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 the *in vivo* effects of intravitreal injection of aflibercept 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 male, 15 female; mean age 66.4±3.4 years) were recruited for the study. All participants received one monthly intravitreal injection of aflibercept (2.0 mg, 0.05 ml) for three consecutive months, and later treatments were applied as needed. The follow-up period was six months. Noncontact specular microscopy was performed on the central cornea of both eyes at the beginning 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 nontreated fellow eyes of patients 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. None of these parameters revealed statistically significant difference over six months after intravitreal injection of aflibercept in either treated and nontreated eyes. There was also no difference in CCT, ECD, CoV, and Hex% between the treated eyes and contralateral nontreated eyes before 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 inceleme.

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 have being 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) for 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, which minimizes the potential for immunogenicity in patients.[6] The prolonged 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 both ranibizumab and bevacizumab have been shown not to have harmful effects on corneal endothelium[11,12], the effect of intravitreal aflibercept on human corneal endothelium has not been reported so far.

Considering the functional importance of the corneal endothelium, particularly in aged population, the present 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

Thirty-four eyes of 34 consecutive patients with neovascular AMD (19 male, 15 female; mean age 66.4±3.4 years; age range 57-76 years) were recruited for this observational prospective study. The study protocol was approved by the Ethics Committee of Numune Eye Training and Research Hospital and adhered to the tenets of the Declaration of Helsinki. All participants signed the informed consent before any study-related procedure. NCT03313401 The inclusion criteria were angiographic and optical coherence tomographic evidence of unilateral neovascular AMD. The exclusion criteria were age more than 80 years, specific corneal conditions such as Fuchs endothelial dystrophy and other corneal endothelial dystrophies, history of ocular and corneal surgery, history of contact lenses use, and ocular and systemic diseases such as diabetes and connective tissue disorders that could effect the corneal endothelium.

All participants received one monthly intravitreal injection of aflibercept for three consecutive months, and later treatments were applied as needed. The follow-up period was six months.

Intravitreal aflibercept injection

The procedure for the intravitreal aflibercept injection was performed using standard aseptic techniques. After providing local anesthesia with proparacaine hydrochloride eye drops (Alcaine, Alcon Laboratories Inc, Fort Worth, Texas, USA), the eyelids and the inferior conjunctival fornix were sterilized 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 1, 3, 6 months after the injection. A blinded observer (S.D.) obtained the corneal endothelial images. The specular microscope automatically 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. Specular microscopy also provided optical pachymetry measurements. The acute toxic of aflibercept on endothelium was evaluated by the the presence of corneal edema and anterior chamber reaction, and intraocular pressure on the first day after the injection. The nontreated eyes served as the control group.

Statistical analysis

Study data were summarized by using descriptive statistics (e.g., mean, median, standard deviation, interquartile range, frequency, and percentage). The normal distribution of the variables were tested using Shapiro Wilk test. For comparison of specular microscopy measurements of treated eye and contralateral eye, Wilcoxon signed rank test was used. 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), 9 patients had minimally classic CNV, and 13 patients had pure occult CNV. The neovascular AMD was diagnosed in the right eye in 18

patients and left eye in 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 were summarized in Table 1.

Specular microscopic measurements for endothelial damage

Intravitreal injection of aflibercept did not have any negative effect in human corneal endothelial cells. The median pre-injection CCT, ECD, CoV, and Hex%, which were determined by specular microscopy, was 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 signicance 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% did not change over six months after intravitreal injection of aflibercept in either treated eyes or nontreated fellow eyes. Likewise, none of these parameters did show statistically significant difference between the treated eyes and contralateral nontreated eyes before and during 6-month follow-up after intravitreal injection (Table 2).

The signs of acute toxic effect of aflibercept on endothelium, which are corneal edema, anterior chamber reaction, or high intraocular pressure, were not recorded in any of the eyes on the first day after the injection.

DISCUSSION

In this 34-patient case-series, we primarily showed that intravitreal injection of aflibercept for treatment of neovascular AMD has 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 antiproliferative and apoptotic activity and expressed negative cellular growth kinetics on the fibroblasts found in choroidal neovascularization in a dose-dependent manner.[14] The VEGF inhibitors currently used in the treatment of neovascular AMD are ranibizumab, bevacizumab, and aflibercept, which have distinct pharmacological properties.[13] Although there are a number of studies on the efficacy and safety of ranibizumab and bevacizumab, data from aflibercept are rather limited. 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 the *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 which indicates 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.[15-17] 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 binding affinity for VEGF than ranibizumab and bevacizumab and requires less-frequent intravitreal injection 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 pigs' eyes, aflibercept completely inhibited VEGF for six hours at a 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 to fluid flow from the aqueous humor to the stroma. It is responsible for maintaining 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, which cause corneal edema, decreased corneal clarity, and loss of visual acuity.[22] Therefore, keeping corneal endothelium healthy has a vital importance particularly for aged patients.[22] Intravitreal injection causes significant concentration of VEGF inhibitors get into contact with human corneal endothelial cells. Previous studies indicated that VEGF and its receptors are expressed in the corneal endothelium, and VEGF inhibitors can be detected in aqueous humour after intravitreal injection, both of which showing that VEGF inhibitors have potential to be cytotoxic to human corneal endothelial cells.[11,23,24]

The effect of the intravitreal injection of ranibizumab or bevacizumab on corneal endothelium has been studied before.[25] Perez-Rico et al. reported that intravitreal injections of ranibizumab did not have significant effect on endothelial cell density, CoV, and Hex% at seven days or six months after the injection. The repeated intravitreal injections of ranibizumab over six months did not also cause substantial changes in the corneal endothelium.[12] Chiang et al. evaluated human corneal thickness change 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 did not affect 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 also agree with these studies showing that neither ranibizumab nor bevacizumab has negative effect on corneal endothelial cells. We found that the CCT, ECD, CoV, and Hex% did not change over

six months after intravitreal injection of aflibercept in either eyes. Furthermore, the CCT, ECD, CoV, and Hex% did not show statistically significant difference between the eye treated with intravitreal aflibercept and contralateral untreated eye before and during 6-month follow-up after intravitreal injection.

The main limitation of the study was the limited sample size, which precludes us reaching a definitive conclusion on the effect of intravitreal aflibercept on human corneal endothelium. Nevertheless, this pilot study provides first evidence that intravitreal aflibercept injections do not have negative effect on human corneal endothelium.

In conclusion, intravitreal injection of clinicaly effective doses of aflibercept for four times on average during the 6-month period do not induce any 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 without any corneal toxicity to treat neovascular AMD.

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