Pharmacokinetics of Bevacizumab and Its Effects on Serum VEGF and IGF-1 in Infants With Retinopathy of Prematurity

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Purpose. To measure serum levels of bevacizumab and to compare serum levels of free vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1) in infants who were treated with either intravitreal injection of bevacizumab (IVB) or laser for type 1 retinopathy of prematurity (ROP).

Methods. Twenty-four infants with type 1 ROP were randomized into three treatment groups: IVB at 0.625 mg per eye per dose, IVB at 0.25 mg per eye per dose, and laser. Blood samples were collected prior to treatment and on posttreatment days 2, 14, 42, and 60. Weekly body weights were documented from birth until 60 days post treatment. Serum levels of bevacizumab, free VEGF, and IGF-1 were measured with enzyme-linked immunosorbent assay (ELISA).

Results. Serum bevacizumab was detected 2 days after the injection, peaked at 14 days, and persisted for up to 60 days with half-life of 21 days. Area under the curve (AUC) analysis showed that systemic exposure to bevacizumab was variable among the subjects and was dose dependent. Serum free VEGF levels decreased in all three subgroups 2 days post treatment, with more significant reductions found in both IVB-treated groups, $P = 0.0001$. Serum IGF-1 levels were lower in both IVB-treated groups.

Conclusions. Clearance of bevacizumab from the bloodstream in premature infants takes at least 2 months. Although serum free VEGF levels decreased following either laser or bevacizumab treatment, the reductions were more significant in the IVB-treated groups. Potential long-term effects of systemic exposure to bevacizumab in infants need to be studied further.

Keywords: retinopathy of prematurity, laser, intraocular injection, bevacizumab, VEGF

Retinopathy of prematurity (ROP) is a leading cause of childhood blindness in the United States and worldwide.[1] Vascular endothelial growth factor (VEGF) is an important component in the pathogenesis of ROP.[2] The goals of ROP treatment include reducing the production of VEGF by the immature retina and eliminating the abnormal growth of new vessels. Currently, laser photocoagulation of the peripheral avascular retina is the treatment standard for type 1 ROP, with a failure rate of approximately 9.1%.[3] Recently, intravitreal injection of the anti-VEGF antibody bevacizumab (IVB) has been used off label as an alternative therapy.[4–9] However, VEGF is necessary for the development of systemic neurons and vessels in premature infants, and blockage of VEGF could potentially inhibit these processes. This concern is supported by a pharmaceutical study demonstrating that bevacizumab was indeed systemically absorbed.[10] It is unclear whether this absorption is dose related and whether there are short- and long-term effects on neurological development. A previous study also indicated that the systemic absorption of bevacizumab resulted in reduced serum VEGF levels[10]; however, further studies are needed due to the lack of a comparative control group in that study.

We hypothesize that the reduction of VEGF may occur in laser-treated infants as well as in IVB-treated infants. The reduction of serum VEGF levels may cause dysregulation of locally and systemically produced cytokines, such as insulin-like growth factor 1 (IGF-1). The role of IGF-1 in the development of ROP has been popularized since it was found to be involved in pathways that increase VEGF activity, such as in promoting hypoxia-inducible factor (HIF)–1α expression via the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathways and in regulating the growth of blood vessels through the P44/42 MAPK pathway.[11] Moreover, IGF-1 is important for postnatal weight gain in premature infants.[12]

In this study, we established the pharmacokinetics of bevacizumab in premature infants treated with different dosages and compared serum levels of free VEGF and IGF-1 before and after either bevacizumab or laser treatment for infants with type 1 ROP.

Table 1. Comparison of Risk Factors and Other Characteristics of Patients With Type 1 Retinopathy of Prematurity in Bevacizumab- and Laser-Treated Groups

	IVB, 0.625 mg, $n = 7$	IVB, 0.25 mg, $n = 10$	Laser, $n = 7$
Birth weight, g	630 ± 116	785 ± 266	714 ± 215
Gestational age, wk	24.2 ± 1.5	25.6 ± 2.4	24.6 ± 1.5
Sex, male (%)	4 (57)	6 (60)	2 (29)
PMA at treatment, wk	34.9 ± 1.7	36.5 ± 1.7	36.7 ± 2.0
Body weight at treatment, g	1776 ± 402	2115 ± 504	2349 ± 632

PMA, gestational age + weeks since birth.

Materials and Methods

Study Subjects

We confirm that the study followed the tenets of the Declaration of Helsinki, informed consent was obtained from parent or legal guardian after explanation of the study and possible consequences, and the research was approved by the Institutional Review Board (IRB) of Baylor College of Medicine.

All infants who had type 1 ROP in both eyes and were treated with laser or IVB from July 2012 to January 2014 were enrolled in this study and randomized into three groups: group 1, conventional laser treatment; group 2, IVB 0.625 per eye per dose; and group 3, IVB 0.25 mg per eye per dose. At beginning of the study, subjects were randomized into either group 1 or group 2 at a 1:1 ratio except those with zone 1 type 1 ROP, whom we treated with IVB. After seven patients were enrolled in each group, all subjects who met type 1 ROP criteria[3] were enrolled in group 3. All patients received treatment in both eyes.

The following data were collected: ROP status at the time of treatment, gestational age (GA), birth weight (BW), primary diagnosis, postmenstrual age (PMA) at time of treatment, body weight at time of treatment, and weekly body weight gain from birth until 2 months post treatment.

Blood samples were collected just before treatment (D0), and on posttreatment days 2 (D2), 14 (D14), 42 (D42), and 60 (D60). For serum collection, whole blood was collected in a microtainer tube with clot activators (Cat. no. 365963; B&D, Franklin Lakes, NJ, USA). Blood cells were removed by centrifuging at 10,000*g* for 15 minutes at room temperature. The supernatant was aliquoted into clean polypropylene tubes and stored in a –80°C freezer until analysis.

Measurement of Serum Bevacizumab, Free VEGF, and IGF-1 Levels

The serum levels of bevacizumab were measured with an enzyme-linked immunosorbent assay (ELISA) as described previously.[10] Briefly, microwell plates (Immuno 96 MicroCell solid plates; Nunc, Roskilde, Denmark) were coated with recombinant human VEGF165 (R&D Systems, Minneapolis, MN, USA) at a concentration of 1.0 μg/mL for overnight at 4°C (100 μL/well). The next day, the plates were washed five times with 1× PBS/0.05% Tween 20 (Sigma-Aldrich Corp., St. Louis, MO, USA), then incubated with 100 μL serum at room temperature for 1 hour. This was followed by 30-minute incubation of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) antibody (Invitrogen, South San Francisco, CA, USA) and a 15-minute reaction with TMB substrate (TMBW-0100-01; BioFX, Eden Prairie, MN, USA) in dark at room temperature. After adding 100 μL stop solution (STPR-0100-01; BioFX), the absorbance was read on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA) at wavelengths of 450 and 540 nm. A standard curve was prepared with bevacizumab ranging from 1 ng/mL to 5000 ng/mL. The background absorbance was subtracted from all values. Each assay was performed twice to obtain an average.

The serum levels of free VEGF and IGF-1 were measured with an ELISA kit (Quantikine ELISA for human VEGF and human IGF-1; R&D Systems). The manufacturer's protocols were followed. The minimum levels detected were 9.0 pg/mL for VEGF and 26 pg/mL for IGF-1. The absorbance was read on the same kinetic microplate reader at wavelengths of 450 and 540 nm. All assays were performed in duplicate.

Statistics

Statistical analyses were performed using IBM SPSS software version 21 (IBM, Armonk, NY, USA) and GraphPad Prism 5 (GraphPad, La Jolla, CA, USA). Bevacizumab serum concentration data were analyzed using noncompartment pharmaclkinetics (PK) model to determine area under the curve (AUC), peak concentration, and half-life. Demographic and clinical variables were summarized with descriptive statistics and are presented as mean ± standard deviation. Generalized estimating equations (GEE) models were used to analyze the relationship among variables. One-way ANOVA with post hoc multicomparison test was used to compare the three groups of data. Regression analysis was used to fit the growth model of body weight. Fisher's exact test was used for proportions. The comparisons of body weight among groups were adjusted for sex, GA, and BW. Confidence interval level was 95%. A P value less than 0.05 was considered statistically significant.

Results

Clinical Characteristics of Patients

Twenty-four infants were enrolled in this study. All infants received treatment for type 1 ROP in both eyes: 7 patients were treated with laser surgery, 7 patients were treated with IVB at 0.625 mg per eye per dose, and 10 patients were treated with IVB at 0.25 mg per eye per dose. The three groups were similar in baseline demographics with regard to mean GA, body weight at birth, mean PMA, and body weight at the time of treatment (Table 1). One-way ANOVA with post hoc multicomparison tests showed that there were no significant differences in BW ($P = 0.4$), GA ($P = 0.5$), PMA at time of treatment ($P = 0.2$), and body weight at the time of treatment ($P = 0.1$). There were noticeable difference for sex among the three groups; however, Fisher's exact test showed that these differences were not significant ($P = 0.3$, laser versus 0.625-mg IVB; $P = 0.2$, laser versus 0.25-mg IVB; and $P = 0.9$, 0.625-mg IVB versus 0.25-mg IVB). Retinopathy of prematurity status at the time of treatment is listed in Table 2.

Serum Concentrations of Bevacizumab

Figure 1A shows serum bevacizumab levels in IVB-treated patients from days 0 to 60 post injection (D0–D60). Bevacizumab was detected in the serum 2 days following injection, reached its peak level at 2 weeks, and persisted for at least 60 days in both IVB-treated groups. Among patients who received a total dose of 1.25-mg IVB (0.625 mg per eye per dose), the serum concentrations (mean ± SD) of bevacizumab on days 0, 2, 14, 42, and 60 were 0, 203.4 ± 115.6, 1002 ± 352.7, 444.4 ± 214.8, and 305.6 ± 190.3 ng/mL, respectively. Among patients who received a total dose of 0.5 mg (0.25 mg

Table 2. Comparison of Status of Type 1 Retinopathy of Prematurity at the Time of Treatment

ROP	IVB 0.625 mg $n = 14$	IVB 0.25 mg $n = 20$	Laser $n = 14$
Zone		*N* (%), eyes	
1	4 (29)	6 (30)	0 (0)
2	10 (71)	14 (70)	14 (100)
Stage		*N* (%), eyes	
2	7 (50)	10 (50)	7 (50)
3	7 (50)	10 (50)	7 (50)
Plus disease	14 (100)	20 (100)	14 (100)

per eye per dose) IVB, the serum concentrations (mean ± SD) of bevacizumab on days 0, 2, 14, 42, and 60 were 0, 72.9 ± 39.9, 424.0 ± 237.8, 172.5 ± 93.5, and 78.7 ± 69.0 ng/mL, respectively. The serum levels were significantly different between these two groups at all time points; *P* values are shown in Figure 1A.

Systemic exposure to bevacizumab over 60 days following the IVB was assessed by AUC_{0-60} analysis. The mean AUC_{0-60} in the 0.625-mg IVB group was 2.4-fold greater than the mean AUC_{0-60} in the 0.25-mg IVB group. Figure 1B shows the distributions of individual AUC_{0-60} among patients in both IVB-treated groups. Generalized estimating equations analysis revealed that there was no identifiable relationship between the serum levels of bevacizumab and body weight at the time of injection or blood collection. However, the mean serum bevacizumab level at each time point strongly correlated to ROP status at the time of treatment. Wald χ^2 analysis demonstrated that mean bevacizumab levels in patients with stage 2 disease were higher than the mean levels in stage 3 disease patients over all time points ($P = 0.003$) in both IVB-treated groups. In addition, the mean levels in patients with zone 2 disease were higher than in those with zone 1 disease across all time points ($P = 0.0001$) in both IVB-treated groups. Bevacizumab remained detectable in the blood for at least 60 days. The half-life for serum bevacizumab was 21 days.

Serum Levels of Free VEGF

At time D0, there were no significant differences in serum VEGF levels among the three groups (laser versus IVB 0.25 mg, $P = 0.6$; laser versus IVB 0.625 mg, $P = 0.9$; IVB 0.625 mg versus IVB 0.25 mg, $P = 0.6$). Serum free VEGF levels decreased in all groups 2 days following treatment, and the reductions were more significant in both IVB-treated groups (Fig. 2). In the laser-treated group, serum free VEGF levels decreased slowly for the first 2 weeks following surgery ($P = 0.002$ between D0 and D2; $P = 0.02$ between D2 and D14) and then plateaued afterward ($P = 0.9$). The two IVB-treated groups showed similar changes in serum free VEGF levels over time. Serum free VEGF levels decreased rapidly 2 days after the injection ($P = 0.0001$ for both groups), then plateaued at very similar levels. Serum free VEGF levels in both IVB-treated groups were approximately 50% of those in the laser-treated group at all time points post treatment. There were no significant differences in serum free VEGF levels between the 0.625 mg and the 0.25 mg IVB-treated groups over time, $P = 0.6$.

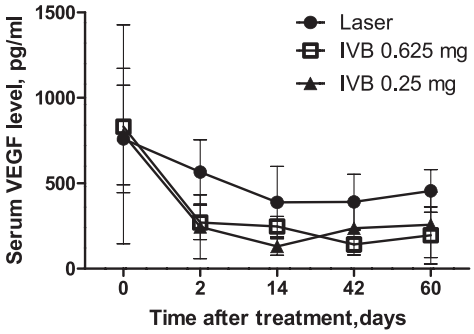

Figure 2. Changes of serum free VEGF concentrations in each of the three subgroups following treatment for type 1 retinopathy of prematurity. VEGF levels decreased significantly in all groups 2 days after injection. *P* values between D0 and D2 are 0.002 for laser-treated group and 0.0001 for both IVB-treated groups.

Serum Levels of IGF-1

Figure 3 shows that serum IGF-1 levels decreased in all three groups 2 days after treatment ($P = 0.5$ in the 0.25-mg IVB group; $P = 0.03$ in the 0.625-mg IVB-treated group; and $P = 0.03$ in the laser-treated group) and then increased over time. The significant increase occurred between D2 and D42 ($P = 0.03$ in the 0.25-mg IVB-treated group; $P = 0.02$ in the 0.625 IVB-treated group; and $P = 0.01$ in the laser-treated group). The changes in IGF-1 levels between the two IVB-treated groups

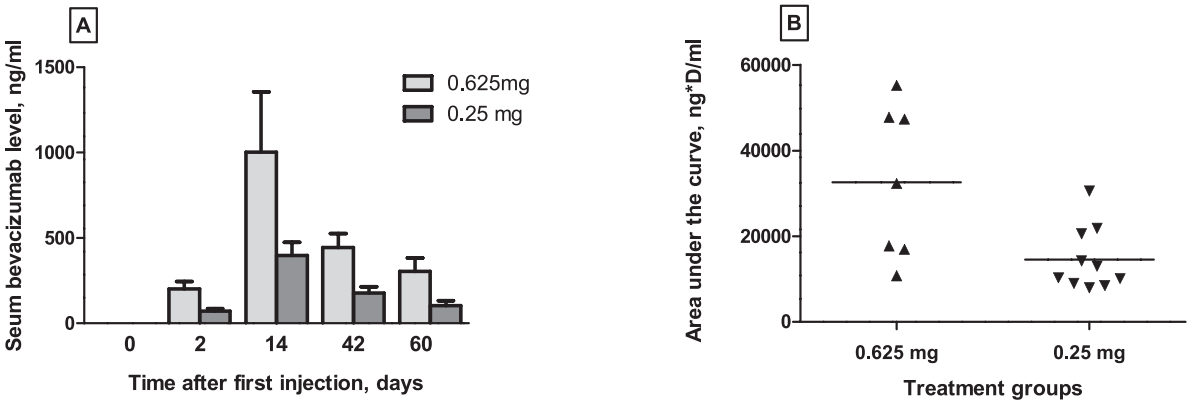

Figure 1. Serum levels of bevacizumab in patients treated with intravitreal injection of bevacizumab at different doses. (**A**) Serum levels at baseline, D2, D14, D42, and D60 post injection. *P* values between two groups at D2, D14, D42, and D60 days are 0.002, 0.05, 0.001, and 0.003, respectively. (**B**) Serum bevacizumab exposed to infants during 60 days following the injection. *Scatter dot plot* shows distribution of the AUC_{0-60} among the patients, with means represented by *horizontal lines* ($P = 0.01$). Mann-Whitney test showed that *P* value for distribution variable was 0.019.

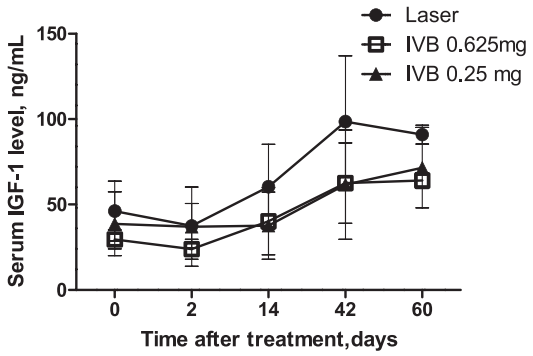

FIGURE 3. The changes of serum IGF-1 levels in patients after receiving different treatments for type 1 retinopathy of prematurity. *P* values between laser- and IVB-treated groups at D14, D42, and D60 days are 0.06, 0.05, and 0.3, respectively.

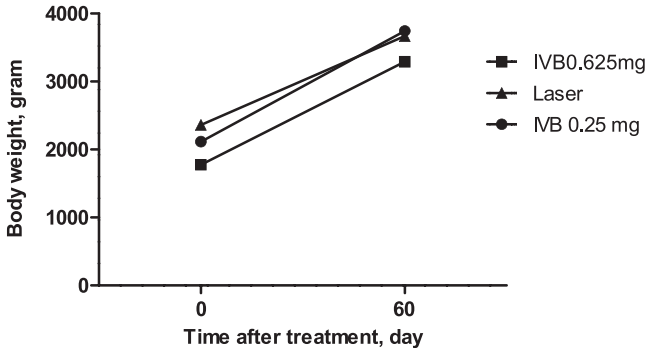

FIGURE 4. Body weight gains in infants from day 0 to day 60 after different treatments. The slopes in IVB 0.625-mg and laser groups are parallel. The slope in the 0.25-mg IVB group is steeper than for the other two groups.

were similar. However, serum IGF-1 levels in both IVB-treated groups were lower than the levels in the laser-treated group, as shown in Figure 3. Up to 42 days following treatment, a strong positive correlation was detected between the IGF-1 serum levels and the infant's body weight on the day that the serum was collected (laser: $r^2 = 0.89$; IVB 0.625 mg: $r^2 = 0.95$; IVB 0.25 mg: $r^2 = 0.74$). Vascular endothelial growth factor level was negatively correlated to IGF-1 level (laser: $r^2 = 0.49$; IVB 0.625 mg: $r^2 = 0.53$; and IVB 0.25 mg $r^2 = 0.51$).

Body Weight Gain

Body weight gains were measured weekly from birth to 60 days following treatment. The growth curve for each group was plotted with mean BW of the same postnatal age. The weight gain followed a linear relationship with age (with $r^2 = 0.92$, 0.96, and 0.98 in 0.25-mg IVB-treated group, 0.625-mg IVB-treated group, and laser-treated group, respectively). The average weight gain prior to ROP treatment was 126.9 g/week (SEM 7.7) in the 0.625-mg IVB-treated group, 121 g/week (SEM 4.6) in the 0.25-mg IVB-treated group, and 128.1 g/week (SEM 4.1) in the laser-treated group. After the treatment, infants in all groups showed continuous increase in body weight as shown in Figure 4. The average weight gain was 158.0 g/week (SEM 2.4) in the 0.625-mg IVB-treated group, 164.9 g/week (SEM 8.9) in the 0.25-mg IVB-treated group, and 157.6 g/week (SEM 4.7) in the laser-treated group. After adjustment for the covariates GA, sex, and BW, the *P* values for the body weight differences at the time of treatments were 0.3 (0.25- IVB versus 0.625-mg IVB), 0.1 (0.25-mg IVB versus laser), and 0.1 (0.625-mg IVB versus laser). The *P* values for body weight differences at 60 days post treatment were 0.1 (0.25-mg IVB versus 0.625 mg IVB), 0.48 (0.25-mg IVB versus laser), and 0.2 (0.625-mg IVB versus laser).

DISCUSSION

A major concern regarding anti-VEGF therapy in ROP is the risk of systemic absorption and its potential side effects. Our results revealed that bevacizumab was absorbed and remained in the bloodstream for at least 2 months following treatment. These findings are consistent with those reported by Sato et al.[10] and Avery et al.[13] In Avery's study, all three anti-VEGF agents (bevacizumab, Ranibizumab, and aflibercept) rapidly moved into the bloodstream after intravitreal injection for age-related macular degeneration (AMD) in adults. However, bevacizumab was cleared relatively slowly from systemic circulation compared to the other two anti-VEGF agents and appeared to accumulate with repeated dosing.[13] The molecular structure of bevacizumab may contribute to its slow clearance from the bloodstream as we observed in this study. Bevacizumab is a recombinant humanized monoclonal IgG-1 antibody containing the Fc domain.[14,15] The majority of IgG elimination occurs via intracellular catabolism, following fluid-phase or receptor-mediated endocytosis.[16] In the bloodstream, Fc-containing IgG antibodies are recycled by binding endothelial cell Fc receptors (FcRn) to protect them from the default degradative pathway within endosomes.[17]

The mechanisms of systemic absorption are unknown. Bevacizumab is larger molecule with molecular weight of 149 kD. There are several ways by which bevacizumab passes through the blood–retina barrier (BRB): simple passive diffusion, via an active transporter/receptor pathway; leakage through extracellular space; or absorptive endocytosis/diapedesis.[18] In infants, 100% of the vitreous is in the gel phase. The retina is primarily a cellular central nervous system tissue with 15- to 20-nm-wide intercellular spaces that do not contain tight junctions (TJs).[19] The BRB has two parts, the endothelial cells of the retinal capillary walls, and the retinal pigment epithelium (RPE). The molecular size cutoff for retinal penetration in the healthy eye is between 100 and 150 Å.[20] Recent animal data suggest that the presence of neonatal FcRn in rat eyes and particularly on the BRB may serve as active transport system for bevacizumab.[21–23] In addition, intravitreal bevacizumab was found in the blood vessel walls of the iris, anterior chamber angle, and ciliary body by immunohistochemistry staining, which suggests that it is transported across the iris vascular endothelial and ciliary body nonpigment epithelial TJs into the bloodstream, and through conventional aqueous humor outflow pathways.[24] Finally, in ROP eyes the endothelial cell gap junction increases. This may allow more drugs to pass through the BRB into the bloodstream. In our study, the serum levels of bevacizumab in the 0.625-mg IVB group were 2.4-fold greater than the levels in the 0.25-mg IVB group, which suggests that the systemic absorption is dose dependent. The difference of mean AUC_{0-60} between the two groups was significant, $P = 0.01$. However, the distribution of AUC_{0-60} in the 0.625-mg IVB group was more variable than that in 0.25-mg IVB group ($P = 0.019$); in addition, there were overlaps of individual AUC_{0-60} between the two groups, as shown in Figure 1B, which could be due to the saturation of FcRn in the retina. Nevertheless, our sample size was small and we cannot draw definite conclusions.

Bevacizumab directly inhibits the binding of VEGF to its receptors, Flt and KDR, on the endothelial cell surface. Vascular endothelial growth factor is critical for the development of blood vessels and neurons.[25,26] Sato et al.[10] found

significantly reduced serum VEGF levels 1 week after 0.5-mg intravitreal bevacizumab administration in infants. Serum VEGF concentrations were also reported to decrease following 1.25-mg intravitreal injections in a small pilot study of diabetic retinopathy patients.[27] In a similar study, intravitreal 1.25-mg bevacizumab injection significantly reduced plasma VEGF levels from 89.7 to 22.8 pg/mL in patients with diabetic macular edema, and from 72.2 to 17.1 pg/mL in patients with exudative AMD after 1 month.[28] Carneiro et al.[29] showed a 42% decrease in circulating VEGF after a third IVB in the treatment of neovascular AMD. A recent study showed that intravitreal bevacizumab appeared to produce significant suppression of free plasma VEGF following the first dose, eventually falling below the lower limit of quantitation between 1 and 7 days following the third dose.[13] Our results clearly showed that serum free VEGF levels decreased in both laser- and bevacizumab-treated groups after treatment. However, the reductions in the bevacizumab-treated groups were more significant than the reductions in the laser-treated group. In fact, the serum levels of free VEGF in the IVB-treated groups were approximately 50% of the levels found in the laser-treated group and remained at a lower level up to 2 months post treatment. The persistent lower levels of serum free VEGF could be due to the slow clearance of serum bevacizumab. The reduction of free VEGF in laser-treated patients could be due to the destruction of retinal cells that secrete VEGF.

In addition to the serum free VEGF levels, we measured the IGF-1 levels in the serum before and after different treatments. The role of IGF-1 has gained attention in the past. Studies have shown that the development of ROP is strongly associated with low levels of IGF-1,[30,31] and it was found to be involved in pathways that increase VEGF activity, such as in promoting HIF-1α expression via the PI3K/Akt and MAPK pathways, and in regulating the growth of vessels through the P44/42 MAPK pathway.[11,32] Our data showed that serum IGF levels increased over time for the first month after the treatment. However, the IGF-1 serum levels in the IVB-treated groups were lower than in the laser-treated group. Insulin-like growth factor-1 is critical for normal vascular development.[29,30] The lower levels of IGF-1 in the IVB-treated group might explain the slow maturation of retinal vessels in IVB-treated ROP patients.

In this study, we assessed the effect of different treatments on body weight gains. We measured the weekly body weight changes from birth to 60 days post treatment. Growth velocity, the change in growth over time, is a more sensitive index of growth than a single measurement of weight. The typical pattern of weight changes in infants is a variable degree of initial weight loss, followed by limited weight gain (70–150 g) between 10 and 20 days. In low BW preterm infants, an absolute growth rate is a reliable indicator of the weight growth pattern during the first year of life, and there is no significant difference between the two sexes.[33] There are some noticeable differences in sex and BW among groups in our study, possibly due to small sample size. In order to eliminate the confounding bias, the statistical analysis of body weight was adjusted for sex, GA, and BW. The weekly body weight gains were similar among groups before the treatment, and the infants continued to grow at similar rates after different treatments. It is comforting to see that although VEGF levels decreased with bevacizumab, the body weight gain was within normal range. However, due to the small sample size, we cannot draw definite conclusions from this study; future studies with larger sample size would be necessary.

This work has direct clinical implications for the treatment of ROP. The findings indicate that systemic absorption of bevacizumab is dose dependent. It is vital to find the lowest effective dose of bevacizumab for the treatment of severe ROP. Our results also demonstrated that bevacizumab in both commonly used dose and a reduced dose remained in the blood system for at least 2 months with reduced serum levels of free VEGF and IGF-1. A long-term large multicenter clinical trial is needed to adequately evaluate the systemic impacts of lower serum free VEGF and IGF-1 levels.

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