

Effect of Bevacizumab on the Viability and Metabolism of Human Corneal Epithelial and Endothelial Cells: An In Vitro Study

Shayan Shokoohi¹, Alfonso Iovieno¹, and Sonia N. Yeung¹

¹ Department of Ophthalmology and Visual Sciences, University of British Columbia Eye Care Centre, Vancouver, BC, Canada

Correspondence: Sonia N. Yeung, Department of Ophthalmology and Visual Sciences, University of British Columbia Eye Care Centre, 2550 Willow Street, Vancouver, BC, Canada V5Z 3N9.
e-mail: sonia.y@gmail.com

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Purpose: To examine the cytotoxic effects of bevacizumab on the viability and metabolism of human corneal epithelial cells (HCEpCs) and human corneal endothelial cells (HCEncs), as well as human retinal pigment epithelial (ARPE-19) cells for comparison.

Methods: Immortalized cell lines of HCEpCs, HCEncs, and ARPE-19 cells were exposed to clinically relevant concentrations of bevacizumab (0.313–5.00 mg/mL). The ApoTox-Glo Triplex Assay was used to assess cell viability, cytotoxicity, and apoptosis, and the Mitochondrial ToxGlo Assay was used to assess cell membrane integrity and adenosine triphosphate (ATP) levels after a 24-hour treatment period.

Results: Across all three cell types, we observed similar results of a decrease in cell viability at 5.00 mg/mL ($P < 0.05$) and an increase in cytotoxicity at 5.00 mg/mL ($P < 0.05$), whereas apoptotic activity remained unchanged ($P > 0.05$), which is a profile consistent with cells undergoing primary necrosis at high concentrations. Additionally, cell membrane integrity was compromised at 5.00 mg/mL ($P < 0.05$), whereas no decrease in ATP levels were observed ($P > 0.05$). Thus, no interference with mitochondrial oxidative phosphorylation in ATP production was seen, and the cells were able to maintain normal metabolic levels at high concentrations of bevacizumab.

Conclusions: HCEpCs, HCEncs, and ARPE-19 cells experience a decrease in viability and undergo primary necrosis when exposed to bevacizumab at a concentration of 5.00 mg/mL; however, they are able to maintain normal metabolism and mitochondrial function at the high concentrations used for the treatment of corneal neovascularization.

Translational Relevance: This study provides safety data on the concentrations of bevacizumab injected intravitreally and complements clinical data showing toxicity of topical bevacizumab on corneal epithelial and endothelial cells.

Introduction

The avascular nature of the cornea is disrupted when corneal neovascularization (NV) causes the formation of new blood vessels on the ocular surface, resulting in opacification and subsequent blindness. Corneal NV is not a diagnosis but a non-specific response to various clinical insults, such as contact lens-related hypoxia, chemical burns, trauma, infectious keratitis, inflammatory disorders, and autoimmune diseases.¹ Vascular endothelial growth factor

(VEGF), a pro-angiogenic factor, plays the most critical role in the development of several retinal neovascular diseases, as well as in corneal NV, and its inhibition by anti-VEGF agents has proven to be successful in the management of these conditions.¹ Bevacizumab (Avastin; Genentech, South San Francisco, CA), a full-length recombinant humanized monoclonal antibody against VEGF, is most widely used and researched due to its cost effectiveness, and, more recently, it has been studied as a topical agent for the treatment of corneal NV.¹

Nevertheless, the off-label use of bevacizumab in ophthalmology is a cause for concern, and its

biocompatibility on the cornea must be ensured, as there is evidence for potential corneal cytotoxicity. It is speculated that topical bevacizumab possibly interferes with the adhesion between the corneal epithelium and the basement membrane, leading to delayed wound healing and stromal thinning, thus causing an increased risk of corneal epithelial defects that make the eye prone to infection, stromal ulceration, perforation, scarring, and significant vision loss.^{1,2} Previous reports have provided conflicting evidence regarding the concentration of bevacizumab that is toxic to human corneal cells, ranging from 1.0 mg/mL up to 5.0 mg/mL, when used topically on the cornea.³⁻⁵

In this *in vitro*, experimental study, we assessed the cytotoxic effects of bevacizumab (Avastin) on the viability and metabolism of two corneal cell lines: human corneal epithelial cells (HCEpCs) and human corneal endothelial cells (HCEncs). Given that published cytotoxicity of bevacizumab on human retinal pigment epithelial (ARPE-19) cells range from 2.50 to 5.00 mg/mL,^{6,7} we also studied ARPE-19 cells for comparison and as a positive control.

Methods

Cell Culture

Human Corneal Epithelial Cells

An immortalized human corneal epithelial cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured on T25 flasks (Corning, Corning, NY) containing corneal epithelial cell basal medium (ATCC) supplemented with a corneal epithelial cell growth kit (ATCC) consisting of 0.2% corneal epithelial growth factor, 5- μ g/mL apo-transferrin, 1.0- μ M epinephrine, 0.4% extract P, 100-ng/mL hydrocortisone hemisuccinate, 6-mM L-glutamine, and 5- μ g/mL recombinant human insulin. The cells were cultured in a 37°C, 5% CO₂ incubator, and the medium was changed every 2 to 3 days until 90% confluence. The cells were then passaged using Gibco 0.25% trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA). Enzyme activity was quenched by a trypsin neutralizing solution (ATCC) containing 10% fetal bovine serum (FBS) in Dulbecco's phosphate-buffered saline. Cells at passage 4 were used for the experiments.

Human Corneal Endothelial Cells

An immortalized human corneal endothelial cell line (B4G12) was purchased from DSMZ (Braunschweig, Germany) and cultured on T75 flasks (Corning) coated with 10- μ g/mL laminin (Sigma-Aldrich, St.

Louis, MO) and 10-mg/mL chondroitin sulfate (Sigma-Aldrich). The medium consisted of human endothelial serum-free medium (Gibco) supplemented with 10-ng/mL basic fibroblast growth factor (Gibco). The cells were cultured in a 37°C, 5% CO₂ incubator, and the medium was changed every 2 to 3 days until 90% confluence. The cells were then passaged using Gibco 0.25% trypsin-EDTA. Enzyme activity was quenched by a protease inhibitor cocktail (Sigma-Aldrich) in a 500-fold dilution with Hanks' balanced salt solution (Gibco). Cells at passage 7 were used for the experiments.

Human Retinal Pigment Epithelial Cells

An immortalized human retinal pigment epithelial cell line was purchased from ATCC and cultured on T75 flasks (Corning) containing Dulbecco's Modified Eagle Medium and Ham's F-12 1:1 mixture (DMEM/F-12; HyClone Laboratories, Logan, UT) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were cultured in a 37°C, 5% CO₂ incubator, and the medium was changed every 2 to 3 days until 90% confluence. The cells were then passaged using Gibco 0.25% trypsin-EDTA. Enzyme activity was quenched by a complete growth medium containing 10% FBS. Cells at passage 13 were used for the experiments.

ApoTox-Glo Triplex Assay

The ApoTox-Glo Triplex Assay was purchased from Promega (Madison, WI) to determine HCEpC, HCEnc, and ARPE-19 cell viability, cytotoxicity, and apoptosis, following the manufacturer's instructions. Two substrates, glycyphenylalanyl-aminofluoroumarin (GF-AFC) and bis-AAF-R110, were added and produced fluorescent signals proportional to viability and cytotoxicity, respectively. Substrate GF-AFC is able to enter cells and is only cleaved by live-cell proteases; it becomes inactive when cell membrane activity has been lost. Conversely, substrate bis-AAF-R110 is not able to enter cells and is only cleaved by dead-cell proteases leaked from cells, which lack membrane integrity. The cleaved substrates produce two products, AFC and R110, respectively, which have different excitation and emission spectra, allowing them to be measured simultaneously at two wavelength sets: 400 nm_{Ex}/505 nm_{Em} (viability) and 485 nm_{Ex}/520 nm_{Em} (cytotoxicity). Subsequently, a caspase-3/7 substrate was added and produced a luminescent signal proportional to the amount of caspase activity present. Substrate caspase-3/7 results in cell lysis, followed by caspase cleavage of the substrate by apoptotic cells, generating a "glow-type"

luminescent signal produced by luciferase. Positive controls included digitonin for the cell viability and cytotoxicity assay and staurosporine for the apoptosis assay. Fluorescence and luminescence were measured with a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT).

Mitochondrial ToxGlo Assay

The Mitochondrial ToxGlo Assay was purchased from Promega to determine HCEpC, HCEnC, and ARPE-19 membrane integrity and adenosine triphosphate (ATP) levels following the manufacturer's instructions. Cells were treated in serum-free RPMI 1640 medium supplemented with 2.0 g/L sodium bicarbonate and 1.8 g/L galactose, as opposed to glucose, which would serve as a non-mitochondrial ATP source, in order to be more responsive to mitochondrial insults and primarily rely on mitochondrial oxidative phosphorylation to produce ATP. First, cell membrane integrity was assessed by measuring a distinct protease activity associated with necrosis using a fluorogenic peptide substrate, bis-AAF-R110, to detect dead cell protease activity. Next, ATP detection reagent was added, causing cell lysis and producing a luminescent signal proportional to the amount of ATP present. Fluorescence and luminescence were measured with a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek Instruments).

Statistical Analysis

All data are expressed as mean \pm standard error of the mean (SEM). Results were analyzed by one-way analysis of variance with Dunnett's post hoc test for multiple comparisons to the untreated control using Prism 8 (GraphPad Software, San Diego, CA). All data are presented as percentage of the control with the control normalized to 100%. $P < 0.05$ was considered statistically significant.

Results

Effect of Bevacizumab on Cell Viability, Cytotoxicity, and Apoptosis

The ApoTox-Glo Triplex Assay was used to assess cell viability, cytotoxicity, and apoptosis of the three cell types in response to clinically relevant concentrations of bevacizumab, ranging from 0.313 to 5.00 mg/mL, after a 24-hour treatment period. Cell viability of HCEpCs was unaffected up to a concentration of 2.50 mg/mL; however, a significant decrease

($P = 0.0015$) was observed at a concentration of 5.00 mg/mL (Fig. 1A). A significant increase ($P = 0.0032$) in cytotoxicity was also seen at a concentration of 5.00 mg/mL (Fig. 1B). Apoptosis, detected by caspase-3/7 activity, remained unchanged across all concentrations (Fig. 1C).

Similarly, the cell viability of HCEncs was unaffected up to a concentration of 2.50 mg/mL; however, a significant decrease ($P = 0.0034$) was observed at a concentration of 5.00 mg/mL (Fig. 2A). A significant increase ($P = 0.0035$) in cytotoxicity was also seen at a concentration of 5.00 mg/mL (Fig. 2B). Apoptotic activity remained unchanged across all concentrations (Fig. 2C).

Finally, cell viability of ARPE-19 was unaffected up to a concentration of 2.50 mg/mL; however, a significant decrease ($P < 0.0001$) was observed at a concentration of 5.00 mg/mL (Fig. 3A). A significant increase ($P = 0.0108$) in cytotoxicity was also seen at a concentration of 5.00 mg/mL (Fig. 3B). Apoptotic activity remained unchanged across all concentrations (Fig. 3C).

Effect of Bevacizumab on Cell Membrane Integrity and ATP Levels

The Mitochondrial ToxGlo Assay was used to evaluate cell membrane integrity and ATP levels of the three cell types in response to clinically relevant concentrations of bevacizumab, ranging from 0.313 to 5.00 mg/mL, after a 24-hour treatment period. Membrane integrity of the HCEpCs diminished, as seen with a significant increase ($P = 0.0032$) in cytotoxicity at a concentration of 5.00 mg/mL (Fig. 4). No significant changes in ATP levels were observed with increasing concentrations of bevacizumab up to 5.00 mg/mL (Fig. 4). These results suggest that bevacizumab does not induce mitochondrial toxicity in HCEpCs at 5.00 mg/mL, as cellular ATP production is not impaired. Therefore, HCEpCs are able to maintain normal metabolism when exposed to bevacizumab up to a concentration of 5.00 mg/mL for 24 hours.

Similarly, the membrane integrity of HCEncs diminished, as seen with a significant increase ($P = 0.0035$) in cytotoxicity at a concentration of 5.00 mg/mL (Fig. 5). ATP levels, however, were not affected by increasing concentrations of bevacizumab up to 5.00 mg/mL (Fig. 5). These results suggest that bevacizumab does not induce mitochondrial toxicity in HCEncs at 5.00 mg/mL, as cellular ATP production is not impaired. Therefore, HCEncs are able to maintain normal metabolism when exposed to bevacizumab up to a concentration of 5.00 mg/mL for 24 hours.

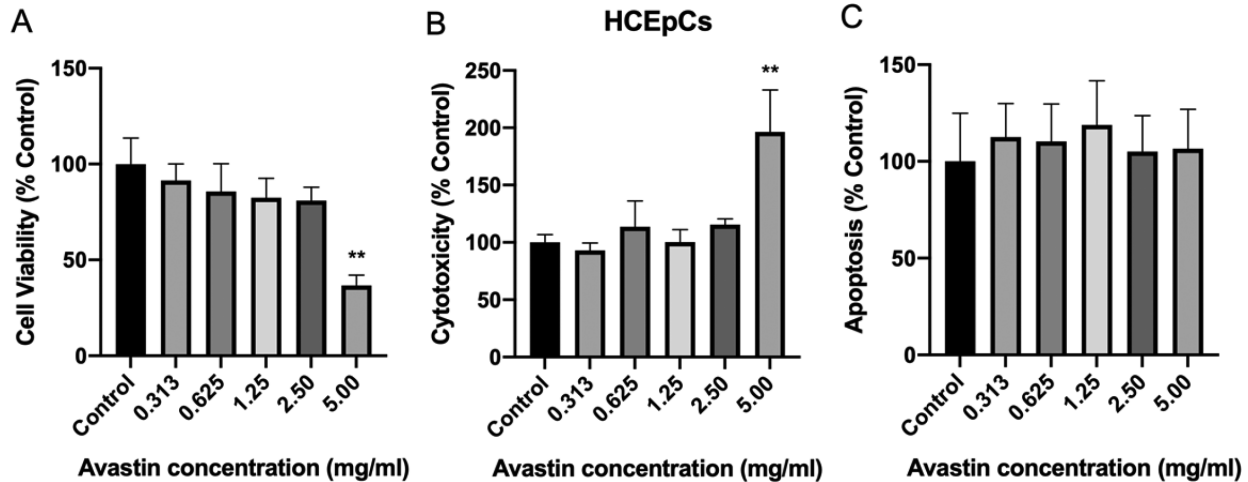


Figure 1. Effect of bevacizumab (Avastin) on HCEpC viability (A), cytotoxicity (B), and apoptosis (C) as detected by the ApoTox-Glo Triplex Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as ** $P < 0.01$.

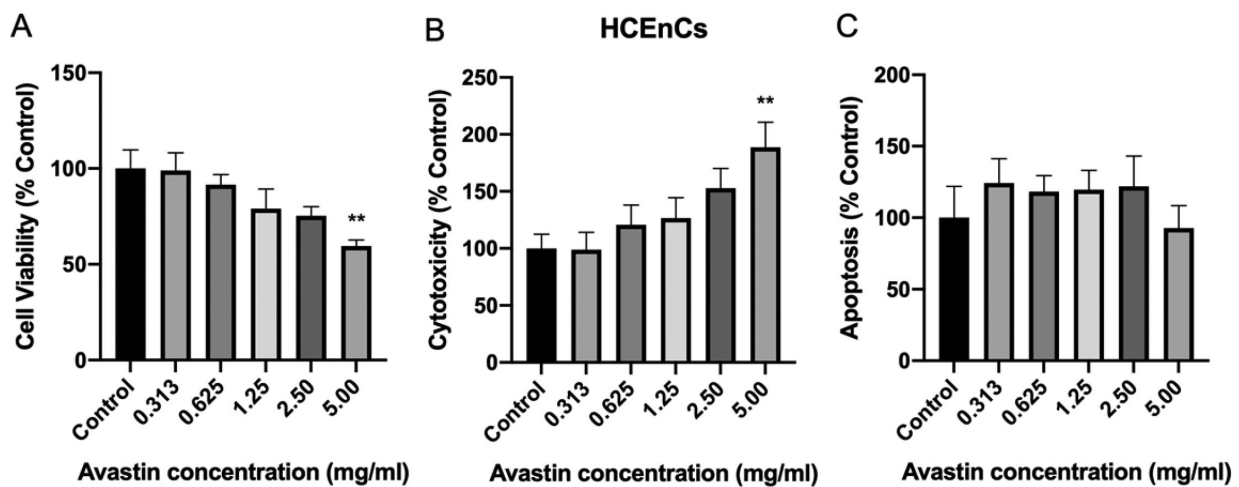


Figure 2. Effect of bevacizumab (Avastin) on HCEnc viability (A), cytotoxicity (B), and apoptosis (C) as detected by the ApoTox-Glo Triplex Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as ** $P < 0.01$.

Finally, the membrane integrity of ARPE-19 cells diminished, as seen with a significant increase ($P = 0.0108$) in cytotoxicity at a concentration of 5.00 mg/mL (Fig. 6). ATP levels remained unchanged with increasing concentrations of bevacizumab up to 5.00 mg/mL (Fig. 6). These results suggest that bevacizumab does not induce mitochondrial toxicity in ARPE-19 cells at 5.00 mg/mL, as cellular ATP production is not impaired. Therefore, ARPE-19 cells are able to maintain normal metabolism when exposed to bevacizumab up to a concentration of 5.00 mg/mL for 24 hours.

Discussion

In this study, we evaluated the cytotoxic effects of bevacizumab on human corneal epithelial cells, endothelial cells, and retinal pigment epithelial cells across various concentrations for a 24-hour treatment period. For all of the experiments, we took the highest concentration of bevacizumab (5.00 mg/mL) that is typically used for the treatment of corneal NV down to the lowest concentration (0.313 mg/mL) that is injected intravitreally (50 μ L of 25-mg/mL stock; diluted in

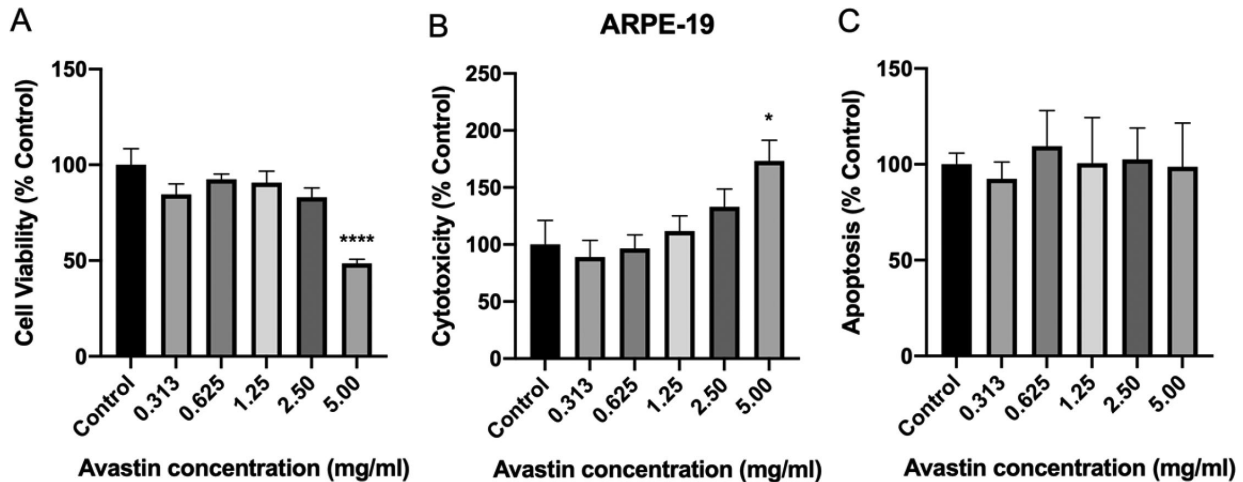


Figure 3. Effect of bevacizumab (Avastin) on ARPE-19 cell viability (A), cytotoxicity (B), and apoptosis (C) as detected by the ApoTox-Glo Triplex Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as * $P < 0.05$ and **** $P < 0.0001$.

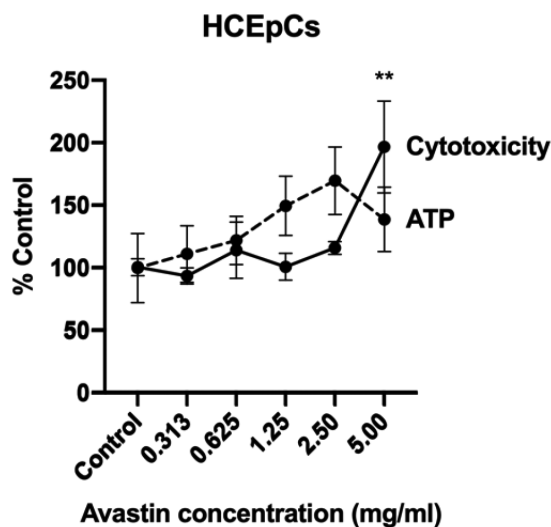


Figure 4. Effect of bevacizumab (Avastin) on HCEpC cytotoxicity (solid line) and ATP levels (dashed line) as detected by the Mitochondrial ToxGlo Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as ** $P < 0.01$.

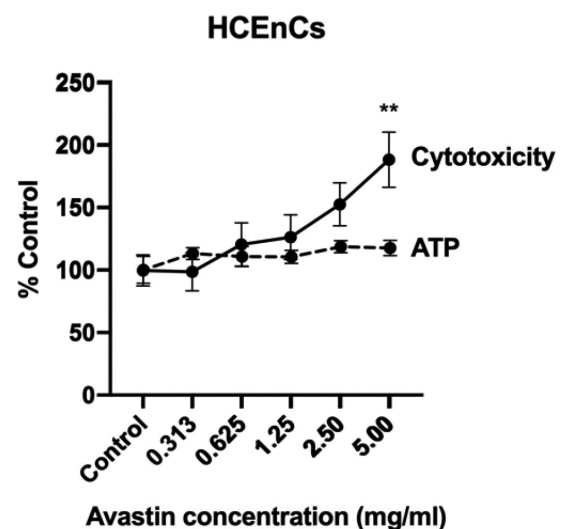


Figure 5. Effect of bevacizumab (Avastin) on HCEnc cytotoxicity (solid line) and ATP levels (dashed line) as detected by the Mitochondrial ToxGlo Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as ** $P < 0.01$.

4 mL of vitreous). Across all three cell types, we observed similar results of a decrease in cell viability at 5.00 mg/mL with a concomitant increase in cytotoxicity at 5.00 mg/mL, whereas apoptotic activity remained unchanged. After a 24-hour treatment period, caspase-3/7 activity was similar to that of the untreated control, a profile consistent with cells undergoing primary necrosis caused by the toxicity of bevacizumab at a concentration of 5.00 mg/mL, leading to non-apoptotic, accidental cell death by a foreign toxin, thus resulting in no increase in caspase-

3/7 activity. Additionally, cell membrane integrity was compromised at 5.00 mg/mL, whereas no changes in ATP levels were observed. Under high concentrations of bevacizumab, we observed cytotoxicity with no mitochondrial dysfunction, as there was no significant decrease in ATP levels. As the final step of the assay, the reagent lyses all the cells and determines the total amount of ATP available both intracellularly and extracellularly; therefore, it is possible that total ATP levels did not diminish, yet more cells had lost their membrane integrity. Nonetheless, we observed no

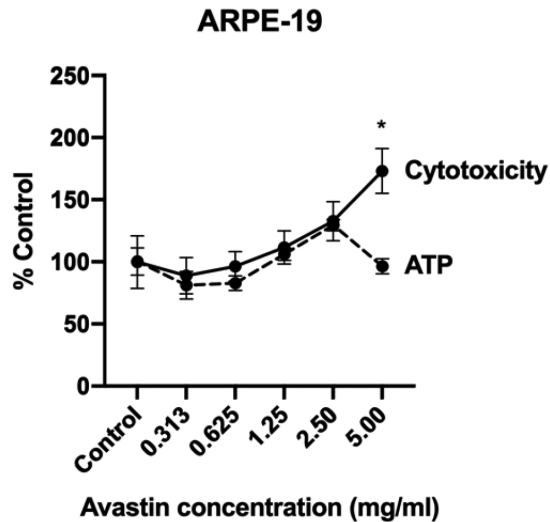


Figure 6. Effect of bevacizumab (Avastin) on ARPE-19 cytotoxicity (solid line) and ATP levels (dashed line) as detected by the Mitochondrial ToxGlo Assay following a 24-hour treatment period. Data are presented as mean \pm SEM ($n = 8$), with significant differences between the treated group and untreated control group designated as * $P < 0.05$.

interference with mitochondrial oxidative phosphorylation in the production of ATP, and the cells appeared to maintain normal metabolic levels and mitochondrial function across the concentrations tested.

Other studies have reported the cytotoxic effects of bevacizumab on human corneal epithelial cells and endothelial cells, although with varying results. Yoeruek et al.⁴ demonstrated that bevacizumab did not cause a significant decrease in cell viability or any morphological changes in HCEpCs up to a concentration of 5.0 mg/mL after 24-hour exposure. Chalam et al.⁵ showed that bevacizumab was non-toxic to HCEpCs up to a concentration of 2.0 mg/mL after 24-hour exposure. Conversely, Kang et al.³ reported that bevacizumab significantly decreased the cell viability of HCEpCs at concentrations of 1.0 mg/mL and 2.0 mg/mL after 24-hour exposure, and they observed a significant reduction in HCEpC wound healing due to decreased expression levels of phosphorylated p38 mitogen-activated protein kinase, a marker for cell migration. In a clinical setting, reports of delayed corneal epithelial healing due to intravitreal bevacizumab have also been published. Colombres et al.⁸ performed a retrospective chart review on 850 eyes of 850 patients receiving intravitreal bevacizumab for various retinal neovascular diseases, and they observed that seven patients who had pre-existing corneal edema developed corneal epithelial defects (CEDs). Importantly, none of the patients without corneal edema developed CEDs.⁸ Therefore, bevacizumab may not be

ideal for treating patients with a pre-existing ocular surface disease (OSD).

One method that has been proposed for the treatment of corneal NV for patients who present with CEDs or OSD is use of the Prosthetic Replacement of the Ocular Surface Ecosystem (PROSE) device as a drug delivery system for bevacizumab. In a recent in vivo study, Yin and Jacobs⁹ evaluated the long-term outcome of using the PROSE device in 13 patients with significant pre-existing OSD and reported that 12 patients had significant corneal NV regression and 10 patients had significant improvement in visual acuity over the 5-year average follow-up period, with no ophthalmic or systemic complications. They speculated that the PROSE device prevented any ophthalmic adverse events because (1) the ocular surface remains nourished and protected underneath the device; (2) a preservative-free, pH-balanced microenvironment is provided by the device to deliver the bevacizumab; (3) bevacizumab, a large molecule, is retained in the reservoir of the device for a longer duration, possibly increasing its bioavailability to the cornea; and (4) the device lowers the susceptibility of the cornea to the many factors that may cause corneal NV, ending the vicious cycle between corneal NV and heightened inflammation.⁹ Thus, the PROSE device may prove to be an effective drug delivery system for treating corneal NV in order to avoid potential corneal cytotoxicity.

Despite evidence that high concentrations of bevacizumab (~ 5.00 mg/mL) may be toxic to the corneal epithelium, very low concentrations of topical bevacizumab are being explored as an effective modality against OSD. In a recent randomized, controlled clinical trial, Kasetsuwan et al.¹⁰ assessed the safety and efficacy of bevacizumab 0.05% (0.5 mg/mL) eye drops as a novel treatment for dry eye disease (DED) in 31 participants, and they concluded that all participants had improvement in tear break-up time, an important diagnostic sign in DED, over the 12-week follow-up period with no adverse events. Thus, this may be considered as an alternative treatment for DED to decrease inflammation as opposed to the use of steroids and cyclosporin-A, which can have adverse events, such as elevated intraocular pressure, cataract, and burning or stinging symptoms.¹¹

Our study does have some limitations. First, the responses of human corneal epithelial and endothelial cells to bevacizumab in vivo may be different from those of immortalized cell lines tested in vitro, as in vivo conditions introduce other environmental factors and results may not be directly transferrable. Second, the treatment period was limited to 24 hours, as cells were treated in starving conditions (serum-free) to study molecular mechanisms, so longer incubation periods

were not possible. Third, we were not able to investigate the effect of repeated topical applications of bevacizumab on corneal cells under in vitro conditions due to limitations of the assay. It is possible that there could be a depot effect or accumulative effect of the drug under in vivo conditions that could contribute to toxicity over time and repeated eye drops. Therefore, in vivo animal studies are necessary to assess how frequency and longevity of topical applications of bevacizumab may affect the safety and efficacy of this treatment for corneal NV.

In conclusion, this in vitro study demonstrates that HCEpCs, HCEncs, and ARPE-19 cells experience a decrease in viability when exposed to bevacizumab at a concentration of 5.00 mg/mL; however, they are able to maintain normal metabolism and mitochondrial function at the high concentrations typically used for the treatment of corneal neovascularization.

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