Comparison of Subconjunctival Aflibercept and Betamethasone for the Treatment of Formed Corneal Neovascularization in a Rabbit Model

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Keywords

Aflibercept · Betamethasone · Chemical burn · Corneal neovascularization · Rabbit model

Abstract

Aim: To compare the efficacy of aflibercept (Eylea®), a potent antivascular endothelial growth factor (VEGF) agent, with betamethasone (Celestone®) and placebo for the treatment of formed corneal neovascularization in a rabbit model. **Methods:** A central corneal chemical burn was created in the right eye of 24 New Zealand albino rabbits. Four weeks later, the rabbits were randomly divided into 4 equal groups for subconjunctival injection of aflibercept, betamethasone, aflibercept+ betamethasone, or saline (control). Digital photographs taken at weekly intervals were rated by 2 masked observers for extent, centricity, and density of corneal neovascularization according to a predefined scale. The percentage of corneal surface involved by neovascularization was quantified by image analysis software (Fiji-J). The change in corneal neovascularization from treatment administration (4 weeks after injury) to 4 weeks later (8 weeks after injury) was assessed. The rabbits were then euthanized, and their eyes were enucleated and processed for histopathological and immunofluorescence studies. **Results:** There was no significant difference in the change in corneal neovasculariza-

tion after treatment among the 4 groups according to the digital images ($p > 0.15$) or histological evaluation with hematoxylin and eosin ($p > 0.08$). On immunofluorescence assay, a lower VEGF concentration was observed in all treatment groups compared to the control group. **Conclusions:** In this rabbit model, corneal neovascularization induced by chemical burn failed to regress with treatment with aflibercept, betamethasone, or their combination.

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Introduction

An equilibrium between proangiogenic and antiangiogenic factors is required to maintain clarity of the cornea [1, 2]. The equilibrium is disrupted in the presence of proangiogenic stimuli from corneal insult such as chemical burn, ischemia, infection, inflammation, and trauma. The resulting corneal vascularization [3, 4] may lead to inflammation, edema, and corneal scarring that block out light and cause irregular astigmatism, thereby impairing visual acuity and leading to the loss of normal corneal functions.

M.E.-M. and E.L. contributed equally to the work.

The treatment of corneal neovascularization is highly challenging. Several treatment modalities are available, but none is widely accepted. They include removing the primary cause of vessel formation, local application of corticosteroids, photodynamic therapy, medication with systemic rapamycin, cyclosporine A, nonsteroidal anti-inflammatory agents, or methotrexate, fine needle diathermy or cryotherapy of the neovascular stem, amniotic membrane transplantation, and laser photocoagulation. Corneal transplantation has a significantly lower success rate in patients with neovascularization than in patients with other indications for transplantation [5, 6].

Vascular endothelial growth factor (VEGF) is an important contributor to normal as well as abnormal angiogenesis. Extensive research has focused on the use of anti-VEGF compounds such as bevacizumab (Avastin®; Genentech, San Francisco, CA, USA and Roche, Basel, Switzerland), ranibizumab (Lucentis®; Novartis, Basel, Switzerland; Genentech and Roche), and aflibercept (Eylea®; Regeneron Pharmaceuticals, Tarrytown, NY, USA, and Bayer, Basel, Switzerland) for the treatment of corneal neovascularization. Aflibercept is a VEGF-trap molecule that acts as a receptor decoy for all VEGF-A isoforms. It is considered the highest-affinity VEGF blocker and is created by combining fragments of VEGFR-1, VEGFR-2, and human IgG Fc [3, 7].

Good results were reported in both animal models and humans when anti-VEGF compounds were administered by subconjunctival injection or topically as eye-drops early during the course of injury [2, 3, 7–13]. However, in the clinical setting, most patients receive treatment only in the nonacute period of corneal injury, when it is intended to induce regression of the fully formed corneal blood vessels in order to improve vision. Bevacizumab is the most commonly used anti-VEGF compound, but only a few studies have investigated its efficacy in reversing fully formed blood vessels in the cornea in either animal models [8, 14–19] or patients [6, 20, 21], and clinical success was variable. One recent study examined the effect of ziv-aflibercept (Zaltrap®) treatment for fully formed neovascularization with positive results [14].

Corticosteroids, which lower tissue inflammation reaction, are a known but unpopular therapy for corneal neovascularization. Results of studies assessing the inhibition of corneal neovascularization with subconjunctival or topical corticosteroids were mixed [18, 22, 23]. The effectiveness of corticosteroids in treating fully formed corneal neovascularization has hardly been investigated, sometimes with positive results [17–19]. Findings for cor-

Color version available online

Fig. 1. Right eye of rabbit no. 25 immediately after creation of a central corneal chemical burn.

ticosteroids combined with bevacizumab were promising, demonstrating a possible additive effect [17, 19, 24].

The aim of this study was to compare the effectiveness of subconjunctival aflibercept, betamethasone, and their combination in the treatment of formed corneal neovascularization in a rabbit model.

Methods

Animals

The study was performed on 24 New Zealand albino female rabbits weighing 2–3.5 kg. Animals were treated and maintained in accordance with the protocols of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and monitored by the Animal Care Committee of our institute.

Central Corneal Burn Creation

General anesthesia was induced by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). Eyes were topically anesthetized with benoxinate hydrochloride 0.4% before each manipulation. A central corneal chemical burn was created as previously described [25]. A round paper disc measuring 6 mm in diameter soaked with 1.0 N sodium hydroxide was briefly placed on the center of the right eye cornea of each rabbit. The remaining chemical reagent was rinsed off with balanced salt solution (Fig. 1). The left eyes were untreated. To decrease the variance, all procedures were performed in the same manner by a single investigator.

Treatment Protocols

We randomly divided the rabbits into 4 equal treatment groups: (1) subcutaneous injection of 0.05 mL aflibercept (Eylea, 40 mg/mL); (2) subcutaneous injection of 0.05 mL betamethasone (Celestone®, Schering-Plough Canada, Inc., Pointe Claire, QC, Canada; 6 mg/mL); (3) subcutaneous injection of 0.05 mL aflibercept (Ey-

lea; 40 mg/mL) combined with 0.05 mL betamethasone (Celestone; 6 mg/mL); (4) subcutaneous injection of 0.05 mL physiological saline 0.9% (control).

The drugs were injected once into the subconjunctival space 4 weeks after creation of the corneal injury when the presence of corneal neovascularization was documented.

Clinical Evaluation of Corneal Neovascularization

Following corneal injury, rabbit eyes were evaluated under general anesthesia at 0, 2, and 4 weeks (before treatment administration) and 5, 6, 7, and 8 weeks (after treatment administration). At each time point, the cornea was photographed with a digital camera mounted on a slit-lamp microscope ($\times 10$ magnification). The photographs were graded independently by 2 masked observers for extent, centricity, and density of corneal neovascularization as defined by Kang et al. [19]: extent – number of clock hours affected by neovascularization (score 1–12); centricity – distance the new vessels extended from the limbus toward the visual axis (1 = up to 2 mm, 2 = 2–4 mm, 3 = 4–6 mm, and 4 = >6 mm); density – average amount of neovascularization on the corneal surface compared with standard photographs presented to the observers (1 = very low, 2 = low, 3 = moderate, 4 = high). The percentage of corneal surface involved by neovascularization was analyzed by a single observer using Fiji-J software (Wayne Rasband at Research Services Branch, National Institute of Health, Bethesda, MD, USA). The area of neovascularization was delineated by thresholding, measured and quantified as a percentage of the total corneal area.

Histological Study and Immunofluorescence Staining

Eight weeks after creation of the corneal injury (4 weeks after administration of treatment), rabbits were euthanized by intravenous administration of sodium pentobarbitone (1–2 mL, 200 mg/mL). The eyes were enucleated, fixed in 10% formaldehyde, trimmed, embedded in paraffin, and sectioned into 5 μ m sections. The presence and density of blood vessels in the corneal tissue were examined in representative mid-sagittal sections stained with hematoxylin and eosin. Blood vessels were counted in 5 high-power fields ($\times 40$) from the limbus or 5 high-power fields in the area of the cornea where they were at highest density.

For VEGF immunofluorescence staining, sections were deparaffinized by sequential washes in ethanol at decreasing concentrations and washed in phosphate-buffered saline for 5 min. Antigen retrieval was performed using 10 mM citric acid (pH 6.0) for 10 min at 95 °C. Sections were blocked in 10% normal donkey serum (Sigma Aldrich, St. Louis, MO, USA) and incubated overnight at 4 °C with mouse alpha human VEGF (Merck Millipore, Burlington, MA, USA) diluted 1:50 in blocker. Thereafter, sections were incubated with Alexa Fluor 488 conjugated donkey anti-mouse IgG (1:100, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Cell nuclei were counterstained with NucBlue fixed stain reagent (Molecular Probes, Eugene, OR, USA). Sections were covered with prolong gold anti-fade reagent (Invitrogen). Images of representative slides from 2 corneas of each treatment group were captured using an Axio Imager Z2 microscope (Zeiss, Oberkochen, Germany).

Statistical Analysis

The clinical scores assigned to the digital photographs taken at treatment administration (4 weeks after injury) and 4 weeks later (8 weeks after injury) were compared. The comparison was con-

ducted for each factor scored by each observer separately. The difference was averaged for each treatment group and compared to the control group using a two-sided *t* test. To compute the *p* value, we used the *spicy.stats* Python package. A *p* value of <0.05 was considered significant.

Results

One eye (No. 21, combined-treatment group) was excluded from the analysis because the rabbit died one day after treatment was administered (presumably a complication of anesthesia).

Clinical comparison of the digital photographs taken at the time of treatment and 4 weeks later revealed spontaneous regression of the blood vessels, mostly in the central cornea, in all 4 groups (Fig. 2). However, on analysis of the scores assigned by each of the masked observers for extent, centricity, and density of corneal neovascularization as well as percentage of corneal surface involved by neovascularization, there was no significant decrease in any of the treatment groups relative to the control group (*p* > 0.15; Fig. 3, 4). Accordingly, histological staining with hematoxylin and eosin that did not reveal a significant decrease in the amount of neovascularization in any of the treatment groups compared to the control group (*p* > 0.08; Fig. 5).

Immunofluorescence staining was performed in only 2 eyes of each treatment group. Therefore, a descriptive and not a quantitative assessment was performed. All treatment groups showed less VEGF staining than the control group (Fig. 6).

Discussion

Corneal neovascularization is the endpoint of a variety of corneal insults, with no accepted treatment modality. Several studies have found anti-VEGF compounds and corticosteroids to be effective in inhibiting corneal neovascularization [2, 3, 7–13, 15–19, 21–24], but only a few sought to determine if their use also leads to the regression of new vessels after they are formed, in either animal models or humans [6, 8, 15–21].

Bevacizumab is the most-studied anti-VEGF agent for the treatment of corneal neovascularization. Papathanassiou et al. [16] showed that subcutaneous bevacizumab successfully prevented neovascularization when it was injected immediately after corneal chemical burn induction in rabbits, with no effect on scarring, and it decreased the

Fig. 2. Photographs of the right eyes of representative rabbits in the 4 study arms: (a + e) aflibercept; (b + f) betamethasone; (c + g) aflibercept + betamethasone; (d + h) saline. Photographs taken at

treatment administration point are marked “before” and photographs taken 4 weeks after treatment administration are marked “after.”

Fig. 3. Box plot showing the difference in extent, centricity, and density of neovascularization before and 4 weeks after treatment administration in the study groups. Positive results represent a reduction in neovascularization. The results are presented as a combination of the findings assessed by 2 masked observers. Definitions: extent – number of clock hours affected by neovascularization (1–12); centricity – distance of the new vessels from the limbus to the visual axis (1 = maximum 2 mm, 2 = 2–4 mm, 3 = 4–6 mm and 4 >6 mm); density – average amount of affected corneal surface compared with standard photographs (1 = very low, 2 = low, 3 = moderate, 4 = high).

area of neovascularization by 42% when it was injected 2 weeks after burn induction. Similarly, in the rat model of Hashemian et al. [18], subcutaneous bevacizumab injected either immediately or 7 days after corneal chemical burn led to a significant decrease in corneal neovascularization compared to betamethasone or control. Contrary results were reported by Lin et al. [15] using a twice-week-

ly schedule of subcutaneous bevacizumab for 1 month in a rabbit model of surgical limb insufficiency. These authors noted a reduction in neovascularization when treatment was administered immediately or 1 week after injury but not when it was administered 1 month after injury, after new vessels had formed. Chen et al. [8] made similar observations in rabbits treated after closed contact

Fig. 4. Box plot showing the difference in percentage of corneal surface involved by neovascularization before and 4 weeks after treatment administration in the study groups. Positive results represent a reduction in neovascularization. Findings were analyzed by a masked observer using Fiji-J software.

Fig. 5. Box plot showing amount of blood vessels counted from the limbus in 5 HPF X40 or 5 HPF in the area of the cornea were blood vessels were at highest density. The presence and density of the blood vessels in the corneal tissue were examined on representative mid-sagittal sections stained with hematoxylin and eosin.

Fig. 6. Photographs of immunofluorescence staining with anti-VEGF antibody (green) compared to hematoxylin and eosin staining in right eyes of representative rabbits from the study groups: (a + e) aflibercept; (b + f) betamethasone; (c + g) aflibercept + betamethasone; (d + h) saline.

lens wear. In clinical studies, bevacizumab given as a subcutaneous injection or topically as eyedrops was shown to lower neovascularization in most cases in the short term [6, 20, 21].

Previous studies by our group comparing bevacizumab with aflibercept, administered either conjunctivally or topically, in a rat model yielded better corneal neovascularization inhibition with aflibercept [2, 3]. Gore et al. [14] conducted the only previous study of the effect of

aflibercept on fully formed neovascularization in an animal model. They showed that a single subcutaneous injection of ziv-aflibercept (Zaltrap) 4 weeks after ocular insult significantly decreased the extent of neovascularization compared to no treatment or weekly subcutaneous bevacizumab injections.

The effectiveness of corticosteroids in decreasing fully formed corneal neovascularization has been examined in only 3 studies. One was the study of Hashemian et al.

[18], mentioned above, wherein unlike subcutaneous bevacizumab, subcutaneous betamethasone had no effect on the extent of neovascularization when injected immediately or 7 days after corneal chemical burn in rats. Accordingly, Kang et al. [19] found that after corneal suture removal in rabbits, subcutaneous bevacizumab treatment lowered corneal neovascularization with no additive effect of subcutaneous triamcinolone injection. Used alone, subcutaneous triamcinolone lowered corneal neovascularization but to a lesser extent than bevacizumab. By contrast, in the chemical burn model of Kadar et al. [17], topical dexamethasone applied 4 times daily starting 4 weeks after injury induction was more effective in reducing neovascularization than topical or subcutaneous bevacizumab and similarly effective to the combination of dexamethasone and subcutaneous bevacizumab.

The present study examined the effect of subcutaneous injections of aflibercept, betamethasone, and their combination on fully formed corneal neovascularization caused by a chemical burn in a rabbit model. On the basis of clinical assessment of weekly digital photographs and histopathological studies, none of the treatment modalities was effective in inducing significant regression of the corneal vessels. The immunofluorescence assay showed a decrease in VEGF antibody staining in all the treated groups compared with the control group. It is possible that treatment indeed lowered the VEGF level, although the reduction did not have a clinical effect on the formed neovascularization.

There are a number of possible explanations for our findings. First, the effect of aflibercept and betamethasone may be limited to inhibition of new-vessel formation in the early stages of corneal insult. This is supported by negative findings in terms of late-formed corneal neovascularization in previous studies of bevacizumab [8, 15] or corticosteroids [18]. When bevacizumab treatment was determined to be effective, it had been administered shortly after corneal injury induction: 14 days in the study of Papathanassiou et al. [16] and 7 days in the study of Hashemian et al. [18]. In the study by Gore et al. [14], showing ziv-aflibercept to be effective in the treatment of formed neovascularization, a sulfur mustard burn model was used and treatment was administered 4 weeks after injury induction. Although we also started treatment after 4 weeks, new-vessel formation peaked earlier in our chemical burn model (4 weeks) than in the sulfur burn model (6 weeks) [14]. Thus, it is possible that our results were limited to the effect of aflibercept on reversing formed vessels, whereas the results of the earlier study in-

corporated also an inhibitory effect on vessels undergoing formation.

Another possible explanation for our findings is the low treatment dose used relative to the studies showing a positive effect of treatment. Kadar et al. [17] administered bevacizumab subcutaneously twice weekly or topically twice daily for 3 weeks, and corticosteroids, 4 times daily for 3 weeks. All schedules were found to be effective. Chen et al. [8] administered bevacizumab subcutaneously once weekly for 4 weeks and found it to be effective for the inhibition but not the treatment of neovascularization. Other studies administered one subcutaneous injection of bevacizumab [16, 18] or corticosteroids [18, 19] or ziv-aflibercept [14] with variable success.

Alternatively, our results could be attributable to an inadequate corneal chemical burn injury. The timing and concentration of the chemical burn injury were correct, and all procedures were performed in the same manner by a single investigator; however, the resulting corneal scars and neovascularization were not uniform among the rabbit corneas. Although chemical burn injury has been used in a number of studies of corneal neovascularization treatment [9, 16, 22, 25], another model that produces more equal corneal burns might have yielded different results.

Finally, different compounds of aflibercept may have different effects. Gal-Or et al. [3], Sella et al. [2], and Gore et al. [14] used ziv-aflibercept (Zaltrap) with good results in a rat model. However, we used Eylea that is commonly applied in clinical ophthalmic treatment. Although both compounds contain the same active material, their different osmolality may have led to different responses.

It is noteworthy that rabbit eyes may have a poorer response to human anti-VEGF compounds than human eyes and may not be representative of expected clinical findings. Several studies have examined the response of corneal neovascularization in rabbit eyes to bevacizumab with good results [8, 9, 16, 18], but studies of aflibercept in rabbit models are very scarce [7, 14].

In conclusion, the present study showed that a single subconjunctival injection of aflibercept (Eylea), betamethasone (Celestone), or their combination is not effective in inducing regression of corneal neovascularization formed following corneal chemical burn injury in a rabbit model. More research is needed to assess whether these treatment modalities are indeed ineffective or whether confounding factors led to a false-negative result. Further studies are also warranted to determine if aflibercept has a role in the clinical setting.

Statement of Ethics

Animal experiments conformed to internationally accepted standards and were approved by the appropriate institutional review body.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All listed authors contributed to the planning, conduct, and reporting of this work.

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