

Subretinal Bevacizumab Detection after Intravitreal Injection in Rabbits

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PURPOSE. To evaluate subretinal detection of bevacizumab 2 hours after intravitreal injection of 1.25 mg in rabbit eyes.

METHODS. Anterior chamber paracentesis using a 30-gauge needle was performed in nine female Dutch-belted rabbits by removal of 0.05 mL of aqueous humor. Transscleral retinal detachment was performed with a modified 25-gauge infusion cannula connected to a bottle of physiologic saline solution (PSS). The animals were divided into experimental group 1, with intravitreal injection of 0.05 mL of (1.25 mg) with a 30-gauge needle ($n = 6$) and the control group 2, with intravitreal injection of 0.05 mL of PSS with a 30-gauge needle ($n = 3$). Two hours after the intravitreal bevacizumab or PSS injection, subretinal fluid was aspirated and immunoassayed to measure the level of bevacizumab. The rabbits were killed by intravenous pentobarbital injection. The eyes were enucleated and fixed in 10% formaldehyde. The pars plana site at which the transscleral cannula was introduced was analyzed by light microscopy, to exclude iatrogenic retinal tears. Eyes with accidental retinal tears were excluded.

RESULTS. Subretinal bevacizumab molecules were detected in the six eyes that received an intravitreal bevacizumab injection. No subretinal bevacizumab was detected in the control eyes. Light microscopy showed no evidence of retinal tears or holes in any rabbits used for the bevacizumab detection and control group.

CONCLUSIONS. Bevacizumab molecules were detected in the subretinal space after intravitreal injection of 1.25 mg of bevacizumab, possibly as the result of diffusion through the retina in a rabbit model. (*Invest Ophthalmol Vis Sci.* 2008;49:1097–1100) DOI:10.1167/iovs.07-1225

Age-related macular degeneration (AMD) is the major cause of irreversible visual loss in patients older than 65 years in developed countries.^{1–4} The disease affects the choriocapillaris, Bruch's membrane, and retinal pigment epithelium. Loss of vision is caused by the development of choroidal neovascularization (CNV) and subretinal fluid as well as photoreceptor

dysfunction and atrophy. The risk factors include age and most likely genetics, smoking, and an associated inflammatory process.^{3,5}

Although a choroidal neovascular membrane develops in only 10% to 20% of eyes, it is the major cause of irreversible visual loss in AMD.^{1–3} Inhibition of vascular endothelial growth factor A (VEGF) is an effective strategy for treating the wet form of AMD.⁶

Bevacizumab (a purified humanized monoclonal antibody produced by recombinant DNA from Chinese hamster ovary; Avastin, Genentech, South San Francisco, CA) has been approved by the U.S. Food and Drug Administration (FDA) for treatment of metastatic colorectal cancer,^{7–9} but it has been used off-label in ophthalmology as a treatment to manage CNV and other ocular vascular diseases.^{10,11}

Bevacizumab is composed of 214 amino acids with a molecular mass of 149 kDa. Experiments in a primate model have suggested that molecules exceeding 100 kDa cannot cross the retinal layers into the subretinal space.¹² However, current clinical use of intravitreal bevacizumab injections worldwide as well as immunohistochemical analysis has shown that the drug has promising effects on CNV, indicating that this drug may diffuse through the retinal layers into the choroidal space.¹³

The objective of this study was to verify whether it is possible to detect bevacizumab in the subretinal space after intravitreal injection in rabbits.

METHODS

Nine eyes of female Dutch-belted rabbits (weight, 1.8–2.0 kg) were used in the study. All animals were treated in accordance with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research and the Rules of Ethics Committee from Federal University of São Paulo. The animals were anesthetized with intramuscular ketamine hydrochloride 35 mg/kg (Phoenix Scientific, Inc., Fort Dodge, IA) and intramuscular xylazine hydrochloride 5 mg/kg (Phoenix Scientific, Inc.). The pupils were dilated with tropicamide 0.5% (Mydracil; Alcon, Fort Worth, TX). A Barraquer wire speculum was used to keep the eyes open. An anterior chamber paracentesis was created with a 30-gauge needle (BD Biosciences, Franklin Lakes, NJ) in all animals, followed by removal of 0.1 mL of aqueous humor.

A transscleral retinal detachment was performed in all animals with a modified 25-gauge infusion cannula 2 mm long (Alcon, Fort Worth, TX) connected to a bottle of physiologic saline solution (PSS; Balanced Salt Solution, or BSS; Alcon) 100 cm above the level of the eye. Before the introduction of the modified cannula for transscleral detachment, a 7-0 Vicryl (polyglactin 910; Ethicon, Norderstedt, Germany) suture was placed 1.5 mm from the limbus. A gentle laminar scleral dissection 0.5 mm deep was performed where the infusion was inserted with a 15° knife (Alcon). The surgical parameter to evaluate the depth of the incision was based on direct observation of the lamina fusca. This anatomic finding indicated that the choroid was positioned at the tip of the knife. Gentle pressure was applied to the adjacent sclera, and no

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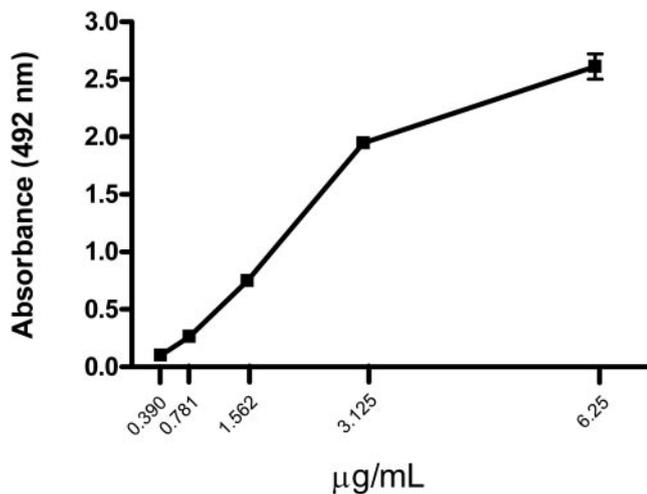


FIGURE 1. Bevacizumab standard curve.

vitreous was observed in the wound. The retinal detachment was directly observed by microscopy to observe whether any retinal tear would occur, which would exclude the eye from the experiment.

The procedure was performed with a surgical microscope and a Machemer lens (Ocular Instruments, Bellevue, WA) positioned on the corneal surface. Coaxial light microscopy was used to observe the retina through the corneal lens, while gentle rotation and counterrotation of the cannula were performed until retinal detachment was achieved. As soon as the detachment reached the central nerve fiber layer area, the cannula was removed, and the wound was closed with the preplaced Vicryl 7-0 suture. This surgical step was performed as soon as possible, to avoid subretinal PSS reflux through the surgical wound.

After the transscleral detachment was created, the animals were divided into two groups. In group 1, six rabbits received an intravitreal injection of 0.05 mL of bevacizumab (1.25 mg/0.05 mL) using a 30-gauge needle (BD Biosciences). In all experiments in group 1, 1.25 mg of bevacizumab was injected into the vitreous cavity. No reflux of bevacizumab and/or vitreous was observed after injection. No retinotomies were performed, to avoid migration of bevacizumab into the subretinal space. In group 2 in three experiments performed in three different rabbit eyes (control eyes), instead of bevacizumab, 0.05 mL of PSS was injected into the vitreous cavity, with a 30-gauge needle (BD Biosciences).

Two hours after the intravitreal bevacizumab injection or saline injection, residual subretinal fluid was aspirated with another 30-gauge cannula that was inserted through the center of the surgical wound (we standardized a timeline of 2 hours to perform the subretinal fluid aspiration to try to reduce the bias of having different quantities of fluid aspiration¹⁴). The samples were subjected to enzyme immunoassay (EIA) to detect bevacizumab.

The rabbits were killed by a 4-mL injection of intravenous pentobarbital. The eyes were enucleated and fixed in 10% formaldehyde. Only one eye of each rabbit was used because the systemic presence of bevacizumab¹⁵ after intravitreal injection theoretically could cause an uncorrected measurement with nonlogical conclusions.

The bevacizumab assay was designed to detect human monoclonal IgG. To determine a standard curve (Fig. 1, Table 1), serial dilutions of bevacizumab in phosphate-buffered saline (PBS; 50 µL/well) were added to a 96-well microplate (Corning, Inc., Corning, NY). Because the minimal volume determined to recover each well was 50 µL and the samples from the experimental and control groups were generally less than this volume (Table 2), PBS was added to increase the volume to 50 µL. The plate was maintained at 37°C overnight. After two washings with 200 µL/well of PBS containing 0.05% Tween 20, 100 µL of PBS-bovine serum albumin (BSA) 1% (blocking solution) was added

TABLE 1. Absorbance of Different Bevacizumab Concentrations by EIA to Determine the Standard Curve

Dilution	Level (µg/mL)	Mean Absorbance (492 nm)
1:4,000	6.250	2.611
1:8,000	3.125	1.946
1:16,000	1.562	0.748
1:32,000	0.781	0.265
1:64,000	0.390	0.101

Bevacizumab serial dilution was used in the solid phase of the EIA microplates and was incubated with HRP-conjugated anti-human IgG and revealed with substrate (H₂O₂-OPD). All dilutions were tested in duplicate, and the absorbances were read in 492 nm. The results represent one of three different and highly reproducible experiments.

to each well for 1 hour at room temperature. The plate again was washed three times and 50 µL of horseradish peroxidase (HRP)-conjugated anti-human IgG (PharMingen, San Diego, CA) or HRP-conjugated anti-mouse IgG diluted 1:1000 in PBS-BSA 1% was added to each well and incubated for 1 hour at 37°C. After three washings, the substrate (hydrogen peroxide and orthophenylene diamine [OPD]) was added. The reaction was stopped after 15 minutes with 50 µL/well of 4 N sulfuric acid, and the absorbance was determined with an EIA reader (Labsystems, Helsinki, Finland) at 492 nm.¹⁶

Bevacizumab was detected in each sample by concentration (in micrograms per milliliter; Fig. 2A, Table 2) based on the standard curve (Fig. 1, Table 1) calculated by a commercial software program (Prism; GraphPad Software, San Diego, CA) and by absolute dose (in nanograms) in the total volume of the residual subretinal aspirate (Fig. 2B, Table 2).

The retinal site where the transscleral cannula was introduced was analyzed by gross pathology, with the Vicryl 7-0 suture previously placed on the sclera used as a reference. The retina was submitted to three serial sections 2 mm apart from the beginning of the retina just around the suture. All samples were stained with hematoxylin and eosin and analyzed by light microscope (Leica, Wetzlar, Germany).

Animals with retinal tears at the site where the transscleral cannula was introduced on histologic evaluation were excluded.

The Student's *t*-test was used to determine differences between groups, with *P* < 0.05 considered significant.

TABLE 2. Bevacizumab Detection by EIA in the Residual Subretinal Aspirate from the Treated (A) or Control (C) Group after Bevacizumab and Saline Intravitreal Injections, Respectively

Samples	Absorbance (492 nm)	Bevacizumab (µg/mL)	Absolute Dose (ng)	Bevacizumab (nM)
A1	0.212	0.38*	5/14 µL*	2.6
A2	0.151	0.24*	5/19 µL*	1.6
A3	0.147	0.23*	7/31 µL*	1.5
A4	0.438	0.88*	19/22 µL*	6.0
A5	1.210	2.60*	130/50 µL*	17.4
A6	2.243	4.91*	157/32 µL*	33
C1	-0.015	0	0/50 µL	0
C2	-0.021	0	0/50 µL	0
C3	-0.011	0	0/50 µL	0

Residual subretinal aspirates were used in the solid phase of the EIA microplates and were incubated with HRP-conjugated anti-human IgG and revealed with substrate (H₂O₂-OPD). Bevacizumab quantification was based on the standard curve according to the software (Prism; GraphPad, San Diego, CA).

* *P* < 0.05, when compared with the control group.

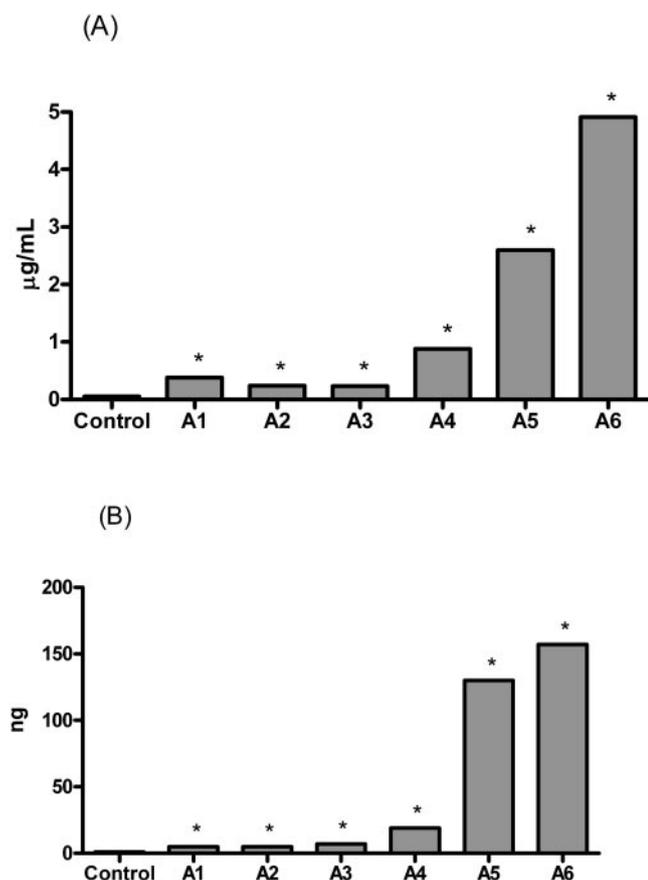


FIGURE 2. Bevacizumab detection in the residual subretinal aspirate by EIA. (A) Concentration; (B) absolute dose. Residual subretinal aspirates were used in the solid phase of the EIA microplates and were incubated with HRP-conjugated anti-human IGG and revealed with substrate (H_2O_2 -OPD). All dilutions were tested in duplicate and the absorbances were read at 492 nm. * $P < 0.05$.

RESULTS

Subretinal bevacizumab molecules were detected in the six eyes that received an intravitreal bevacizumab injection. No subretinal bevacizumab was detected in the control group that received an intravitreal saline injection.

The presence of bevacizumab was determined by standardized enzyme immunoassay (EIA). A direct EIA (tested in the solid phase) was used as the antigen as well as different concentrations of bevacizumab, to establish a standard curve (Table 1, Fig. 1) in samples from both groups. Based on the standard curves, samples with absorbances higher than 0.100 were considered positive.

The concentrations of bevacizumab in all samples varied from 4.91 to 0.23 µg/mL, which was significant ($P < 0.05$) compared with the control group with no bevacizumab (Table 2, Fig. 2A). Once the volume of the residual subretinal aspirate from each animal was different, the absolute dose in each sample was calculated and the result indicated that the immunoassay was sensitive. Bevacizumab concentrations from 157 ng/32 µL (A6) up to 5 ng/14 µL (A1) were detected (Table 2, Fig. 2B).

Light microscopy showed no evidence of retinal tears or holes in any rabbits. The retinal structure was intact in all examined samples, which excluded the possibility of bevacizumab reaching the subretinal space through iatrogenic holes

in these animals. The area submitted for histologic evaluation was the retinal site of the transscleral detachment.

DISCUSSION

These results of direct EIA showed that bevacizumab molecules reached the subretinal space. Because the drug is a humanized monoclonal antibody against VEGF-A, bevacizumab was used as an antigen, which was detected with anti-human IgG conjugated with peroxidase in the ELISA dosage method; the process was revealed using hydrogen peroxide and OPD. The presence of bevacizumab was compared by using an anti-mouse IgG-peroxidase, as bevacizumab is a monoclonal antibody synthesized from mouse spleen cells; however, no positive reaction was obtained (data not shown). Despite the sensitivity and specificity of the EIA, we believe that recombinant VEGF-A used as antigen (in the EIA solid phase) will increase these properties. Nevertheless, the most likely mechanism of subretinal migration of bevacizumab is by direct retinal penetration as previously reported by Shahar et al.¹³

The exact manner of the migration of the subretinal bevacizumab is unknown. The absence of retinal tears excludes the possibility of direct iatrogenic bevacizumab migration through the subretinal space. In addition, the bevacizumab molecule is probably heterogeneously distributed inside the vitreous cavity.¹⁷

In contrast to the theory that molecules larger than 100 kDa cannot pass through the retinal layers in a primate model,¹⁵ alternative mechanisms of bevacizumab migration into the subretinal space in rabbit models have been postulated.^{10,13} One is the possibility of vascular absorption of bevacizumab that leaked as the result of the needle insertion into the subconjunctival space and the migration into the subretinal space via the vascular pathway or by another pathway, because systemic concentrations of bevacizumab have been described after intravitreal injection.¹⁵

In the present study, we tried to use the full retina as a semipermeable membrane to analyze the transretinal migration into the subretinal space 2 hours after intravitreal injection. We hypothesized that bevacizumab could induce an antiangiogenic effect in the CNV a few hours after injection. However, we cannot postulate that bevacizumab crossed through the retina into the subretinal space either by an active or passive mechanism.

The differences in the amount of bevacizumab detected among the animals may have resulted from irregular absorption of the subretinal bleb. Based on a study showing that all subretinal blebs become plane (without subretinal fluid) 24 hours after subretinal fluid injection,¹⁵ we standardized a timeline of 2 hours to perform the subretinal fluid aspiration to try to reduce the bias of having different quantities of aspirated fluid.¹⁴ This quantity could change the concentration values. However, the rates of absorption varied (Table 2).

Another factor possibly related to the subretinal variability in the level of bevacizumab is the probable irregular distribution of bevacizumab inside the vitreous cavity. This molecule may be more concentrated in regions without direct contact with the retinal surface, which may affect the absorption rate.

The actual therapeutic concentration of subretinal bevacizumab is still unknown. A recent in vitro study, however, suggested that a molar ratio of 2.6:1 of bevacizumab to homodimeric VEGF₁₆₅ is necessary for maximum inhibition of endothelial cell proliferation. In this neovascular model, 500 ng/mL (3.40 nM) of bevacizumab was sufficient to block 50 ng/mL (1.3 nM) of VEGF completely, thereby preventing VEGF-induced endothelial cell growth.¹⁸

The animal model used in the present study had limitations: (1) Inflammatory mechanisms of the choroidal neovascular complex have been theorized to play a role in bevacizumab migration in human eyes.¹¹ However, in the present study, a model of normal rabbit eyes was tested. (2) The rabbit retina is avascular, and the behavior of the bevacizumab molecule in human eyes may differ from that in rabbit eyes.¹⁵ (3) Compared with the human retina, the rabbit retina is thinner and has no fovea. Therefore, this rabbit experiment is a model without anatomic similarity to human eyes or primate models.

The results of the present study support the need for experimental studies in animals with anatomic structures similar to human retinas and indicate the need for the development of a quantitative method of bevacizumab administration. Additional experiments using variations in the interval of subretinal fluid aspiration (e.g., aspiration at 2, 4, and 6 hours after intravitreal injection) may estimate the rate of bevacizumab transretinal migration and optimize the dose of intravitreal bevacizumab injection in human eyes.

A combination of well-established methodology of transretinal migration and quantitative dosage and an animal model of the vascular retina will allow comparisons of subretinal concentrations and transretinal migration rates in bevacizumab and other anti-VEGF drugs, to optimize the use of an ideal VEGF inhibitor in human eyes. Additional studies in other animal models and using different VEGF inhibitors are necessary to optimize the use of these drugs in human eyes.

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