVB or IVR. Patients with Type 1 ROP, as defined by the ETROP study,[14] who received IVB or IVR were enrolled. Patients who underwent laser treatment previously, laser treatment after IVB or IVR, or transfusions of whole blood before or after IVB or IVR were excluded. This study was conducted from February 2013 to December 2014 at the Chang Gung Memorial Hospital in Taoyuan, Taiwan, and it was approved by the Institutional Review Board of the hospital (IRB100-3476A3 and IRB100-4294A3). The research adhered to the tenets of the Declaration of Helsinki. The status of the off-label use of IVB and IVR for ROP treatment was explained to the parents of the patients in detail. The choice of IVB or IVR treatment was made by the parents. The parents were well informed about the efficacy and possible complications with both forms of treatment, including the risks of retinal detachment, endophthalmitis, and systemic VEGF suppression and the possible neurodevelopmental impact after anti-VEGF treatment. Adverse events reported with the systemic administration of anti-VEGF monoclonal antibodies, including thromboembolic events, myocardial infarction, stroke, kidney disease, hypertension, and gastrointestinal perforations, were monitored after the intravitreal injections in these ROP patients.[15–18] Neither of the treatments was covered by the national insurance, and the parents had to pay for the treatments. All the parents provided informed consent before the administration of IVB or IVR, and written informed consent was obtained from the parents for enrollment of their children in the study.

Intravitreal Injection of Antivascular Endothelial Growth Factor Drugs

The technique used for intravitreal injection of anti-VEGF agents was as previously described.[2] The anesthesia involved an intravenous injection of midazolam (Dormicum; Cenexi SAS, Fontenay-sous-Bois, France) or fentanyl (Fentanyl-Fresenius; Bodene Limited, Port Elizabeth, South Africa) to sedate the infant before treatment. Vital signs were monitored throughout the whole procedure. The eyes were prepared in a standard fashion using 5% povidone/iodine and topical antibiotics; 0.625 mg (0.025 mL) of bevacizumab or 0.25 mg (0.025 mL) ranibizumab was injected intravitreally ~1.5 mm posterior to the limbus via the pars plicata under intravenous sedation. The injection was performed initially with a 30-gauge needle directed perpendicularly to the globe and then directed slightly toward the center of the globe after the tip of the needle passed the lens equator. Care was taken to prevent damaging the lens or retina. After the injection, retinal artery perfusion was checked, and the patients received the topical antibiotic levofloxacin (Cravit; Santen Pharmaceutical Co, Osaka, Japan) for 7 days.

Vascular Endothelial Growth Factor Measurement After Intravitreal Injections of Bevacizumab or Ranibizumab

Blood samples were collected 1 to 2 days before intravitreal injection and at 2, 4, 8, and 12 weeks after IVB and IVR. Baseline blood samples were drawn 1 to 2 days before intravitreal injection. The tested serum target was VEGF, which was measured with ELISA. The procedures were performed according to a previous study with some modifications.[9] The blood samples were collected in sterile tubes and centrifuged at 3,000 rpm for 10 minutes until a clear separation between the serum and the cell components was seen. The serum was then transferred to sterile tubes and stored at −20°C until the assay. The serum concentration of VEGF was measured with an ELISA kit for human anti-VEGF (Human VEGF Immunoassay; R&D Systems, Minneapolis, MN), which was able to detect the 121 and 165 isoforms of VEGF according to the manufacturer's protocol. The minimum detectable level of the test was 9.0 pg/mL.

All the measurements were performed twice to obtain average values.

Statistical Analysis

Data are presented as the median (range) or the mean ± standard deviation (SD). We used Wilcoxon signed-rank test to compare differences at each time point in serum levels of VEGF before and after treatment. Additionally, the trend in VEGF level changes at various time points within a group was evaluated with Friedman test. The Mann–Whitney *U* test was conducted to compare the differences in baseline VEGF levels and the proportion of the reduction in VEGF levels from baseline to each follow-up time point between the 2 treatment groups. Statistical Analysis System (SAS) software, version 9.2 (SAS Institute Inc, Cary, NC), was used for all the data analyses. A *P* value <0.05 was considered to indicate statistical significance. The level of significance was pre-specified in the study protocol.

Results

Twelve patients were included in the study; however, 1 patient had a severe cardiopulmonary disorder and an unstable clinical course so that the pediatricians judged that it was not appropriate to draw additional blood 4 weeks after IVB. Thus, this patient was excluded. One patient received IVR first, but no positive response was observed. The patient later received IVB for the progression of ROP. This patient was excluded as well. In total, 10 patients (6 boys and 4 girls) with Type 1 ROP were enrolled in this study. The median time from blood collection at baseline to injection was 0 days (range, 0–1 day). Among these 10 study patients, 7 (70%) had a history of transfusions of packed red blood cells. Five patients received blood transfusions before intravitreal injection, and three patients received blood transfusions after intravitreal injection. The median time between blood transfusion and blood drawing was 12 days (range, 6–22 days). These transfusions were given at least 6 days before the blood samples were drawn. All the patients received transfusions of packed red blood cells, rather than the transfusion of whole blood. They should have had no or a minimal effect on the components of serum VEGF proteins.[19] The demographics of the patients are summarized in Table 1. Six patients received IVB, and four patients received IVR. Five patients (83%) received IVB in both eyes, and 1 patient (17%) received IVB in 1 eye. Four patients (100%) received IVR in both eyes. The mean gestational age of the infants was 27.2 ± 1.7 weeks (range, 24.1–29 weeks), and the mean birth weight was 1,024.6 ± 183.9 g (range, 786–1,320 g). All the patients received IVB or IVR as the primary treatment, and none of the infants underwent laser photocoagulation of the

Table 1. Demographic Characteristics of the Infants With ROP and Treatment

Patient No./Sex/GA, Weeks	Birth Weight, g	Study Eye	ROP Stage	Zone	Plus Disease	TVL	IVH	PDA	RDS	PPHT	BPD	NEC	Sepsis	Anemia	Pneumonia	Tx Group	PMA at Tx, Weeks	ROP Regression
1/F/28	1,136	Both	3	II	+	−	1	1	1	0	1	0	0	1	1	IVB	35	Yes
2/M/26.7	991	Both	3	II	+	+	1	0	1	0	1	0	1	1	0	IVR	33	Yes
3/M/27	1,020	Both	3	II	+	+	1	1	1	0	1	0	1	1	1	IVB	35	Yes
4/M/24.1	813	Both	3	I	+	+	0	0	1	0	1	0	1	1	1	IVB	33.1	Yes
5/M/29	1,250	Both	3	I	−	−	0	1	1	0	0	0	0	1	0	IVR	39.4	Yes
6/F/25.6	786	Both	3	II	+	−	0	0	1	0	1	0	1	1	1	IVR	39.9	Yes
7/M/28.7	1,320	Both	3	II	+	−	0	1	1	0	0	0	0	1	0	IVR	39.9	Yes
8/F/29	1,115	Both	2	II	+	−	0	0	1	0	0	0	0	1	0	IVB	35.3	Yes
9/F/25.6	995	Both	3	II	+	+	1	1	1	0	1	1	1	1	1	IVB	35	Yes
10/M/28.4	820	Right	3	II	+	+	0	0	1	0	1	0	0	0	0	IVB	39.6	Yes

− or 0, absent; + or 1, present; BPD, bronchopulmonary dysplasia; GA, gestational age; IVH, intraventricular hemorrhage; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; PMA, postmenstrual age; PPHT, primary pulmonary hypertension; RDS, respiratory distress syndrome; TVL, tunica vasculosa lentis; Tx, treatment.

peripheral avascular retina before IVB or IVR. The mean postmenstrual age at initial IVB or IVR was 37.5 ± 5.0 weeks. All the eyes had complete resolution of abnormal neovascularization of ROP and continued vascularization toward the peripheral retina after a single IVB or IVR treatment. At the end of follow-up, all the eyes had resolution of ROP, and none of the eyes showed recurrence of ROP. No obvious adverse systemic complications were noted in the patients after 17.1 ± 4.5 months (range, 9–22 months) of follow-up.

In patients receiving IVB only, the median (range) serum VEGF level was 440.6 (156.5–807.5) pg/mL before IVB (n = 6), which decreased to 58.5 (44.6–83.3) pg/mL at 2 weeks (n = 6; P = 0.03), to 58.8 (37.2–97.4) pg/mL at 4 weeks (n = 6; P = 0.03), to 79.2 (42.3–95.8) pg/mL at 8 weeks (n = 6; P = 0.03), and to 94.9 (56.3–163.2) pg/mL at 12 weeks after IVB (n = 5; P = 0.06). The serum VEGF level significantly decreased between baseline and up to 8 weeks in the ROP patients who underwent IVB treatment (P = 0.007). The results are shown in Table 2.

In patients receiving IVR only, the median (range) serum VEGF level was 351.8 (281.9–634.4) pg/mL before IVR (n = 4), which changed to 357.7 (162.7–704.1) pg/mL at 2 weeks (n = 4; P = 1.0), to 274.1 (162.3–619.3) pg/mL at 4 weeks (n = 4; P = 0.38), to 209.9 (164.9–459.3) pg/mL at 8 weeks (n = 4; P = 0.13), and to 197.6 (144.1–628.2) pg/mL at 12 weeks after IVB (n = 4; P = 0.13). There was no significant difference in the serum VEGF level between baseline and up to 8 weeks in the ROP patients who underwent IVR treatment (P = 0.212). The results are shown in Table 3 and Figure 1.

There was no significant difference in baseline VEGF levels between IVB and IVR (P = 0.91). A comparison of the proportion of serum VEGF reduction in patients treated with IVB or IVR at up to 12 weeks is shown in Table 4. Compared with baseline levels, serum VEGF was found to be more suppressed in patients with Type 1 ROP who received IVB treatment, compared with the levels in patients who received IVR treatment (P = 0.01, P = 0.03, and

Table 2. VEGF Changes From Baseline Up to 3 Months After Bevacizumab Treatment

Time	N	Median (Range), pg/mL	Mean ± SD, pg/mL	P^*
Baseline	6	440.6 (156.5–807.5)	419.7 ± 29.8	
2 weeks	6	58.5 (44.6–83.3)	61.8 ± 15.0	0.03
4 weeks	6	58.8 (37.2–97.4)	64.1 ± 25.7	0.03
8 weeks	6	79.2 (42.3–95.8)	76.5 ± 20.2	0.03
12 weeks	5	94.9 (56.3–163.2)	106.7 ± 44.8	0.06

*P value was evaluated by Wilcoxon signed-rank test.

Table 3. VEGF Changes From Baseline Up to 3 Months After Ranibizumab Treatment for Patients With ROP

Time	N	Median (Range), pg/mL	Mean ± SD, pg/mL	P^*
Baseline	4	351.8 (281.9–634.4)	405.0 ± 161.4	
2 weeks	4	357.7 (162.7–704.1)	395.6 ± 235.3	1.00
4 weeks	4	274.1 (162.3–619.3)	332.4 ± 207.7	0.38
8 weeks	4	209.9 (164.9–459.3)	261.0 ± 134.6	0.13
12 weeks	4	197.6 (144.1–628.2)	291.9 ± 226.4	0.13

*P value was evaluated by Wilcoxon signed-rank test.

P = 0.03, respectively, at 2, 4, and 8 weeks after intravitreal injection).

Discussion

Although the IVR group had a higher percentage of bilateral treatments than the IVB group, the IVB group had more significant serum VEGF suppression than the IVR group after anti-VEGF treatment. Our results showed that the serum VEGF decreased significantly for 2 months in patients with ROP after IVB treatment (P = 0.007; Friedman test). In contrast, serum VEGF did not change significantly after IVR treatment (P = 0.212; Friedman test). Although the safe range of VEGF serum concentrations in premature babies remains unknown, our data suggested that bevacizumab had a greater impact on systemic VEGF than ranibizumab, and clinicians should be cautious with its use in ROP patients.

During the study period, one female patient (not included in the study) with birth weight of 680 g received IVR initially for Stage 3, Zone II ROP, but the condition was found to have worsened after this treatment. She was then treated with IVB 2 weeks after
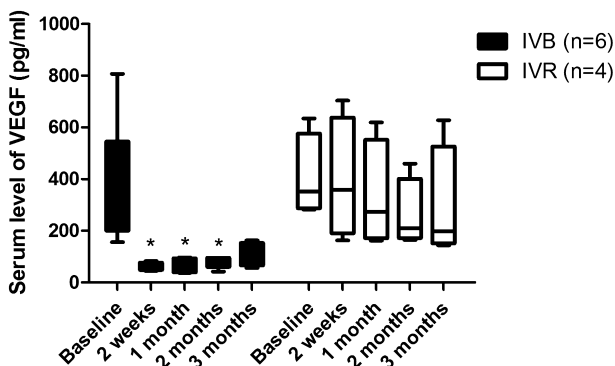

Fig. 1. Box plot showing the serum level of VEGF after IVB and IVR in patients with ROP. At baseline before IVB, the mean serum VEGF levels in the patients with IVB and IVR were comparable. After IVB, the serum level of VEGF decreased significantly for 2 months. However, the VEGF levels were not significantly different from baseline in the patients after IVR. *P < 0.05 compared with the data at baseline.

Table 4. Comparison of Bevacizumab and Ranibizumab on Proportion of Serum Vascular Endothelium Growth Factor Reduction From Baselines Up to 12 Weeks

Differences in Level of VEGF Between Different Time and Baseline	Bevacizumab (n = 6)		Ranibizumab (n = 4)		P*
	Median (Range)	Mean ± SD	Median (Range)	Mean ± SD	
2 weeks-baseline	−0.82 (−0.93 to −0.70)	−0.81 ± 0.09	−0.10 (−0.42 to 0.45)	−0.04 ± 0.40	0.01
4 weeks-baseline	−0.82 (−0.93 to −0.57)	−0.80 ± 0.13	−0.16 (−0.59 to 0.15)	−0.19 ± 0.33	0.03
8 weeks-baseline	−0.80 (−0.92 to −0.39)	−0.74 ± 0.20	−0.29 (−0.59 to −0.25)	−0.36 ± 0.16	0.03
12 weeks-baseline†	−0.67 (−0.90 to −0.24)	−0.62 ± 0.29	−0.38 (−0.57 to −0.01)	−0.33 ± 0.25	0.27

*Mann–Whitney U test.
†N = 5 in the bevacizumab group.

IVR, and her ROP regressed after IVB. The serum VEGF changes after IVR and IVB treatments are shown in Figure 2. Compared with the VEGF level at baseline, IVR caused little VEGF suppression, and IVB caused greater VEGF changes. This patient further strengthened our study results finding that IVB caused more significant VEGF suppression than IVR.

Similar outcomes have been reported in adult patients. Avery et al[20] found that both ranibizumab and bevacizumab rapidly moved into the bloodstream after intravitreal injection, but ranibizumab very quickly cleared, whereas bevacizumab demonstrated greater systemic exposure and produced a marked reduction in plasma-free VEGF. Carneiro et al[21] noted that the median serum level of VEGF was reduced by 42% in patients with age-related macular degeneration 28 days after receiving the third monthly IVB treatment, in contrast to no changes in patients treated with IVR. In the "alternative treatments to Inhibit VEGF in age-related choroidal neovascularization" (IVAN) study,[22] the serum level of VEGF in patients treated with bevacizumab was suppressed to approximately one-half of that in patients receiving ranibizumab. Compared with adults, the suppression of systemic serum VEGF in newborns lasts even longer, up to 2 months after intravitreal administration of bevacizumab.[10] This phenomenon should be considered in these young patients with rapidly developing systemic organs, and VEGF might participate in the process of organogenesis.

Various pharmacokinetic mechanisms of anti-VEGF therapeutics are possible causes of the observed significant suppression of systemic serum VEGF in patients with ROP after bevacizumab treatment. Bevacizumab is a larger, full-length immunoglobulin G (IgG) molecule with slower retinal clearance and therefore prolonged diffusion into the systemic circulation.[23] In contrast, the systemic half-life of a Fab molecule, such as ranibizumab, is a few hours, whereas that of a full-length IgG is up to 3 weeks in the general circulation.[24] Because of these structural differences, a much longer systemic half-life has been noted with bevacizumab compared with that of ranibizumab after intravitreal injection (20 days vs. 2 hours for bevacizumab and ranibizumab, respectively).[4,5] Finally, the neonatal Fc receptor (FcRn) plays a role in modulating IgG transportation and protecting against IgG catabolism, resulting in the prolonging of the serum half-life of IgG. Ranibizumab has no Fc portion, whereas bevacizumab contains the Fc portion structurally. The presence of FcRn in the retina could help to explain why bevacizumab is able to cross the blood–retina barrier.[25] Fc-containing molecules are recycled by binding to endothelial cell FcRn receptors to prevent them from entering the degradative pathway within endosomes.[26] This recycling decreases the systemic clearance of Fc-containing molecules, such as bevacizumab.

Sato et al[9] found that serum VEGF levels were suppressed for at least 2 weeks after IVB in ROP patients. Hong et al[27] found that IVB reduced plasma VEGF in infants with threshold ROP over a 7-week period. Our recent study showed that VEGF suppression persisted for up to 8 weeks after IVB.[10] Presently, there has
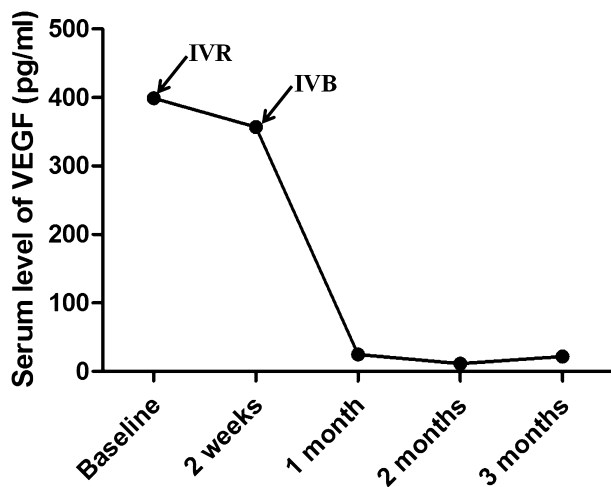

Fig. 2. The serum VEGF changes in one patient who initially received IVR, followed later by IVB. Compared with the VEGF level at baseline, IVR caused little VEGF suppression, and IVB caused more VEGF changes in this patient.

been only one case report of the serum level of VEGF in a patient receiving bilateral ranibizumab for ROP. In that patient, the serum VEGF level was suppressed for 3 weeks and returned to the original level 4 weeks later.[28] Zhou et al[29] recently found that IVR reduced plasma VEGF levels 1 day after injection in infants with ROP. This phenomenon disappeared 1 week after the injection. They concluded that IVR did not induce prolonged systemic VEGF suppression. Our data further confirmed that IVB resulted in more profound serum VEGF suppression than IVR.

Vascular endothelial growth factor is vital in angiogenesis, in maintaining organ health, in repairing wounds after injury, and in the development of various vital organs in the body.[30–38] Because VEGF concentrations are highly elevated in advanced ROP and because VEGF has been found to be a driving force in neovascularization,[39–41] it is a reasonable approach to block VEGF with anti-VEGF agents, such as bevacizumab, for the treatment of ROP. However, the inhibition of VEGF raises concerns that these important physiologic effects associated with VEGF will be inhibited, leading to abnormal organogenesis or neurodevelopment. Based on our current data, the selection of an anti-VEGF with less systemic VEGF interference or reducing the dose of anti-VEGF agent or using an anti-VEGF agent only once in ROP patients seems to be a safer choice because of the concerns mentioned above.

Vascular endothelial growth factor is stored in the alpha granules[42] and is released in platelet activation during clotting.[42,43] Therefore, serum VEGF levels are usually higher than plasma VEGF levels, and the wide confidence interval in the measurement of serum VEGF (median [range] of serum VEGF at baseline, 440.6 [156.5–807.5] and 351.8 [281.9–634.4] pg/mL in patients receiving IVB and IVR, respectively) could be related to this factor. Although plasma VEGF might contribute less to platelet clotting, plasma VEGF levels are very low and usually lie at the lower detection limit of currently available ELISA, rendering reliable measurement of plasma VEGF difficult. Therefore, both serum and plasma are important for the measurement of VEGF levels in the peripheral blood. A recent study of age-related macular degeneration also showed a similar trend of changes in serum and plasma VEGF levels after aflibercept (Eylea; Regeneron, Tarrytown, NY) or ranibizumab treatment.[44]

There were several limitations to this study, including fewer patients and fewer blood samples available at each time point. It is challenging to enroll patients with severe, acute ROP in prospective trials and to obtain adequate blood samples because the systemic condition of a newborn might not always be suitable to allow for blood samples to be drawn safely. The small

number of patients might have contributed to the wide confidence intervals in the measurement of serum growth factors in this study. Additionally, the choice of treatments was not random in the current study. Despite these limitations, our results showed that inhibition of VEGF was more significant with IVB than with IVR in the treatment of ROP.

In conclusion, IVB for Type 1 ROP was found to cause significant systemic serum VEGF suppression. In contrast, IVR for ROP resulted in no or barely detected suppression of systemic VEGF, compared with IVB. The suppression of systemic VEGF in this pediatric population was even longer than that found in adult patients. These findings suggested that IVB should be used with caution in the treatment of ROP. We do not know whether such VEGF alterations after IVB treatment in ROP patients are associated with poorer long-term neurodevelopment outcomes. Before we have the answer to this question, IVR could be a safer choice than IVB in the treatment of Type 1 ROP. In contrast, lower serum VEGF has been shown to reduce the incidence of intraventricular hemorrhage[45,46] but increase the incidence of necrotizing enterocolitis.[47,48] Future studies comparing the efficacy and the corresponding serum VEGF changes of a lower dose of bevacizumab than that used in this study and evaluating the long-term developmental outcomes of these patients are warranted.

Key words: intravitreal injections of bevacizumab, intravitreous injections of ranibizumab, retinopathy of prematurity, serum, vascular endothelial growth factor.

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