·Basic Research·

Short term apoptotic activity of intravitreal bevacizumab on rabbit retina

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Abstract

• **AIM: To evaluate the safety and the short term apoptotic activity of intravitreal bevacizumab in rabbit eyes by histopathological analysis.**

• **METHODS: Twenty -eight eyes of 14 rabbits were divided into three groups: 8 rabbits in group 1 and 3 rabbits in each of group 2 and group 3. Intravitreal bevacizumab (1.25mg/0.05mL) was applied to the right eyes of each subject in group 1 and group 2 (11 eyes) and the same volume of saline was applied to the left eyes of each subject in group 1 and group 3 (11 eyes). The left eyes in group 2 and the right eyes in group 3 were left untreated and used as control. Enucleated eyes were used for histopathologic analyses.**

• **RESULTS: After immunohistochemical staining with caspase-3 and p53, there was no histological evidence of toxicity to the retina and the optic nerve in any of the sections that were analyzed in all three groups. In addition, vascular endothelial cells located at the retina and the optic nerve tissues in all groups showed a similar staining pattern with caspase-3 and p53.**

• **CONCLUSION: Our study showed that intravitreal bevacizumab with the dose of 1.25mg/0.05mL caused no histological signs of toxicity or apoptotic activity on the rabbit retina.**

• **KEYWORDS:** apoptotic activity; bevacizumab; retina; toxicity
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INTRODUCTION

Bevacizumab is a humanized murine anti-vascular endothelial growth factor (VEGF) monoclonal antibody that is specific to human VEGF and blocks all its isoforms[1,2]. It is the first antiangiogenic agent to be approved by the FDA for the treatment of metastatic colorectal cancer, advanced non-small cell lung cancer and metastatic breast cancer[3,4]. Recently, intravitreal injection of bevacizumab has been extensively used for the treatment of macular edema and intraocular neovascularization in diseases such as diabetic retinopathy, age-related macular degeneration (ARMD), retinal venous occlusion, neovascular glaucoma, and many other conditions[5-10].

In the normal retina, the primary sources of VEGF are ganglion cells, Müller cells, and retinal pigment epithelium[11]. Retinal pigment epithelium derived VEGF molecule is essential for the development and the maintenance of the choriocapillaris integrity and mainly secreted from the basal side of retinal pigment epithelium [12]. The absence of VEGF molecule has been shown to cause an atrophy of the choriocapillaris and results in a loss of endothelial cell fenestrations [13]. An increase in the retinal and intravitreal VEGF concentration has been shown in patients and laboratory animals with ischemic retinopathies induced by oxidative damage and hypoxia[14-18].

Some safety studies have reported the lack of toxicity of intravitreal injection of bevacizumab in rabbits[19-22]. However, recent studies have demonstrated ultrastructural changes after intravitreal injection of bevacizumab in rabbits and primates [23-25]. In the present study, we aimed to evaluate the safety and the short term apoptotic activity of intravitreal injection of bevacizumab in rabbit eyes by histopathological analysis.

MATERIALS AND METHODS

Materials Prior approval of the experimental protocol was obtained from Ankara Numune Training and Research Hospital Research Committee. The animals were treated and

maintained in accordance with the tenets of Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research.

On the day before drug administration, the 28 eyes of the 14 male New Zealand white rabbits weighing 1.5-2.5kg were divided into three groups: 8 rabbits in group 1 and 3 rabbits in each of group 2 and group 3. We applied 1.25mg/0.05mL intravitreal bevacizumab (Avastin, Genentech, San Francisco, CA, USA) to the right eyes of each subject in group 1 and group 2 (11 eyes) and the same volume of saline to the left eyes of each subject in group 1 and group 3 (11 eyes). The left eyes in group 2 and the right eyes in group 3 were left untreated and used as control.

Methods All eyes were clinically examined before the injection, then 1d after and finally at the end of the study (14th day). Ophthalmic examination included an indirect ophthalmoscopy with a 20-D aspherical lens to exclude any possible vitreoretinal disorders and an external inspection of the anterior segment structures with an external light source and a magnifying lens to record the baseline appearance of the conjunctiva, cornea, and lens. Before the administration of bevacizumab, the rabbits were anesthetized by subcutaneous injection of a mixture containing ketamine hydrochloride (Fort Dodge, Inc, Fort Dodge, Indiana, USA) (25mg/kg) and xylazine hydrochloride (Phoenix Scientific Inc, St Joseph, Missouri, USA (6mg/kg) solution. The pupils were dilated with topical application of 2.5% phenylephrine hydrochloride (Mydfrin, Alcon, USA) and 0.5% tropicamide (Tropamide, Bilim, Turkey). Intravitreal injections were performed under sterile conditions. The eyes were washed with several drops of 5% povidone iodide. Topical proparacaine hydrochloride (Alcain; Alcon, Fort Worth, TX, USA) drops were applied for additional topical anaesthesia. A speculum was used to keep the eyelids open. A 27-gauge needle attached to a 1mL syringe was transconjunctivally introduced into the vitreous cavity 1.5mm posterior to the superotemporal limbus. After slowly injecting the drug into the eye, the needle was held in place for 10s before withdrawal to prevent reflux from the entry site. At the end of the procedure, topical antibiotic ointment was applied and 0.3% ofloxacin eye drop was administered from this time point four times per day for five days. Examinations were performed after injection of the central retinal artery occlusion.

Histopathological analysis The animals were euthanatized on the 14th day by intracardiac injection of 2mL pentobarbital (Beuthanasia-D Special; Schering-Plough Animal Health Corp, Kenilworth, New Jersey, USA). The eyes were immediately enucleated with careful manipulation to preserve globe integrity and fixed in neutral formalin solution for 2d. Specimens were obtained from cuts through the whole globe oriented perpendicular to the medullary wings then embedded in paraffin blocks. Sections of 5μm thickness obtained by a microtome were stained with hematoxylin and eosin (H&E), caspase-3, and p53. All sections were examined by light microscope (Olympus BX5/TF, Tokyo, Japan) and photographed with a digital camera (Nikon Digital Sight DS-L1, Tokyo, Japan).

Immunohistochemical staining Sections were deparaffinized with xylol and rehydrated with graded alcohol concentrations and they were distilled with water after being incubated at 60℃ for 45min. Then, they were boiled in the citrate buffer (pH 6.0) and washed with tries buffered saline (TBS) for 5min and hydrogen peroxide for 10min. Primary rabbit polyclonal caspase-3 (CPP32, Gene Tex, Inc.) and monoclonal p53 (BP53-12, Gene Tex, Inc.) antibodies were administered to the washed blocks for 2h. The specimens were washed with TBS, treated with biotin-labeled goat anti-polyvalent for 20min and incubated for 10min after being stained with 3-Amino-9-ethylcarbazole (AEC) chromogen. Then they were washed with distilled water and waited in Mayer's hematoxylin for 2min. Finally, they were washed 3-4 times with distilled water and covered with aqueous mount (low viscosity).

Statistical Analysis Statistical analyses were performed using software SPSS version 16.0 (SPSS Inc, Chicago, Illinois, USA). Chi-square test was used to compare the groups.A P value <0.05 was considered statistically significant.

RESULTS

Ophthalmic examination revealed no evidence for external ocular inflammation, cataract formation, or retinal abnormality in any of the eyes during the follow-up period. Light microscopy of sections revealed an existence of inflammatory cell infiltration that includes plasma cells in 3 of 11 eyes which received bevacizumab, in 4 of 11 eyes which received saline and 2 of 6 eyes which were left untreated. There was no significant difference between the groups (P=0.89). In all groups, we observed similar amount of vacuolization in the ganglion cell layer (Figure 1) and some distinctive dispersion and detachment of the retinal layers, including the rod and the cone cells and the retinal pigment epithelium. After immunohistochemical staining with caspase-3 (Figure 2) and p53 (Figure 3), there was no histological evidence of toxicity to the retina and the optic nerve in any of the sections that were analyzed in all three groups. In addition, vascular endothelial cells located at the retina and the optic nerve tissues in all groups showed a similar staining pattern with caspase-3 and p53.

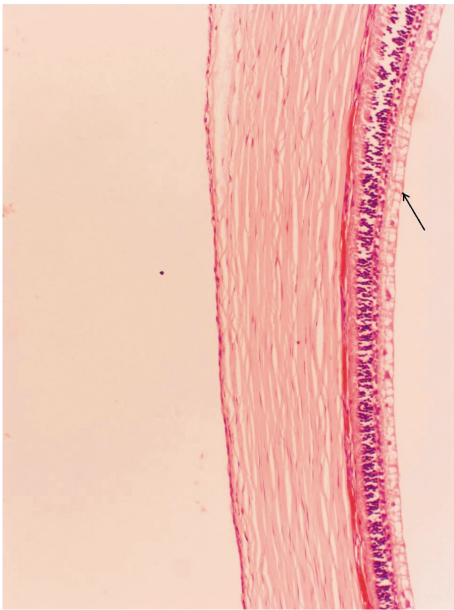

Figure 1 Vacuolization in the ganglion cell layer in bevacizumab injected group (arrow) (×200).

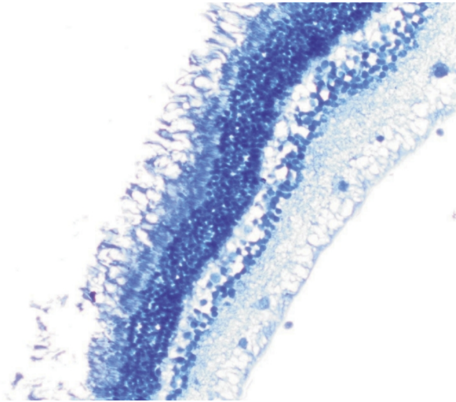

Figure 2 There was no staining in retinal layer with p53 in bevacizumab injected group (×200).

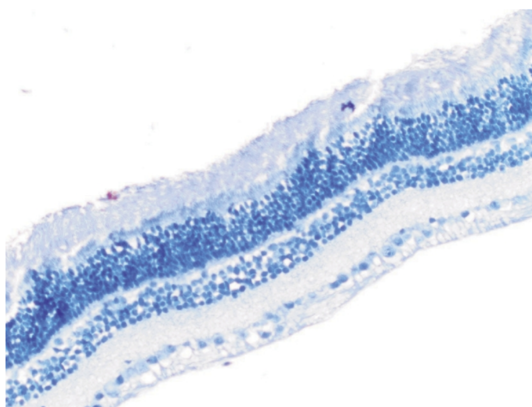

Figure 3 There was no staining in retinal layer with Caspase-3 in bevacizumab injected group (×200).

DISCUSSION

Our study showed that intravitreal injection of bevacizumab (1.25mg/0.05mL) caused no histological sign of toxicity or apoptotic activity on the rabbit retina. In all groups, we observed similar amount of vacuolization in the ganglion cell layer and some distinctive dispersion and detachment of the retinal layers including the rod and the cone cells and the retinal pigment epithelium.

It has been shown that the administration of VEGF inhibitors results in changes not only in the pathological microvasculature but also in normal vessels[18]. Withdrawal of VEGF leads to endothelial cell apoptosis[26]. VEGF displays a protective effect on apoptotic retinal cells in a dose-dependent manner [27]. VEGF acts as a potent survival factor by protecting endothelial cells from apoptosis *via* activation of protein kinase C or phosphatidylinositol 3OH-kinase/Akt pathways and upregulation of anti-apoptotic proteins such as bcl-2, XIAP and survivin [25,28]. VEGF also inhibits the expression of p53, the caspases and other apoptotic/cell death-related genes (Bak, Bax, Bcl2,TNF-α, *etc.*) [29]. The p53 gene is a well defined tumor-suppressor gene which produces a 53-kDa phosphoprotein that regulates cell proliferation and apoptosis in response to DNA injury [30-32]. It also suppresses angiogenesis [33]. In addition, it targets bax which promotes apoptosis and on the other hand, it down regulates the expression of bcl-2 that prevents apoptosis. Caspase-3 is an important mediator of apoptosis in a variety of cells and tissues. It is responsible for the cleavage of poly-ADP-ribose polymerase, which is activated during apoptosis[34-36].

Some experimental studies have shown that intravitreal bevacizumab had short-term retinal safety and favourable short-term results, in accordance with our study [19-21]. Manzano *et al* [19] evaluated the retinal toxicity of varying doses (500μg/0.1mL, 1.0mg/0.1mL, 2.5mg/0.1mL and 5.0mg/0.2mL) of intravitreal bevacizumab in rabbits. Histological examination with light microscopy and electroretinography results in all groups showed no retinal toxicity. However, some inflammatory cells were found in the vitreous at the 5mg dose. In another study, 3 repeated 1.25-mg intravitreal bevacizumab injections did not cause a toxic effect on cornea and uveoretinal tissue [37]. Similarly a single 1.25mg or 2.5mg intravitreal bevacizumab injection did not lead to toxic damage within 90-days in the neurosensorial retina and retinal pigment epithelium[38].

Xu *et al* [39] used the TUNEL method (*in situ* terminal-deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling) for detection of DNA fragmentation in nuclei of apoptotic cells in the nuclear layers of rabbit eyes. They evaluated the potential toxicity of repeated intravitreal injections (three sequential, biweekly) of bevacizumab in doses of 2.5mg/0.1mL or 5.0mg/0.2mL and reported that only very few TUNEL-positive cells were detected in the eyes that received 2.5mg/0.1mL of

bevacizumab. Avci *et al* [40] compared the apoptotic activity after intravitreal injections of bevacizumab and pegaptanib sodium in an experimental rabbit model. They reported that a significant nuclear DNA fragmentation in the outer retinal layers shown by the TUNEL method was evident with higher doses of bevacizumab at 14d. On the other hand, Xu *et al* [39] showed that TUNEL-positive cells were detected abundantly in the outer nuclear layer at 1 week after all the injections bevacizumab. However, the number of TUNEL-positive cells was found to be decreased at 4 weeks after all injections with no statistically significant difference among groups at this time point. In our study, on the 14th day neither caspase 3 nor p53 staining patterns were observed in any of the groups.

Main limitations of our study were the lack of electrophysiological testing and comparison of different dose schedules.

In conclusion, intravitreal bevacizumab with the dose of 1.25mg/0.05mL caused no histological sign of toxicity or apoptotic activity on the rabbit retina. As VEGF plays its anti-apoptotic activity specifically in ocular diseases related with tissue hypoxia, such as diabetic retinopathy, vascular occlusions and retinopathy of prematurity, we suggest investigating the apoptotic activity of these drugs could be more determinative in animal models with the aforementioned ocular disorders.

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