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The effects of intravitreally injected bevacizumab on the retina and retina pigment epithelium: experimental *in-vivo* electron microscopic study in intact *versus* vitrectomized eyes

Research Article

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Abstract: To analyze the retinal toxicity of bevacizumab at various doses both in vitrectomized and non-vitrectomized rabbit models. Twenty- eight rabbits were included in the study. Twenty- four rabbits were assigned to six groups, with 4 of the rabbits in the control group. The animals in Groups 1, 2 and 3 received bevacizumab at a dose of 0.3 mg, 0.5 mg and 1.5 mg /eye, respectively. The rabbits in Groups 4, 5 and 6 received intravitreal bevacizumab of 0.3 mg, 0.5 mg and 1.5mg/eye, respectively, after gas compression vitrectomy. Two weeks after the procedure, the rabbits were euthanized. Retina tissue samples were then obtained and examined with both light and electron microscopes. In Groups 1, 2 and 3 after bevacizumab injection, toxic degeneration in the photoreceptor and retinal pigment epithelium cells was observed via electron microscopic examination. The findings in Groups 4 and 5 were normal as compared to the control group. In Group 6, toxicity in the bipolar neurons and photoreceptor cells was noticed. Increased toxicity and retinal penetration were noticed in all administered doses of bevacizumab in the presence of vitreous. In addition, ocular toxicity occurred through the injection of the highest dose of bevacizumab after vitrectomy. It is possible that the bevacizumab dose and the,vitreous are as important as the drug half-life in the vitreous.

Keywords: *Anti-VEGF • Bevacizumab • Electron microscopy*

1. Introduction

Bevacizumab (Avastin, Genentech Inc, San Francisco, California, USA) is a recombinant humanized monoclonal IgG1 antibody that binds all isoforms of vascular endothelial growth factor (VEGF) (VEGF110, VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206) and works to neutralize its biological activity [1-4]. It consists of two antigen-binding regions that are named F_{ab} and F_c; upon binding to these regions, bevacizumab interferes with the binding of VEGF to its respective receptors, thereby inhibiting its signal [5]. The drug is approved by the Food and Drug Administration

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for intravenous use in combination with 5-fluouracil based chemotherapy for metastatic colorectal cancer [6].

It has been administered off-label as an intravitreal treatment in VEGF-mediated diseases such as choroidal neovascularization [7-9], central retinal vein occlusion [10], proliferative diabetic retinopathy [11,12] and pseudophakic cystoid macular edema [13]. In general, healthy retinal tissue limits the penetration of materials above 70 kDa in molecular weight; however, bevacizumab, which weighs approximately 148 kDa, was present in the retinal tissue [2]. Despite its large molecular size, previous analysis had shown the easy penetration of bevacizumab into pathologic retinal tissue [2,3]. Clinically to date while limited safety data are available, no retinal toxicity has been reported after intravitreal injection of bevacizumab [2,3,14]. Previous groups have evaluated the safety of intravitreal injection of bevacizumab in rabbits using electrophysiological testing [15] and histopathological analysis [3]. Recently, several studies have reported beneficial therapeutic effects of intravitreal injection of bevacizumab as well as documenting its penetration into the deeper retinal layers [2,7,9].

The purpose of our study was to analyze the retinal toxicity of bevacizumab at various doses both in vitrectomized and non-vitrectomized rabbit models.

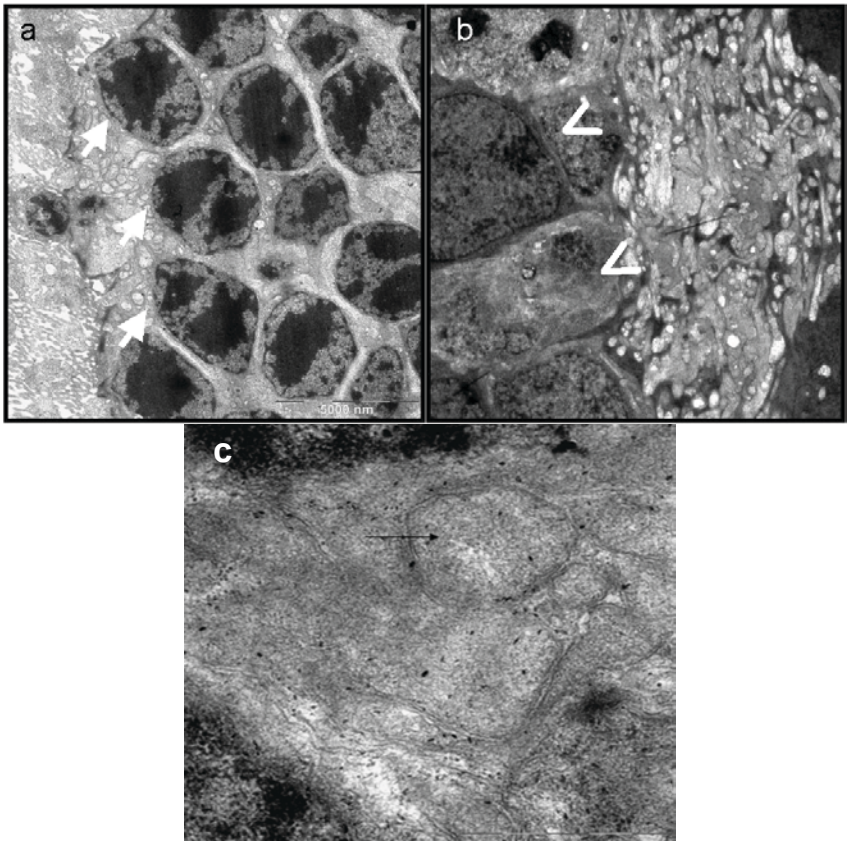
2. Material and Methods

Twenty eight adult New Zealand albino rabbits each weighing 2.5-3.0 kg were included in the study. All experiments were conducted in accordance with the Animal Care and Use Committee and the Association for Research in Vision and Ophthamology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbits were housed under (12/12) hours light-dark cycle and allowed free access to water and food. Four of the twenty eighht rabbits were used in the control group. On the day before drug administration, 24 rabbits were assigned to 6 groups (n=4 in each Group). Various concentrations of bevacizumab were prepared: 0.3 mg/0.1 ml, 0.5 mg/0.1 ml and 1.5 mg/0.1 ml. The animals in Groups 1, 2 and 3 received bevacizumab with a dose of 0.3 mg, 0.5 mg and 1.5 mg/eye, respectively, through a 30-gauge needle. The rabbits in Groups 4, 5 and 6 received intravitreal bevacizumab of 0.3 mg, 0.5 mg and 1.5 mg/eye, respectively, after gas compression vitrectomy. The rabbits in the control group received a volume of 0.1 ml sterile balanced saline solution. The right eyes received the drug as study eyes. Before all intravitreal injections and vitrectomies were performed, the rabbits were anesthetized by an intramuscular combination of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (6 mg/kg). The pupils were dilated with 2.5% phenylephrine hydrochloride and 10% tropicamide. After ocular surface anesthesia was administered with a topical instillation of proparacaine hydrochloride, the eyes were washed with several drops of 5% povidone iodide. A 30-gauge needle was introduced into the vitreous cavity, 2 millimeters (mm) posterior to the superotemporal limbus. The syringe was directed under visual control using an indirect ophthalmoscope towards the center of the vitreous above the optic disk. A volume of 0.1 ml was then slowly injected. After the procedure, chloramphenicol ointment was applied to the eyes. Tobramycin 0.3%, dexamethasone sodium phosphate 0.1% and 10% tropicamide were applied into the inferior conjunctival fornices 3 times a day during the post-operative period.

The study eyes were examined clinically before, 3 days after intravitreal procedure, and at the end of the study. According to the protocol, the following parameters were recorded: corneal clarity, transparency, appearance of the lens and retina, conjunctival reaction, and cells in the anterior and posterior segment of the eye. The anterior chamber and anterior vitreous were examined by slit lamp under the highest magnification for evaluation of cellular reaction and flare. Before and after the procedures were conducted, the crystalline lens was examined to assess any opacity that might have occurred because of the effects of intravitreal bevacizumab. At baseline, immediately after and three days after the procedure, all study eyes were examined by indirect ophthalmoscopy and a 20-D aspherical lens to ensure clear and sharp imaging of the retina. These procedures thereby exclude any possible disease and enable the detection of retinal injury.

After 2 weeks, the rabbits were euthanized by an intravenous injection of an overdose of sodium pentobarbital (80 mg/kg body weight). The eyes were enucleated with careful manipulation to preserve globe integrity. Each eye was immediately placed in neutral formalin solution for light microscopic examination and in 2.5% glutaraldehyde in 0.1 M phosphate buffer for electron microscopic examination. Retinal tissue samples were cut into small sections. They were fixed in 2.5% phosphate buffered gluteraldehyde for 2 hours and postfixed in 1% osmium tetroxide, dehydrated in serial alcohol, and then embedded in araldite. The semi-thin sections were stained with toluidine blue and examined with a light microscope (BH2 Olympus). After the selection of appropriate specimens, thin sections were obtained and stained with uranyl acetate and lead citrate. They were examined under an electron microscope (Carl Zeiss Libra 120).

Figure 1. The laminas of retina were normal in control group. (a) photoreceptor cells (white arrows) (b) bipolar neurons (white arrowheads) X2000, 5000 nm, (c) normal mitochondrial ultrastructure (arrow) X12.500, 1000nm.



3. Results

After intravitreal injections and vitrectomy as well as during the follow-up period, the conjunctiva and cornea were apparently normal with anterior segment examinations. No conjunctival hyperemia or corneal epithelial, stromal, or endothelial changes were observed in either early or late term. In all study eyes, there was no inflammatory response, with the lens and vitreous appearing clear and the fundus intact. Light microscopy showed normal retinal histologic findings for the sterile-balanced saline solution injection of the control group (Figure 1).

Electron Microscopy: Decreased pigment granules of retinal pigment epithelium and vacuolisation in cytoplasma were observed in the 0.3 mg bevacizumab-injected group (Group 1). Irregularity to lamellar form and lamellar dissection in the photoreceptor cells, dissection between the nucleus's inner and outer membrane at the outer nuclear layer, mitochondrial cristolysis and mitochondrial swelling were observed in bipolar neurons. Normal morphology in the ganglion cells and other retinal layers was observed. The findings for the 0.5 mg and 1.5 mg bevacizumab-injected groups (Group 2- 3) were similar to those with Group 1 (Figure 2).

The findings for the vitrectomized groups injected with 0.3 mg and 0.5 mg bevacizumab (Groups 4 and 5) were similar to that of the control group. The nucleus morphology of pigment epithelium cells, the outer and inner segments of the photoreceptor cells were normal in appearance. The bipolar neurons and the other retinal layer showed congruity with the findings of the control group (Figure 3).

Retinal pigment epithelium cells in Group 6, which received 1.5 mg bevacizumab after vitrectomy, were normal as observed via electron microscopy. While outer and inner segments were normal in appearance, a different electron density was observed in nucleus of the photoreceptor cells. In addition, although a group of the cells' nuclei were observed to be electron-dense, the other nuclei were evaluated to be more electron-pale. In the same group, even though bipolar neurons with necrotic nuclei were observed, a more marked dissection between the inner and outer membrane of some neuronal nuclei was observed (Figure 4).

Figure 2. (a,b) The group of non-vitrectomized. Nuclear inner and external membran were separated in external nuclear lamina (white arrows), (c,d,e) mitochondrial cristolysis and mitochondrial swelling in bipolar neurons (*) X2000, 5000 nm, (f) mitochondrial cristolysis and loosing of mitochondrial matrix (arrows) X12.500, 1000nm .

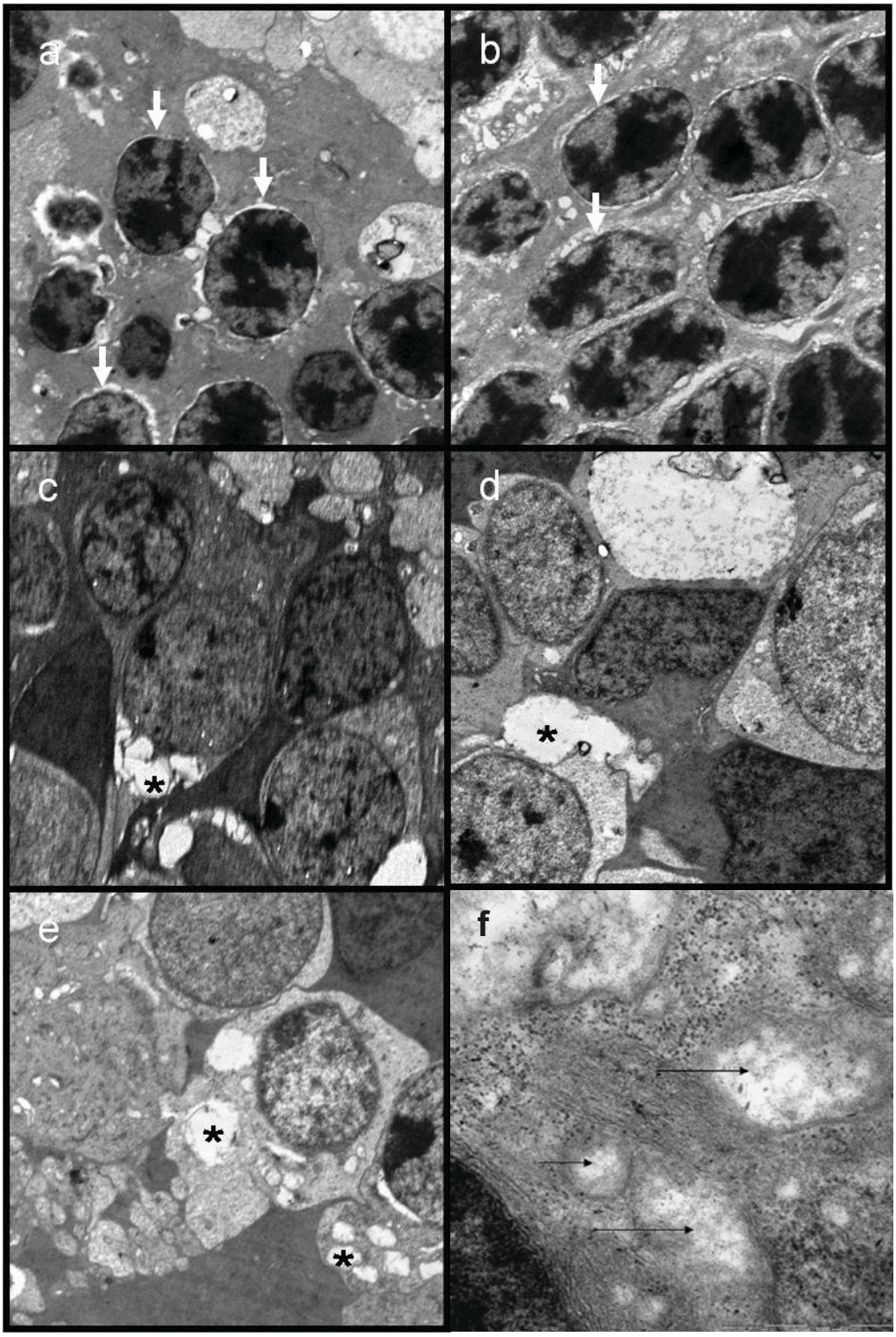


Figure 3. The group of vitrectomized with bevacizumab injection of 0.3 mg and 0.5 mg (a,c). The nuclei of photoreceptor cells were observed as normal (white arrows), (b,d) nuclei and cytoplasmic material were normal in bipolar neurons (white arrowheads) X2000, 5000 nm.

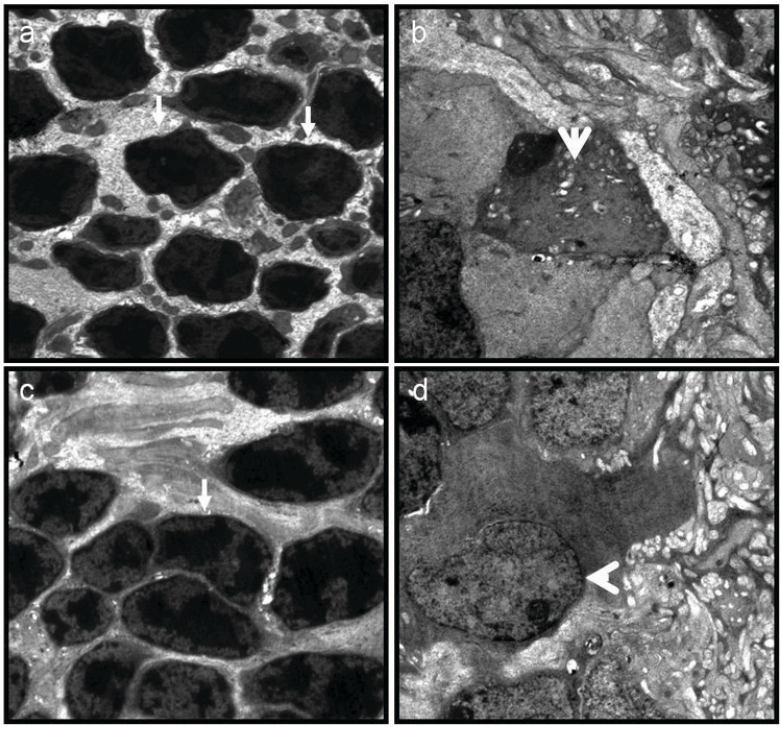
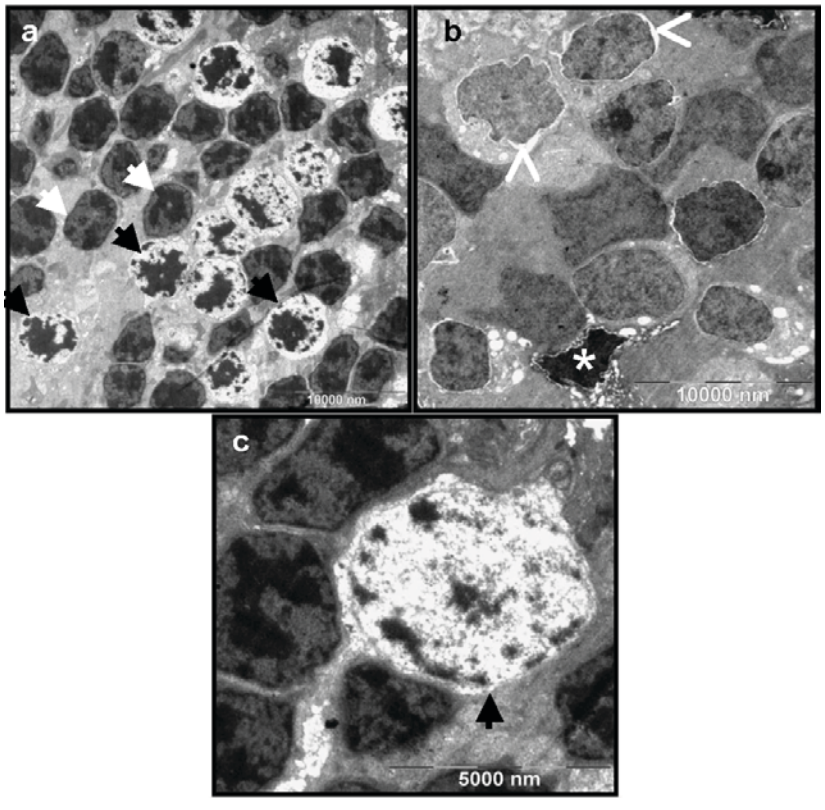


Figure 4. The group of vitrectomized with bevacizumab injection of 1.5 mg (a,c) pale photoreceptor cell nuclei (black arrows), dark photoreceptor cell nuclei (white arrows), (b) necrotic bipolar neurons (*) X2000, 5000 nm.



4. Discussion

In our study, we analyzed the possible retinal toxicity of bevacizumab at various doses in both vitrectomized and non-vitrectomized rabbit eyes and performed electron microscopic examination to determine any structural and ultrastructural changes in the retinal layers. In Groups 1, 2 and 3 after injection of bevacizumab at doses of 0.3 mg, 0.5 mg and 1.5 mg, respectively, toxic degeneration in the photoreceptor and retinal pigment epithelium cells was found. This toxic degeneration also showed that the chemical and structural characteristics of the vitreous may have caused prolonged intravitreal duration and delayed the filtration of injected bevacizumab. In Group 6, which received the highest dose of bevacizumab (1.5 mg/0.1 ml) after vitrectomy, toxic degeneration in the photoreceptor cells were noticed with electron microscopic examination although normal apperance of retinal pigment epithelium cells was observed.

Bakri et al. [1] described the histologic changes with light microscope after intravitreal bevacizumab in pigmented rabbits. They administred 1.25 mg (0.05 ml) and 2.50 mg (0.1 ml) of intravitreal bevacizumab in rabbit eyes. These doses showed no evidence of inner or outer retinal or optic nerve toxicity by light microscopy at one month in the rabbit model. They also mentioned that the volume of the human vitreous is approximately 4 ml, and the volume of the vitreous in a Dutch-belted rabbit is approximately 1.5 ml. Therefore, a certain amount of drug injected into a rabbit eye is equivalent to a concentration of 2.7 times that injected into a human eye. In spite of this overdose, no toxicity in retinal layers was noticed in their study. Although, we showed normal retinal histologic findings under light microscope as Bakri et al. [1], toxic degeneration in the photoreceptor and retinal pigment epithelium cells in Groups 1, 2, 3, and 6 was noticed via electron microscopy.

The serum half-life of bevacizumab is 20 days. It has the longest serum half-life when compared with the other anti-VEGF drugs (pegaptanib and ranibizumab) [4]. Although the exact half-life of bevacizumab in the eye is uncertain, the half-life of antibodies with similar molecular weight is approximately 5.6 days [16]. In this study, we thought that, due to the rapid elimination of bevacizumab in vitrectomized eyes, the duration and half-life of the injected drug became shorter in Group 4, 5 and 6. The electron microscopic findings in Groups 4 and 5 were normal in terms of retinal pigment epithelium and photoreceptor cells and bipolar neurons as the control group. We thought that the main reason for this result was the lack of vitreous tissue in the vitrectomized eyes. In spite of the normal findings of retinal layers in Groups 4, 5, and 6, the latter receiving the highest dose of bevacizumab (1.5 mg/0.1 ml) after vitrectomy, toxicity in the bipolar neurons and photoreceptor cells was noticed via electron microscopic examination. In the light of these study results, the study drug dose and the presence of vitreous are as important as the half-life in the vitreus both for bevacizumab's effectiveness and the toxicity.

In experimental toxicity studies based on electrophysiology and light microscopy in the literature, no toxicity was noticed after injection with bevacizumab [2,5,15]. Iriyama et al. [3] determined the potential toxicity of intravitreal bevacizumab and the inhibition of VEGF signalling, they used anti-rat VEGF antibody or bevacizumab in rats and evaluated their toxicity to retinal layers, and to retinal ganglion cells (RGCs) both *in vivo* and *in vitro*. They showed that bevacizumab and anti-rat VEGF antibody exhibited no retinal toxicity using a rat model *in vitro* and *in vivo* at a dose that completely blocks the biological function of VEGF; as a result, they suggested that acute toxic effects of bevacizumab on RGCs might be negligible. Therefore, their study supported that a single intravitreal injection of bevacizumab showed no toxicity on RGCs, but it remains unknown whether repeated injection of bevacizumab with a longer follow-up would clinically demonstrate toxicity on RGCs. Shahar et al. [2] evaluated the possible toxicity of intravitreally injected bevacizumab in a rabbit model and tested possible bevacizumab toxicity to the ganglion cells as well as to distal retinal layers. At the end of the study, they showed that intravitreal bevacizumab could penetrate beyond the internal limiting membrane into the retina. They also noticed that the dose injected in the study was twofold greater than that currently used in humans. Furthermore, the drug was injected into a smaller vitreous volume (less than 2 ml) in the rabbit, as opposed to a volume of 4-5 ml in humans. Based on electrophysiology studies, they showed no evidence for retinal toxicity resulting from a single intravitreal injection of bevacizumab in a rabbit model. It was reported that bevacizumab at concentrations of 0.125, 0.25, 0.50, and 1.0 mg/ml was safe in the short term for human cells (in particular, human microvascular endothelial cells) and rat neurosensory retinal cells *in vitro* [14]. No toxicity to retinal cells was reported even with 5.0 mg intravitreal bevacizumab using light microscopy [5,15].

Inan et al. [11] evaluated the possible toxicity of bevacizumab at various doses in rabbit eyes. Although electrophysiologic investigation and light microscopy showed normal retinal function and structure, mitochondrial disruption in the inner segments of photoreceptors was detected by electron microscopy, and apoptotic expression was detected after the injection

of intravitreal bevacizumab. They thus mentioned that intravitreal bevacizumab might cause toxicity by apoptosis in the photoreceptor layer. These electron microscopic findings were similar to our study results.

Based on our knowledge, this is the first study to analyze the retinal toxicity of bevacizumab at various doses in both vitrectomized and non-vitrectomized rabbit models. We showed increased toxicity and retinal penetration in all administered doses in the non-vitrectomized groups (Groups 1, 2 and 3). In addition, ocular toxicity occurred in Group 6 that received the highest dose of intravitreal bevacizumab injection after vitrectomy. The limitations of this study were the lack of electrophysiologic investigations before and after the procedures as well as the lack of the measurement of duration and half-life of bevacizumab in both non-vitrectomized and vitrectomized rabbit eyes. Therefore, intravitreal bevacizumab may cause toxicity by apoptosis in the photoreceptor layer. In future studies, quantitative analysis should be performed to determine the extent of possible apoptosis. Further studies are needed to clarify the toxic side effects of intravitreal bevacizumab, especially for cumulative effects at repeated doses, before a definitive conclusion can be drawn about its safety.

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