Effect of Aflibercept on Human Corneal Endothelial Cells in Neovascular Age-Related Macular Degeneration: A Pilot Study

Neovasküler Tip yaşa Bağlı Makula Dejenerasyonunda Uygulanan İntravitreal Aflibercept Tedavisinin Korneal Endotel Hücreleri Üzerine Etkisi: Pilot Bir Çalışma

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ABSTRACT

Purpose: We aimed to evaluate *in vivo* effects of intravitreal aflibercept injection on human corneal endothelial cells in patients with neovascular age-related macular degeneration (AMD).

Methods and Materials: Thirty-four eyes of 34 consecutive patients with unilateral neovascular AMD (19 men, 15 women; mean age 66.4±3.4 years) were recruited to the study. All participants received monthly intravitreal aflibercept injections (2.0 mg, 0.05 ml) for three consecutive months and if needed thereafter. The follow-up period was six months. Noncontact specular microscopy was performed on the central cornea of both eyes at baseline and during follow-up, including the central corneal thickness (CCT), the endothelial cell density (ECD), the coefficient of variation of the cell size (CoV), an objective measure of polymegathism, and the percentage of the hexagonal cells (Hex%), an index of pleomorphism. The untreated contralateral eyes served as the control group.

Results: The median number of intravitreal injections per patient was 4 (range, 3-6). The median pre-injection CCT, ECD, CoV, and Hex% was 534.0µm, 2266.0 cells/mm^2, 34.5 and 46.0%, respectively. No significant difference was detected in these parameters in either treated and untreated eyes during 6 months after intravitreal aflibercept injection. There was also no difference in CCT, ECD, CoV, and Hex% between the treated eyes and contralateral untreated eyes at baseline and during 6-month follow-up.

Conclusion: Repeated intravitreal injections of 2.0 mg aflibercept do not cause any harmful effect on the corneal endothelium evaluated by specular microscopy in patients with neovascular age-related macular degeneration (AMD).

Key Words: Aflibercept, age-related macular degeneration, cornea endothelium, specular microscopy, vascular endothelial growth factor.

ÖZ

Amaç: Bu çalışmada neovasküler tip yaşa bağlı makula dejenerasyonu (YBMD) hastalarında uygulanan intravitreal aflibercept tedavisinin kornea endotel hücreleri üzerine *in vivo* etkisini incelemek amaçlandı.

Gereç ve Yöntemler: Bu çalışmaya tek taraflı neovasküler tip YBMD tanısı mevcut olan 34 hastanın (19 erkek, 15 kadın; ortalama yaş; 66.4±3.4 yıl) 34 gözü dahil edildi. Bütün hastalara ilk üç ay ardışık intravitreal aflibercept (2.0 mg, 0.05 ml) uygulanmasının ardından takiplerde gerektikçe tedavi uygulandı. Takip süresi 6 ay olarak belirlendi. Non-kontakt speküler mikroskopi ile başlangıçta ve takiplerde her iki gözdeki santral kornea kalınlığı (SKK), endotel hücre yoğunluğu (hücre/mm^2), değişkenlik katsayısı (%) ve hekzagonalite (%) parametreleri değerlendirildi. Hastaların tedavi almayan diğer gözleri kontrol grubu olarak belirlendi.

Sonuçlar: Çalışmada hastalara uygulanan ortanca enjeksiyon sayısı 4 (min:3-max:6) idi. Başlangıçta tedavi alan gözlerde ortanca SKK, endotel hücre yoğunluğu, değişkenlik katsayısı ve hekzagonalite değerleri sırasıyla 534.0µm, 2266.0 hücre/mm^2, 34.5 ve 46.0% olarak belirlendi. Başlangıçta ve 6 aylık takipler boyunca tedavi uygulanan ve uygulanmayan gözlerde ayrıca tedavi alan gözler ile tedavi almayan gözler arasında SKK, endotel hücre yoğunluğu, değişkenlik katsayısı ve hekzagonalite açısından istatistiksel olarak anlamlı fark saptanmadı.

Sonuç: Yaş tip YBMD hastalarında tekrarlayan dozlarada uygulanan 2.0 mg intravitreal aflibercept tedavisinin speküler mikroskopi ile değerlendirildiğinde kornea endotel hücreleri üzerine herhangi bir toksik bir etkisi saptanmamıştır.

Anahtar Kelimeler: Aflibercept, yaşa bağlı makula dejenerasyonu, kornea endoteli, speküler mikroskopi, vasküler endotelyal büyüme faktörü.

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INTRODUCTION

Intravitreal injections of vascular endothelial growth factor (VEGF) inhibitors is increasingly used in the treatment of neovascular age-related macular degeneration (AMD) in ophthalmic practice.[1-4] The most commonly used VEGF inhibitors are bevacizumab (Avastin®, Genentech, San Francisco, California, USA), ranibizumab (Lucentist®, Genentech, San Francisco, California, USA) and aflibercept (Eylea®, Regeneron Pharmaceuticals Inc., Tarrytown, New York, USA) among which aflibercept and ranibizumab were approved by the Food and Drug Administration (FDA) in this indication.[1-4]

Aflibercept is the most recently developed VEGF inhibitor with a recombinant fusion protein consisting of human VEGF receptor extracellular domains from receptors 1 and 2 (VEGFR1 and VEGFR2) fused to the Fc domain of human IgG.[5] This protein contains all human amino acid sequences, minimizing the potential for immunogenicity in patients.[6] The longer intravitreal half-life of aflibercept compared with ranibizumab can translate to a lower treatment load in terms of injections, monitoring, and medical visits.[7,8] Several *in vitro* studies have shown that aflibercept, at the concentration usually used for treating retinal disorders, had no toxicity to the ocular cells.[9,10] However, although it was also shown that both ranibizumab and bevacizumab have to have harmful effects on corneal endothelium[11,12], the effect of intravitreal aflibercept on human corneal endothelium has not been reported so far.

Given the functional importance of the corneal endothelium, particularly in elder population, this study was designed to evaluate the *in vivo* toxicity of aflibercept on human corneal endothelial cells in patients with neovascular AMD.

MATERIALS AND METHODS

Study design and population

This observational study included 34 eyes of 34 consecutive patients with neovascular AMD (19 male, 15 female; mean age 66.4 ± 3.4 years; age range 57 - 76 years). The study protocol was approved by the Ethics Committee of Numune Eye Training and Research Hospital. The study was conducted in accordance to Declaration of Helsinki. All participants gave written informed consent before any study-related procedure (NCT03313401). The inclusion criteria were evidence of unilateral neovascular AMD on angiography and optical coherence tomography. The exclusion criteria were age >80 years, presence of specific corneal conditions such as Fuchs endothelial dystrophy and other corneal endothelial dystrophies, history of ocular and corneal surgery, history of contact lens use, and comorbid ocular and systemic diseases that could affect the corneal endothelium such as diabetes and connective tissue disorders.

In all participants, monthly intravitreal aflibercept injections were administered for three consecutive months and if needed thereafter. The follow-up period was six months.

Intravitreal aflibercept injection

The intravitreal aflibercept injection was performed using standard aseptic techniques. After provision of local anesthesia with proparacaine hydrochloride eye drops (Alcaine, Alcon Laboratories Inc, Fort Worth, Texas, USA), the eyelids and the inferior conjunctival fornix sterilization was achieved with 5% povidone iodine. Aflibercept (2.0 mg, 0.05 ml) was injected through the pars plana (4 mm behind the limbus) using a 27-gauge needle.

Evaluation of corneal endothelial cells

Noncontact specular microscopy (Tomey EM-3000 Specular Microscope, Tomey Corp, Japan) was performed on the central cornea before the first intravitreal aflibercept injection and on months 1, 3, 6 after the injection. A blinded observer (S.D.) obtained the corneal endothelial images. The specular microscope evaluated the endothelial cell density (ECD), the coefficient of variation of the cell size (CoV), an objective measure of polymegathism, and the percentage of the hexagonal cells (Hex%), an index of pleomorphism,in automated manner. Specular microscopy also provided optical pachymetry measurements. The acute toxic of aflibercept on endothelium was evaluated by the presence of corneal edema and anterior chamber reaction, and intraocular pressure on the first day after the injection. The untreated eyes served as the control group.

Statistical analysis

Study data are summarized by using descriptive statistics (e.g., mean, median, standard deviation, interquartile range, frequency, and percentage). The normal distribution was assessed using Shapiro Wilk test. The Wilcoxon signed rank test was used for comparison of specular microscopy measurements of treated eye and contralateral eye. The effect of time on specular microscopy measurements of treated eye and contralateral eye was evaluated by Friedman test.

The analyses were performed by using the IBM SPSS software package (Statistical Package for Social Sciences, version 17.0, IBM Corporation, Armonk, New York, USA). Bonferroni correction was used to adjust p value for multiple comparisons (p=0.05/number of comparisons).

RESULTS

Among 34 participants, 12 patients had predominantly classic choroidal [neovascularization (CNV) while 9 patients had minimally classic CNV, and 13 patients had pure occult CNV. The neovascular AMD was diagnosed in the right eye of 18

patients and in the left eye of 16 patients. All patients completed six months of follow-up after intravitreal aflibercept injection. The mean number of intravitreal injections per patient was 4 (range, 3-6). Demographic and clinical characteristics of patients are summarized in Table 1.

Specular microscopic measurements for endothelial damage

Intravitreal - aflibercept injection had no negative effect in human corneal endothelial cells. At baseline, median CCT, ECD, CoV, and Hex% values as rated by specular microscopy, were 534.0µm, 2266.0 cells/m^2, 34.5 and 46.0%, respectively (Table 2). The CCT,

Table 1. *Demographic and clinical characteristics of study patients.*

Parameters	Result (n=34)
Age (years), *mean ± SD (range)*	66.4±3.4 (57-76)
Gender, *n (%)*	
Male	19 (55.9%)
Female	15 (44.1%)
Treated eye, *n (%)*	
Right	18 (52.9%)
Left	16 (47.1%)
Number of intravitreal injections per patient, *median (range)*	4 (3-6)

Table 2. *The specular microscopic measurements of central corneal thickness, endothelial cell density, coefficient of variation of the cell size, and percentage of the hexagonal cells in the treated eyes and contralateral untreated eyes over 6-month follow-up.*

	Treated eyes	Untreated eyes (control)	p[a]
Central corneal thickness (µm)			
Pre-injection	534.0 (516.0-545.2)	526.0 (514.0-545.0)	0.55
Month 1	533.0 (523.0-549.0)	529.0 (517.0-545.0)	0.37
Month 3	534.0 (524.0-548.0)	531.0 (518.0-548.0)	0.26
Month 6	535.0 (521.0-549.0)	535.0 (519.0-544.0)	0.66
p[b]	0.23	0.54	
Endothelial cell density (cells/mm^2)			
Pre-injection	2266.0 (2209.0-2666.0)	2274.0 (2266.5-2812.0)	0.13
Month 1	2265.0 (2182.0-2669.0)	2263.0 (2219.0-2801.0)	0.09
Month 3	2268.0 (2184.0-2665.0)	2262.0 (2217.0-2799.0)	0.28
Month 6	2259.0 (2185.0-2662.0)	2257.0 (2214.0-2801.0)	0.07
p[b]	0.26	0.11	
CoV			
Pre-injection	34.5 (34.0-36.0)	37.5 (34.0-39.0)	0.41
Month 1	37.0 (35.0-38.0)	37.0 (36.0-38.0)	0.52
Month 3	36.0 (35.0-38.0)	37.0 (36.0-38.0)	0.21
Month 6	35.5 (34.0-37.0)	39.0 (36.0-39.0)	0.59
p[b]	0.07	0.09	
Hex%			
Pre-injection	46.0 (45.0-48.0)	47.0 (44.0-50.0)	0.92
Month 1	47.5 (43.0-48.0)	49.0 (42.0-49.0)	0.51
Month 3	47.0 (43.0-48.0)	46.5 (43.0-48.0)	0.71
Month 6	48.0 (44.0-50.0)	49.0 (43.0-49.0)	0.19
p[b]	0.32	0.43	

Data are presented as median (interquartile range).

CoV, coefficient of variation of the cell size; Hex%, percentage of the hexagonal cells.

[a]Wilcoxon signed rank test for comparison of injection eye and contralateral eye at each evaluation point. After Bonferroni correction (p=0.05/4), p<0.0125 is accepted as statistically significant.

[b]Friedman test for significance of change in specular microscopy measurements with time. After Bonferroni correction (p=0.05/2), p<0.025 is accepted as statistically significant.

ECD, CoV, and Hex% showed no change over six months after intravitreal aflibercept in either treated eyes or untreated contralateral eyes. Similarly, no significant difference was observed in these parameters none of these parameters between the treated eyes and contralateral untreated eyes at baseline and during 6-month follow-up after intravitreal injection (Table 2).

No signs for acute toxic effect of aflibercept on endothelium including corneal edema, anterior chamber reaction, or high intraocular pressure, were recorded on the first day after the injection.

DISCUSSION

In our case series including 34 patients, we primarily showed that intravitreal aflibercept injection for treatment of neovascular AMD had no harmful effect on corneal endothelium during 6-months of follow-up. The vascular endothelial growth factor plays a key role in the angiogenesis and pathophysiology of neovascular retinal diseases including neovascular AMD.[13] Intravitreal injection of VEGF inhibitors can treat neovascular AMD and improve visual function by inhibiting neovascularization and decreasing vascular permeability. It has been shown that VEGF inhibitors including aflibercept show high anti-proliferative and apoptotic activity, and express negative cellular growth kinetics on the fibroblasts found in choroidal neovascularization in a dose-dependent manner.[14] Currently, VEGF inhibitors used in the treatment of neovascular AMD are ranibizumab, bevacizumab, and aflibercept, which have distinct pharmacological properties.[13] Although there are numerous studies on the efficacy and safety of ranibizumab and bevacizumab, data are rather limited for aflibercept. Due to their different pharmacological properties, the safety and efficacy data from one cannot be extrapolated to the others. Therefore, in the present study we evaluated *in vivo* effects of aflibercept on human corneal endothelial cells in patients with neovascular AMD.

Aflibercept is a recently approved anti-VEGF offering a new therapy for treatment of neovascular AMD. It is a fusion protein of VEGF receptors 1 and 2.[5] It has higher affinity to VEGF compared with ranibizumab or bevacizumab, indicating longer duration of action for aflibercept.[7,8] In experimental and clinical trials, the efficacy of aflibercept in treatment of AMD has been shown to be comparable to that of ranibizumab and bevacizumab.[15,16,17] In studies comparing aflibercept with bevacizumab or ranibizumab, aflibercept had fewer negative effects on retinal cell lines such as change in cell morphology, apoptosis or permanent decrease in cell viability, cell density or proliferation.[9,18] Aflibercept has greater affinity to VEGF than ranibizumab and bevacizumab and requires less frequent intravitreal injections than ranibizumab and bevacizumab.[8] The vitreous half-life of aflibercept is shorter than bevacizumab, but longer than ranibizumab.[19,20] In retina pigment epithelium and retina cultures prepared from pig eyes, aflibercept completely inhibited VEGF for six hours at the minimal concentration, and displayed a prolonged VEGF inhibition compared to bevacizumab and ranibizumab.[21] However, this advantage of aflibercept raises concerns about possible side effects of long-term usage.

The corneal endothelium is a barrier for fluid flow from the aqueous humor to the stroma. It accounts for maintenance of corneal transparency by regulating stromal hydration. The endothelial cell density decreases with age, and further damage to corneal endothelium by disease, trauma or drugs, may lead to its loss of function, resulting in corneal edema, decreased corneal clarity, and loss of visual acuity.[22] Therefore, maintaining corneal endothelium healthy is of important, particularly in aged patients.[22] Intravitreal injection causes exposure of human corneal endothelial cells to significant concentration of VEGF inhibitors. Previous studies indicated that VEGF and its receptors are expressed in the corneal endothelium, and VEGF inhibitors can be detected in aqueous humor after intravitreal injection, both of which show that VEGF inhibitors have potential cytotoxic effects to human corneal endothelial cells.[11,23,24]

The effect of the intravitreal injection of ranibizumab or bevacizumab on corneal endothelium has been studied previously.[25] Perez-Rico et al. reported that intravitreal injections of ranibizumab have no significant effect on endothelial cell density, CoV, and Hex% within first 7 days or six months after the injection. The repeated intravitreal injections of ranibizumab over six months caused no substantial change in the corneal endothelium.[12] Chiang et al. evaluated changes in human corneal thickness and corneal endothelial cell density up to six months after intravitreal injection of 2.5 mg bevacizumab and found that intravitreal bevacizumab has no harmful effects on the corneal endothelium.[11] *In vitro* studies also showed that bevacizumab is not toxic to corneal cells of human origin.[26] Furthermore, although intracameral injection of ranibizumab caused deterioration in endothelial cell morphology in rabbit cornea[27], intracameral injection of bevacizumab had no effect on endothelial cell viability or morphology in the rabbit or human cornea[27,28,29]. Bevacizumab did not induce apoptosis or necrosis in human corneal endothelial and fibroblast cells *in vitro*.[30,31] In a recent study by Guzel et al.; it was reported that monthly intravitreal bevacizumab or ranibizumab injections for three consecutive months does not affect corneal morphology and has no harmful effects on the endothelium in patients with diabetic macular edema.[32] Our findings are also in agreement with these studies showing that neither ranibizumab nor bevacizumab has negative effect on corneal endothelial cells. We found that there was no change in CCT, ECD, CoV, and Hex% over

six months after intravitreal aflibercept injection in either eyes. Furthermore, the CCT, ECD, CoV, and Hex% showed no significant difference between the eye treated with intravitreal aflibercept and contralateral untreated eye at baseline and during 6-month follow-up after intravitreal injection.

The main limitation of the study was the limited sample size, which precludes us to make a definitive conclusion on the effect of intravitreal aflibercept on human corneal endothelium. Nevertheless, this pilot study provides first evidence that intravitreal aflibercept injections have no negative effect on human corneal endothelium.

In conclusion, intravitreal aflibercept injections at clinically effective doses for four times on average during the 6-month period induce no harmful effect on human corneal endothelium evaluated by specular microscopy. Further prospective, large-scale, prolonged studies are needed to confirm that intravitreal aflibercept can be used safely with no corneal toxicity to treat neovascular AMD.

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