

Evaluating the safety of intracameral bevacizumab application using oxidative stress and apoptotic parameters in corneal tissue

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• **CONCLUSION:** This study revealed that intracameral bevacizumab injections seemed to be systemically safe but may have elicited local toxic effects in the corneal tissue, as indicated by the oxidative stress parameters and histopathological evaluations.

• **KEYWORDS:** apoptosis; cornea; intracameral bevacizumab; oxidative stress

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Abstract

• **AIM:** To investigate the possible effects of intracameral bevacizumab on oxidative stress parameters and apoptosis in corneal tissue.

• **METHODS:** In total, 30 rats were assigned randomly into the following three groups of 10 rats each: a sham group (Group 1; $n=10$), a control group [Group 2; balanced salt solution (BSS) was administered at 0.01 mL; $n=10$], and a treatment group (Group 3; bevacizumab was administered at 0.25 mg/0.01 mL; $n=10$). The total antioxidant status (TAS) and the total oxidant status (TOS) in the corneal tissue and blood samples were measured, and the oxidative stress index (OSI) was calculated. Additionally, corneal tissue histopathology was evaluated for caspase -3 and 8 staining and apoptotic activity.

• **RESULTS:** In the blood samples, the TAS, TOS, and OSI levels were not significantly different (all $P>0.05$). Compared with the sham and control groups, the TOS and OSI levels in the corneal tissues were significantly different in the bevacizumab group (all $P<0.05$). No statistically significant differences were observed between the sham and control groups (all $P>0.05$). However, compared with the sham and control groups, greater immunohistochemical staining for caspases -3 and 8 and an elevated level of apoptotic activity were observed in the bevacizumab group.

INTRODUCTION

Toxicity to corneal endothelial cells can lead to the loss of corneal transparency and, ultimately, blindness. The pharmacokinetics, safety, and dose-dependent toxicity of intracamerally administered bevacizumab, which is an anti-vascular endothelial growth factor (VEGF), to corneal endothelial cells have not been established. Several reports have demonstrated that this drug can be injected safely and effectively into the eye to treat various ocular neovascular disorders. However, adverse drug-related events associated with bevacizumab treatment have also been reported in retrospective studies, though in a limited number of patients^[1-3].

Generally, bevacizumab may be delivered topically, intracamerally, or intravitreally. Among the different modes of delivery, intracameral delivery has been used least frequently. Intravitreal bevacizumab injection is associated with the risk of complications such as endophthalmitis and retinal pigment epithelial tears. Currently, the intracameral injection of bevacizumab is being used more widely in clinical trials and is thus considered to be safe for the corneal endothelium. Although the intracameral injection of bevacizumab may be less invasive than intravitreal delivery, the safety of this route of administration remains unclear^[1,4-6]. The generation of oxygen-derived free radicals has been suggested to be responsible for injuries to various organs, and the induction of apoptosis by oxidative stress is well

established [7-11]. It has been shown that oxidative stress is a central mechanism of cellular damage that affects all organs and tissues, and free radicals are known to cause cellular damage and to be generated by some ophthalmic preparations [12-15]. Indeed, it remains controversial, and there is little reported information regarding whether the use of bevacizumab is safe and reliable when administered to the cornea because several studies have reported possible toxic effects and safety issues associated with bevacizumab injections [16-18].

Measuring different oxidant and antioxidant molecules is impractical, and oxidant and antioxidant effects are additive. Because there are numerous oxidants and antioxidants in the body, measuring the total oxidant-antioxidant status is more valid and reliable. When only a few parameters are measured, the levels may remain unchanged or decrease despite increases or decreases in the actual oxidant status [19,20]. Beyond the toxic effects of intravitreal administrations of bevacizumab on cornea that have been mentioned above, we hypothesized that intracameral bevacizumab might also have toxic effects on the cornea. In this experimental study, we aimed to investigate the possible effects of intracameral bevacizumab on oxidative stress parameters using the total oxidant status (TOS), total antioxidant status (TAS) and apoptosis in the corneal tissue.

MATERIALS AND METHODS

Animals In total, 30 adult male Wistar Albino rats aged 48wk and weighing 180-200 g were used. The animals were housed under continuous observation in appropriate cages in a quiet, temperature- (21°C ±2°C) and humidity (60%±5%)-controlled room and were maintained on a 12/12h light-dark cycle. The animals were housed five per cage and were provided with commercial standard diet and water *ad libitum*. All experiments in this study were performed in accordance with the "principles of laboratory animal care". The experiments were approved by the Ethical Committee on Human and Animal Research at Harran University, Sanliurfa, Turkey.

The rats were randomly assigned to the following three groups of 10 rats each: 1) a sham group (Group 1; $n=10$); 2) a control group [Group 2; balanced sterile salt solution (BSS) was administered at a dose of 0.01 mL; $n=10$]; and 3) a treatment group (Group 3; bevacizumab was administered at a dose of 0.25 mg/0.01 mL; $n=10$).

The TAS and TOS in the corneal tissue and blood samples were measured, and the oxidative stress index (OSI) was calculated. Corneal tissue histopathology was assessed in terms of caspase-3 and 8 staining. Apoptotic activity was also evaluated.

Intracameral Injection Technique Prior to the initiation of the intracameral injection procedure, the rats were anesthetized *via* intramuscular injections of ketamine (50 mg/kg; Ketalar; Parke Davis, Eczacibasi, Istanbul,

Turkey) and xylazine (10 mg/kg; Rompun; Bayer AG, Leverkusen, Germany) under aseptic conditions. Topical anesthetic (0.5% proparacaine) was dropped into the eyes of the rats 10min prior to the injections.

The globe was fixed from the edge of the limb using horizontal angled conjunctival forceps with teeth. The anterior chamber injections that were tangential to the limbus were performed at 3 o'clock using an insulin syringe (0.30×8-mm, 30 G×5/16", Ayset Medical Products Industry Co., Adana, Turkey). The injections were performed using a YZ20T9 operating microscope (Nanjing, Redsun Optical Co., Ltd., Jiangsu Province, China). No injections were performed in Group 1. BSS [0.01 mL; Industria Farmaceutica Galenica Senese Materino d'Arbia (SI), Italy] was injected intracamerally in Group 2. Bevacizumab (0.25 mg/0.01 mL; Altuzan, Roche Diagnostics GmbH, Mannheim, Germany) was injected intracamerally in Group 3. At 7d, two rats in Group 2 and one rat in Group 3 had died.

At 7d, the rats were deeply anesthetized again prior to enucleation. To reach the back of the globe, pressure was applied on the edge of the limb using conjunctival forceps, and enucleation was performed using corneoscleral scissors. At the end of enucleation, the bulbus oculi was removed, and the animals were euthanized by exsanguination. Round cornea specimens were taken with a limbal incision using a 15° corneal blade. Pathological specimens were placed in 10% formaldehyde, and biochemical specimens were placed into dry boxes. Finally, a 3-cm midline abdominal incision was made, and a 1.5 mL blood sample was taken from the inferior vena cava. The tissue and blood samples were stored at -70°C until the measurements of TAS and TOS activities [11,12].

Caspases-3 and 8 staining The materials were fixed with 10% formaldehyde, and 4-µm-thick sections cut from paraffin wax blocks were stained with a standard streptavidin-biotin immunoperoxidase method using anti-caspase-3 (cleaved; clone: N/A, catalog no. PP 229 AA, Biocare Medical) and anti-caspase-8 (clone: C502S, catalog no. GTX59555, Gene Tex) antibodies. Tonsillar tissue was used as a positive control. All specimens were evaluated by light microscopy (Olympus BX51TF; Olympus Corp., Tokyo, Japan). The immunohistochemical study was evaluated semi-quantitatively using the following scale: negative staining was scored as "0", weak staining was scores as "1", moderate staining was scored as "2", and intense staining was scored as "3" [11,12].

Measurement of Total Antioxidant Status The TAS of each supernatant fraction was determined using the novel automated measurement method developed by Erel [21,22]. In this method, hydroxyl radicals, *i.e.* the most potent biological radical, are produced. In the assay, the ferrous ion solution present in Reagent 1 is mixed with hydrogen peroxide, which is present in Reagent 2. The sequentially produced radicals,

Table 1 Biochemical oxidative stress parameters

Variables	Sham group (n=10)	Control group (n=8)	Bevacizumab group (n=9)	P ¹
TAS (mmol Trolox equiv/L)	0.97 (0.80, 1.12)	1.17 (0.83, 1.50)	0.88 (0.76, 1.36)	0.223
TOS (μmol H ₂ O ₂ equiv/L)	40.25 (29.71, 55.08)	43.49 (11.86, 72.37)	40.53 (16.16, 74.10)	0.988
OSI (arbitrary units)	4.20 (3.00, 6.56)	3.70 (1.42, 4.87)	4.36 (2.09, 6.10)	0.368

Variables are expressed as medians, minimums and maximums. ¹The Kruskal-Wallis test was used. TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

Table 2 Corneal tissue oxidative stress parameters

Variables	Sham group (n=10)	Control group (n=8)	Bevacizumab group (n=9)	P ¹
TAS (mmol Trolox equiv/L)	0.40 (0.20, 0.45)	0.47 (0.18, 0.84)	0.48 (0.17, 1.31)	0.165
TOS (μmol H ₂ O ₂ equiv/L)	5.30 (2.88, 8.84)	6.21 (3.42, 8.79)	11.58 (3.57, 15.48) ^{a,b}	0.014
OSI (arbitrary units)	1.39 (1.31, 2.71)	1.76 (1.36, 2.34)	3.66 (1.65, 6.10) ^{c,d}	0.001

Variables are expressed as medians, minimums and maximums. ¹The Kruskal-Wallis test was used. ^{a,b,c,d}A Mann-Whitney *U* test was used if statistical significance was found. ^aBevacizumab vs Sham: *P*=0.013; ^bBevacizumab vs Control: *P*=0.036; ^cBevacizumab vs Sham: *P*=0.001; ^dBevacizumab vs Control: *P*=0.002. TAS: Total antioxidant status; TOS: Total oxidant status; OSI: Oxidative stress index.

including the brown-colored dianisidiny radical cation produced by the hydroxyl radical, are also potent radicals. Using this method, the antioxidant effect in the sample against these potent-free radical reactions that are initiated by the produced hydroxyl radicals is measured. The assay exhibited excellent precision values below 3%. The results are expressed as nmol Trolox equiv/mg protein.

Measurement of Total Oxidant Status The TOS of each supernatant fraction was determined using a novel automated measurement method that was also developed by Erel^[21,22]. The oxidants in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which is measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in nmol H₂O₂ equiv/mg protein.

Oxidative Stress Index The OSI was defined as the ratio of the TOS to the TAS level. For these calculations, the TAS units were changed to mmol/L, and the OSI was calculated according to the following formula:

$$\text{OSI (arbitrary units)} = \text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equiv/L}) / \text{TAS (mmol Trolox equiv/L)}$$

Repeatability of the Experimental Results Concerning whether the repeatability of these experimental results is sufficiently repeatable in different trials, this method does not involve the use of any different techniques, which helps improve the stability and repeatability of the experimental results.

Statistical Analysis All statistical analyses were performed using the SPSS software (ver. 17.0 for Windows; SPSS Inc., Chicago, IL, USA). Nonparametric independent group comparisons were performed. The data are expressed as medians, minimums, and maximums. For multiple comparisons, the Kruskal-Wallis was used to compare the

Table 3 Immunohistochemical staining for caspase-3

Groups	0	+	++	+++	Total
Sham group	9	1	-	-	10
Control group	7	1	-	-	8
Bevacizumab group	0	3	4	2	9

Table 4 Immunohistochemical staining for caspase-8

Groups	0	+	++	+++	Total
Sham group	9	1	-	-	10
Control group	7	1	-	-	8
Bevacizumab group	1	3	4	1	9

groups, and the Mann-Whitney test was used when statistical significance was found. A two-sided *P* value < 0.05 was considered to indicate statistical significance.

RESULTS

The TAS, TOS, and OSI results for both the blood samples and corneal tissues are presented in Tables 1 and 2. In the blood samples, the TAS, TOS, and OSI levels were not significantly different (all *P*>0.05). Compared with the sham and control groups, the TOS and OSI levels in the corneal tissue were significantly higher in the bevacizumab group (all *P*<0.05). However, no statistically significant difference was found between the sham and control groups (all *P*>0.05). Moreover, compared with the sham and control groups, greater immunohistochemical staining for caspases-3 and -8 and increased apoptotic activities were observed in the bevacizumab group (Tables 3, 4; Figures 1, 2).

DISCUSSION

To our knowledge, this is the first study to evaluate the safety of intracameral bevacizumab for corneal tissue using oxidative stress parameters and apoptotic activity. The main findings of this study are as follows: 1) intracameral bevacizumab had no apparent systemic effect, and 2) intracameral bevacizumab may have locally toxic effects on the corneal tissue.

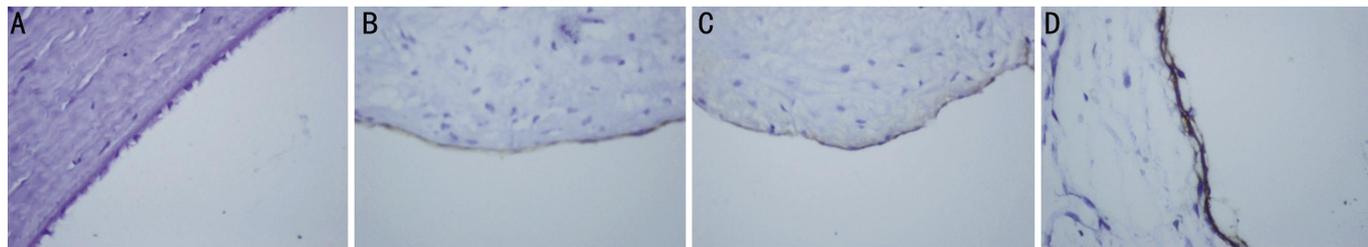


Figure 1 Immunohistochemical staining of endothelial cells for caspase-3 (×400) A: Negative; B: Weak; C: Moderate; D: Intense.

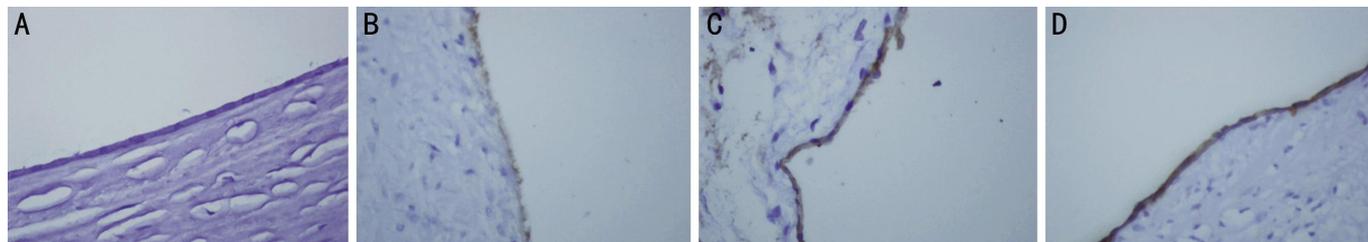


Figure 2 Immunohistochemical staining of endothelial cells for caspase-8 (×400) A: Negative; B: Weak; C: Moderate; D: Intense.

Several reports have suggested that low doses of bevacizumab that are delivered topically or subconjunctivally to treat corneal neovascularization and pterygia do not cause serious adverse effects [23-27]. Further, intravitreal injections of bevacizumab do not to have harmful effects on the corneal endothelium. These findings are consistent with other reports of the effects of bevacizumab on cultured corneal cells [28-31]. Finally, intracameral injections have recently been used more frequently in preclinical and clinical studies. Park *et al* [5] used a rabbit model and found that intracameral bevacizumab injection had no effects on corneal endothelial cells or corneal thickness. Rusovici *et al* [1] reported similar results in a cell culture model. Shin *et al* [32] performed intracameral injections of bevacizumab in both rabbit eyes and neovascular glaucoma patients for 1mo and observed no toxic effects on the corneal endothelium. Lim *et al* [33] performed intracameral bevacizumab injections in neovascular glaucoma patients, and corneal toxicity did not occur in the short-term follow-up period of that study. Moreover, the Tübingen Bevacizumab Study Group [34] performed intracameral administrations of bevacizumab in three selected iris rubeosis patients with associated neovascular glaucoma and observed no morbidity over a 1-mo follow-up period. Additionally, Chalam *et al* [35] demonstrated that bevacizumab was nontoxic to human corneal epithelial, corneal fibroblast, and human umbilical vascular endothelial cells at various doses.

In contrast, several *in vivo* and *in vitro* studies have reported opposing results, *i.e.* possible toxic effects of bevacizumab injections [16-18]. Specifically, with intraocular injections, infectious or noninfectious intraocular inflammatory reactions may occur [36]. Kim *et al* [37] showed that the use of a high concentration of bevacizumab as a topical treatment can cause cornea epithelial defects. There are some reports of intraocular inflammatory responses, such as endophthalmitis,

retinal detachments, and suprachoroidal hemorrhages, following intravitreal bevacizumab injections [38-42]. In a study by Colombres *et al* [43], the intravitreal injection of bevacizumab caused spillage over the ocular surface and created the same effect on the edematous corneas that was observed in our seven patients. In a retrospective study of 1200 patients who were treated with intravitreal bevacizumab, corneal infiltrative keratitis and corneal stromal edema were noted in 1.1% of patients [44]. Although Hosny *et al* [45] demonstrated that intracameral bevacizumab injections elicited no adverse effects on corneal endothelial cells, these authors reported a significantly increase in the rate of corneal endothelial cell loss at the end of the fourth month of follow-up [3.95%±6.78% (range 1.54-23.22)].

In the literature, only a few studies have been performed to investigate the effects of bevacizumab on ocular tissue using oxidative stress markers alone or in combination with assessments of apoptotic activity. Sari *et al* [36] evaluated the effects of repeated 1.25-mg intravitreal bevacizumab injections on rabbit corneas and uveoretinal tissues using histological and biochemical analyses in an experimental study. No inflammation in the aqueous humor and no signs of corneal or uveoretinal toxicities were observed in the bevacizumab-injected eyes. In the corneal tissue, the activity of the caspase 3 enzyme did not exhibit any significant change, whereas the differences in the activity of the caspase 8 enzyme between the bevacizumab-injected group and the control and sham groups were statistically significant. Corneal oxidative stress markers, including catalase activity, glutathione levels, and malondialdehyde content, did not exhibit any significant differences. Sancho-Tello *et al* [46] studied the histopathological, biochemical and functional effects of intravitreal bevacizumab on rat eyes with a special emphasis on its immediate pro-inflammatory features and the eventual association of these features with cellular oxidative

burden. For this purpose, these authors performed bevacizumab injections (75 mg/rat eye) and performed biochemical analyses of oxidative stress-related markers, including malondialdehyde and glutathione peroxidase, at 24h, 1wk and 4wk; they observed no changes in any of the oxidative stress markers at any of the time points after the injections. Additionally, Xu *et al*^[47] evaluated the potential toxicity of repeated intravitreal injections of bevacizumab in rabbit eyes. These authors performed three sequential, biweekly, intravitreal injections of bevacizumab at doses of 2.5 mg/0.1 mL or 5.0 mg/0.2 mL. The eyes were enucleated at 1 and 4wk after the last intravitreal injection and subsequently underwent light and electron microscopic evaluations and testing for apoptotic activity. These authors found that the biweekly, multiple intravitreal injections of bevacizumab did not result in evidence of toxicity in regular clinical and functional observations at either the 2.5 mg or 5.0 mg doses and further revealed that the 5.0 mg dose may have induced transient inflammation, ultrastructural abnormalities, and apoptosis. In our study, we analyzed the toxicity of bevacizumab using oxidative stress parameters and apoptotic activity and observed increases in the oxidative stress parameters and apoptotic activity in the corneal tissue. Given this background, the majority of the previously reported clinical and experimental studies have shown that the intracameral administration of this drug is safe for corneal tissue. However, controversy remains regarding whether the bevacizumab is safe when administered *via* other intraocular routes. Our results are not fully consistent with those of previous studies regarding the toxic effects of bevacizumab when it is administered intracamerally to the corneal tissue. These inconsistencies might have resulted from the methods that we employed in the present study. For example, we used rat model in contrast to the other studies. Specifically, we found no systemic effects, as determined via assessments of oxidative stress parameters in the blood samples. However, we identified increased oxidative stress parameters and apoptotic activities in the corneal tissue samples by assessing oxidative stress markers and caspase-3 and -8 staining. As mentioned above, oxidative stress is considered to be responsible for cellular damage, and the use of the more valid and reliable oxidative stress parameters in the present study contrasts with the methods utilized in many previous studies. In conclusion, our evaluations revealed showed that the intracameral administration of bevacizumab is systemically safe but may have local toxic effects in the corneal tissue, as indicated by the histopathological evaluations. Our study has some limitations that should be noted. First, this study used an animal model, and we did not assess temporary effects or the effects of different doses of bevacizumab on endothelial cell function. Second, no clinical or experimental toxicities were observed to be associated with the intracameral administration of bevacizumab; however, these studies did

not use electron microscopy or quantitate apoptotic activity with methods such as TUNEL. Additional detailed information would be gained via electron microscopic and quantitative apoptotic activity assessments. Our investigation has perhaps provided deeper insight into the toxic effects of intracameral bevacizumab. However, additional studies are needed to address these issues.

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