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RESEARCH ARTICLE

Inhibition of Corneal Neovascularization by Subconjunctival Injection of Ranibizumab and Bevacizumab in Rabbit Cornea

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Summary

The aim of this study was to evaluate the effects of subconjunctival ranibizumab and bevacizumab injection on angiogenesis in the rabbit cornea. The corneas of 24 New Zealand rabbits were cauterized with silver nitrate to induce neovascularization. The eyes were irrigated with 10 ml of 0.9% saline solution. The alkaline burns were similar in all the rabbits. At the 24 h after cauterization, the rabbits were divided into three groups of eight animals each: The first group (GC) received 0.02 ml 0.9% saline solution as a control group whereas second (GR) and third (GB) groups received 0.5 mg ranibizumab and 1.25 mg bevacizumab by subconjunctival injection, respectively, on days first and 7 after lesion. The rabbits' corneas were extracted on the 14th day. Digital photographs of the corneas were obtained and the newly formed vessels were analyzed in a computerized system (google sketch-up program). The rates of these vessels were compared between the groups. Ranibizumab and bevacizumab were both effective on inhibition of angiogenesis, in comparison to 0.9% saline solution ($P<0.05$). Ranibizumab was found to be statistically more effective to reduce corneal neovascularization than bevacizumab ($P<0.05$). Bevacizumab and ranibizumab were found to be effective in inhibiting the corneal neovascularization in the rabbit cornea. Ranibizumab seemed more effective than bevacizumab on inhibiting corneal neovascularization in the rabbit cornea.

Keywords: *Ranibizumab, Bevacizumab, Rabbit cornea, Corneal neovascularization, Angiogenesis*

Korneal Neovaskularizasyonun Subkonjonktival Ranibizumab ve Bevacizumab Enjeksiyonu ile Tavşan Korneasında İnhibisyonu

Özet

Bu çalışmanın amacı; subkonjunktival olarak uygulanan ranibizumab and bevacizumab'ın tavşan korneasında anjiyogenezis üzerine etkilerini değerlendirmekti. Yirmi dört adet Yeni Zelanda tavşanının korneaları neovaskülarizasyon oluşturmak için gümüş nitrat ile koterize edildi. Gözler 10 ml %0.9 salin solusyonu ile yıkandı. Tüm gözlerde eşit miktarda kimyasal yanık oluşturulmasına dikkat edildi. Koterizasyon sonrası 24. saatte tavşanlar üç gruba ayrıldı: Kontrol grubuna (GC) (n=8) subkonjunktival 0.02 ml %0.9'luk salin solüsyonu; Grub Ranibizumab'a (GR) (n=8) subkonjunktival olarak 0.5 mg ranibizumab ve Grup Bevasizumab'a (GB) (n=8) ise subkonjunktival olarak 1.25 mg bevacizumab 1. ve 7. günlerde uygulandı Topical antibiotik tedavisi sonrası, tavşan korneaları 14. günde çıkarıldı. Korneaların digital fotoğrafları alınarak yeni oluşmuş damarlar bilgisayar programı (google sketch-up programı) yardımıyla analiz edildi. Yeni oluşan damar yapılarının alanının kornea alanına oranları gruplar arasında değerlendirildi. Ranibizumab ve Bevasizumab'ın her ikisi de anjiyogenez inhibisyonunda %0.9 salin solüsyonuna kıyasla ($P<0.05$) daha etkili olduğu saptandı.Ranibizumab'ın korneal neovaskülarizasyon azaltıcı etkisi Bevasizumab'tan istatistiksel olarak anlamlı ($P<0.05$) derecede fazla bulundu. Bevasizumab ve Ranibizumab'ın tavşan korneasında neovaskularizasyonu inhibe etmekte etkili olduğu tespit edildi. Ranibizumabın tavşan korneasında neovaskülarizasyonu inhibe edici etkisinin Bevasizumab'tan daha fazla olduğu saptandı.

Anahtar sözcükler: *Ranibizumab, Bevasizumab, Tavşan korneası, Korneal neovaskularizasyon, Anjiyogenez*

INTRODUCTION

Corneal avascularity is an essential element of corneal transparency and optimal vision [1]. The cornea has the unique feature of being normally avascular, but under pathologic conditions vessels invade the cornea from the limbal vascular plexus. A wide variety of insults including infection, inflammation, ischemia, degeneration, trauma, and loss

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of limbal cell barrier can cause corneal neovascularization (CNV) [2].

Corneal neovascularization is under the control of local, pro- and anti-angiogenic factors [3]. The natural balance of these factors maintains corneal avascularity. The overall process of angiogenesis involves the degradation of the extracellular matrix and the vascular basement membrane by matrix metalloproteinases (MMP), allowing endothelial cells to invade and form vessels [4]. Under inflammatory conditions, the invasion of endothelial cells into the cornea is largely stimulated by the actions of macrophages which enhance inflammation through the recruitment of additional macrophages while also producing pro-angiogenic factors [5]. The most significant role of macrophages in CNV is their secretion of Vascular Endothelial Growth Factor (VEGF) [6]. VEGF factor plays a key role in angiogenesis in the human cornea.

Comprised of 5 isoforms, VEGF promotes several steps within normal vascular growth including the induction of angiogenesis, endothelial cell proliferation, enhanced inflammatory response, proteolytic activities and increased vascular permeability [2]. Several cellular components within the human cornea have been found to excrete VEGF when under duress or inflammation, including corneal endothelial and epithelial cells, fibroblasts, macrophages, and limbal vascular endothelial cells [7]. VEGF antagonists disrupt these pathways, thus preventing and regressing corneal neovascularization. Blockage of VEGF with bevacizumab and ranibizumab has been a successful treatment in decreasing visual morbidity associated with abnormal vascular conditions of the choroid and retina [8].

More recently, topical anti-VEGF agents have been used to treat abnormal vascular conditions of the cornea. Vascularization from conditions such as chemical injury, Stevens-Johnson Syndrome, ocular cicatricial pemphigoid, interstitial keratitis, post infectious keratitis, and corneal graft failure has been shown to regress with the use of topical and subconjunctival bevacizumab [9-12]. It is presented that subconjunctival bevacizumab treatment to chemical burns of rat corneas decreased inflammatory cell infiltration and cytokines [13].

The aim of this study was to evaluate the effects of subconjunctival injection of ranibizumab 0.5 mg and bevacizumab 1.25 mg on angiogenesis in the rabbit cornea that was chemical injury by cauterized with silver nitrate crystal.

MATERIAL and METHODS

Animals

A total of 24, ten-twelve month-old, New Zealand white albino male rabbits (average weight: 3-3.5 kg) supplied by the Experimental Animals Unit at Kafkas University (Kars, Turkey), were used in the study.The study was carried out in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Kafkas University Animal Care and Use Committee (Approval Number: KAÜ-HADYEK/2011-002). The rabbits were kept under standard conditions (20±1°C, 12 h light/12-h dark cycles) and were fed 160 g pelleted rabbit diet (Ankara Feedstuff Industry, Ankara, Turkey) daily and water was available *ad libitum*. After alkaline burns formed, they were randomly divided into three groups of eight animals each: control group (GC), bevacizumab group (GB) and ranibizumab group (GR), respectively. A complete blood count was performed for each rabbit on days 0, 7, and at the end of the study.

Anesthesia Procedure

The animals were withheld food 12 h prior to operation. General anesthesia was performed with combination of xylazine HCl (Rompun® vial, 23.32 mg/ml, Bayer Turkish Chemistry Industry, Istanbul, Turkey) (5 mg/kg, IM) and ketamine HCl (Ketalar® vial, 50 mg/ml, Pfizer, Istanbul, Turkey) (50 mg/kg, IM) intramuscularly. Before corneal burns were achieved, propacaine HCl 0.5% (Alcaine®, Alcon) as a topical anesthetic eye drop was also applied to form a more analgesic effect as defined by Mahoney and Waterbury [14].

Creating the Alkaline Burn Model

Alkaline burn was achieved in the right eye by contacting the silver nitrate applicator sticks (75% silver nitrate, 25% potassium nitrate) (*Flexible Caustic Applicator 6", Bray Group Ltd., UK*) to all corneal surface for 2 min. Thus, alkali-induced corneal neovascularization model was performed as decscribed by Mahoney and Waterbury [14] with some modifications. Excess silver nitrate was removed by rinsing the eyes with approximately 10 ml of a 0.9% saline solution. A single investigator (M.E.) cauterised all animals for similarity of process model *(Fig. 1)*.

Treatment Protocol

On the first and seventh days after the formation of alkaline corneal burn, subconjunctival anti-VEGF agent ranibizumab 0.5 mg (0.05 ml) (Lucentis®, Genentech/Novartis, South San Francisco, California, USA) and bevacizumab 1.25 mg (0.05 ml) (Avastin®; Genentech/Novartis, South San Francisco, California, USA) were injected to the righ eye of rabbits in group GR and GB, respectively. Group GC (n=8) received a subconjunctival injection of 0.05 ml of 0.9% saline solution. After the formation of alkaline burn, tobramycin topical antibiotic ointment once a day and tobramycin eye drop four times a day (Tobrased®, Bilim İlaç, Istanbul-TURKEY) were used for 14 days in all groups.

Evaluation of Corneal Neovascularization

On the 14th day of the study, following the clinical

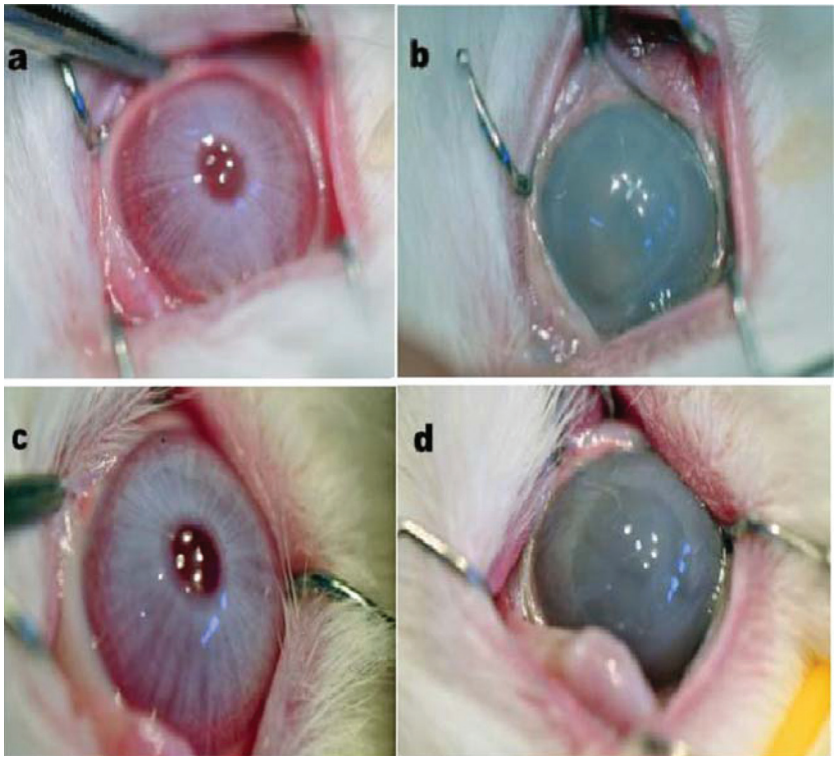

Fig 1. The cornea samples before and after cauterization **a,c-** Normal corneas, **b,d-** Corneas after alkali burn was performed

Şekil 1. Korneaların koterizasyondan önce ve sonrasında görünümü **a,c-** Normal kornealar, **b,d-** Alkali yanık oluşturulduktan sonraki kornealar

examination, the rabbit corneas were totally extracted with 360 degree incision under general anesthesia, as described above. After then, eviseratio bulbi was done and conjunctival tissue was stitched with 6/0 polyglactin 910 suture (*Vicryl*® Ethicon Inc. UK) and antibiotic ointment was applied for 5 days in all animals. The surgery was performed by the same surgeon. The animals in all the study groups were delivered to the Laboratory Animal Resource Center, Kafkas University (Kars, Turkey) to be used for other experimental studies. The corneas in each group were digitally photographed for the status of corneal neo vascularization under standard conditions by Nikon D90 SLR camera, with camera lens distance of 85 mm, shooting distance of 29 cm, digital zoom rate of 100%, and with artificial light source. Then, corneas samples were passed all of fixed procedure and sections (thickness 5 μm) stained with Hematoxylen & Eosin for histological studies. H&E stain slides evaluated and photographed under ligth microscope (Olympus BX-51). The ratio of the neovascularization zone to all corneal area was calculated via Google sketch-up program in all groups *(Fig. 2)*. The corneas were placed in concordance to their anatomical curvatures on a round shaped light source *(Fig. 2A)*. Total corneal areas were measured *(Fig. 2B)*. The neovascularized areas were marked with the Google sketch-up program *(Fig. 2C)*. The total neovascularization areas were calculated and the ratio of the neovascularized areas to total corneal areas were obtained.

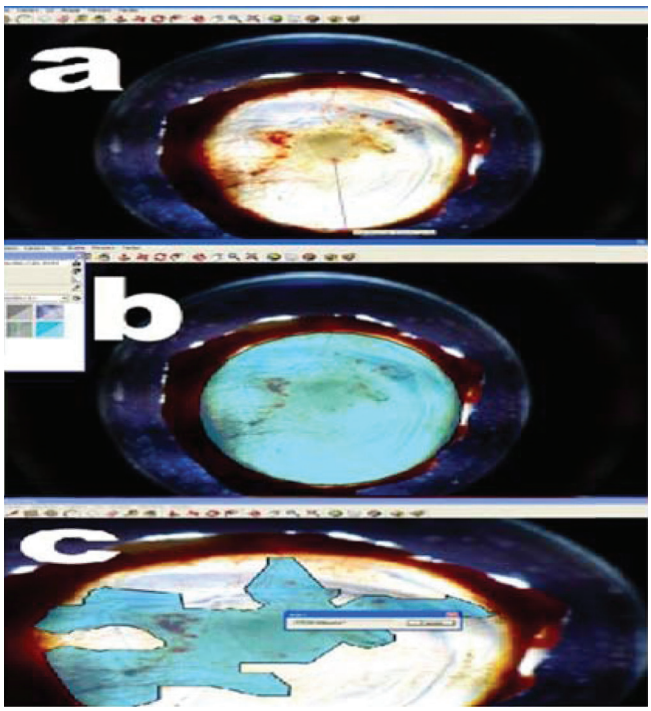

Fig 2. The application scheme of the Google SketchUp program **a-** The photograph of the cornea on the round shaped light source, **b-** The measurement of the total corneal area, **c-** The calculation of the total neovascularization area with the Google SketchUp program

Şekil 2. Google SketchUp programının uygulanış şeması **a-** oval aydınlatılmış yüzeye konmuş korneanın görünümü, **b-** Total korneal alanın ölçülmesi, **c-** Google SketchUp programı ile total neovaskularizasyon alanının ölçülmesi

Statistical Analysis

After assessing the normality in groups by using One Sample Kolmogorov Smirnov test, comparisons between groups were completed using a non-parametric Kruskal-Wallis Test for continuous data. $P<0.05$ was considered statistically significant.

RESULTS

Corneal neovascularization that induced by chemical cauterization with silver nitrate was supported with the histological findings such as the presence of intense inflammatory cells (neutrophil leukocyte and eosinophil leukocyte) and newly formed blood vessels *(Fig. 3)*.

The digital photographs of the corneas which were taken on the 14th day after treatment for all studygroups were presented in *Fig. 5*. When the corneal neovascularization rates were assessed by using Google sketch-up program, the mean rate of control group was found as 69.54±16.48% (40.83-92.00), 31.56±3.06% (25.41-35.25) in the ranibizumab group and 42.95±5.94% (34.02-51.29) in the bevacizumab group *(Table 1)*.

The percentage of CNV in ranibizumab (GR) and bevacizumab (GB) groups were statistically significant difference lower than the control group (GC). On the other hand, ranibizumab was found to be statistically more effective to reduce CNV than bevacizumab ($P<0.05$).

No adverse effects such as corneal melting, descemetocele, or corneal perforation were observed in external ophthalmic examination in all study groups.

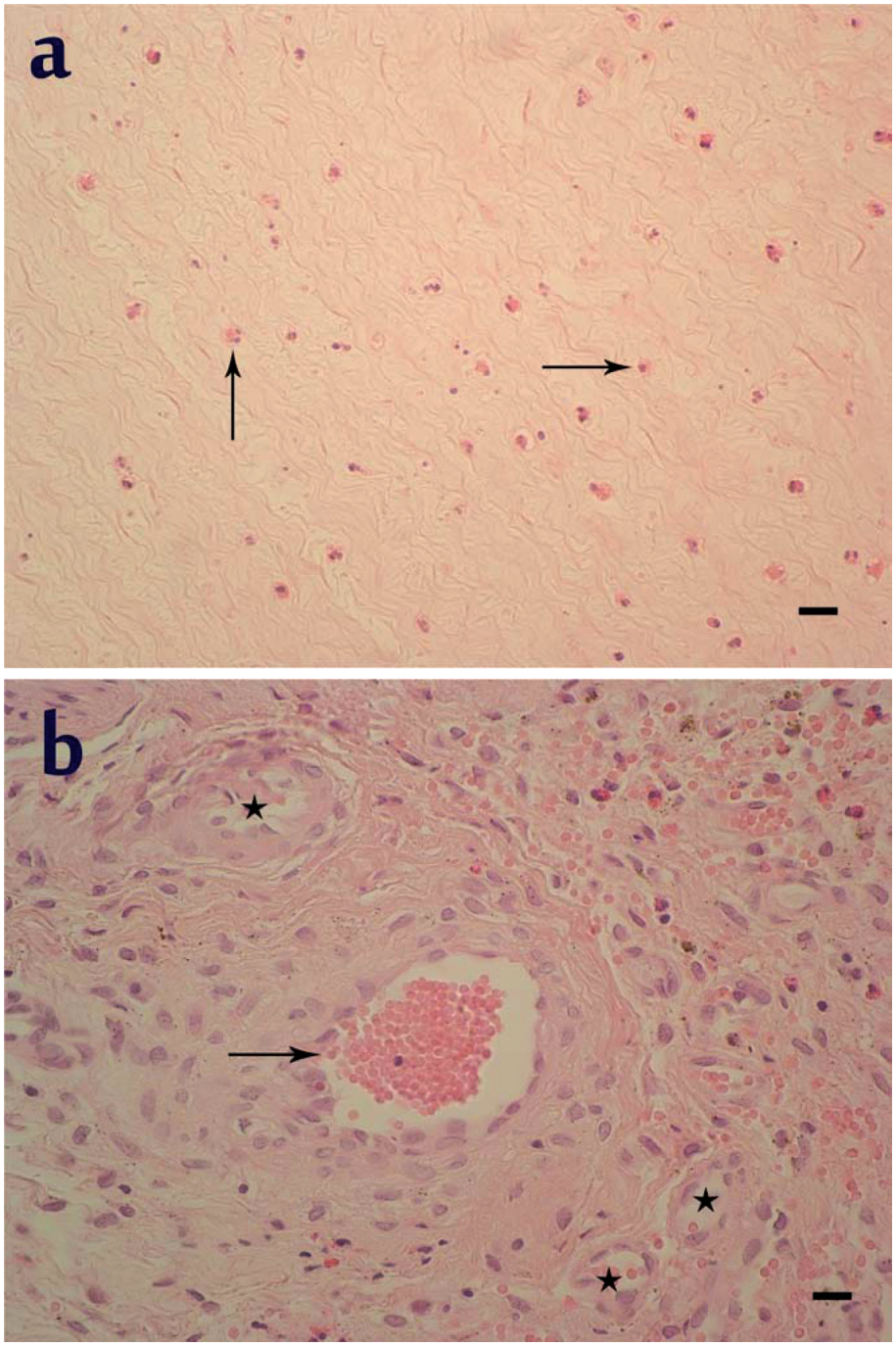

Fig 3. Histological sections of the neo vascularized rabbit corneas **a-** Neutrophil leukocytes *(vertical arrow)* and eosinophil leukocytes *(horizontal arrow)*, **b-** Newly formed vein *(arrow)* and arteries *(stars)*, H&E, Bar=50 μm

Şekil 3. Neovaskularize tavşan korneasından histolojik kesitler **a-** Nötrofil lökositler *(dikey ok)* ve eosinofil lökositler *(yatay ok)*, **b-** Yeni oluşmuş ven *(ok)* ve arterler *(yıldız)*, H&E, Bar=50 μm

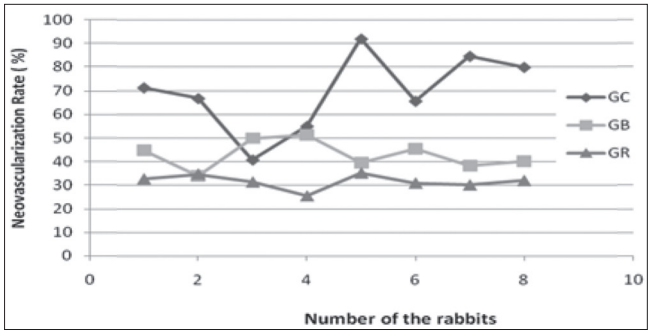

Fig 4. The corneal neovascularization rates in the study groups **GB-** Bevacizumab Group, **GR:** Ranibizumab Group, **GC-** Control Group

Şekil 4. Çalışma gruplarında korneal neovaskularizasyon oranları **GB-** Bevacizumab Grup, **GR:** Ranibizumab Grup, **GC-** Control Grup

and bevacizumab in corneal neovascularization induced by alkali burn. Although we found that both agents were effective on reducing CNV, ranibizumab was found to be more effective than bevacizumab ($P<0.05$). Recently, both anti-VEGF agents are commonly used for retinal vascular disorders and macular diseases, such as macular neovascular degeneration, diabetic retinopathies, retinal neovascularization due to retinal vascular disorders, retino-pathy of prematurity, and neovascular glaucoma [15]. Even though there are many reports involving the effect of bevacizumab on inhibiting angiogenesis of the anterior segment of the eye in the literatüre [16-21], studies about the use of ranibizumab for the same purpose are rare [22,23].

Table 1. *The corneal neovascularization rates in the study groups*
Tablo 1. *Çalışma gruplarındaki korneal neovaskularizasyon oranları*

Groups	Number of Eye	Neovascularization Rate (%)		
		Minimum	Maximum	Average±Standard Deviation
GB	8	34.02	51.29	42.95±5.94
GR	8	25.41	35.25	31.56±3.06
GC	8	40.83	92.00	69.54±16.48

GB: *Bevacizumab Group,* ***GR:*** *Ranibizumab Group,* ***GC:*** *Control Group*

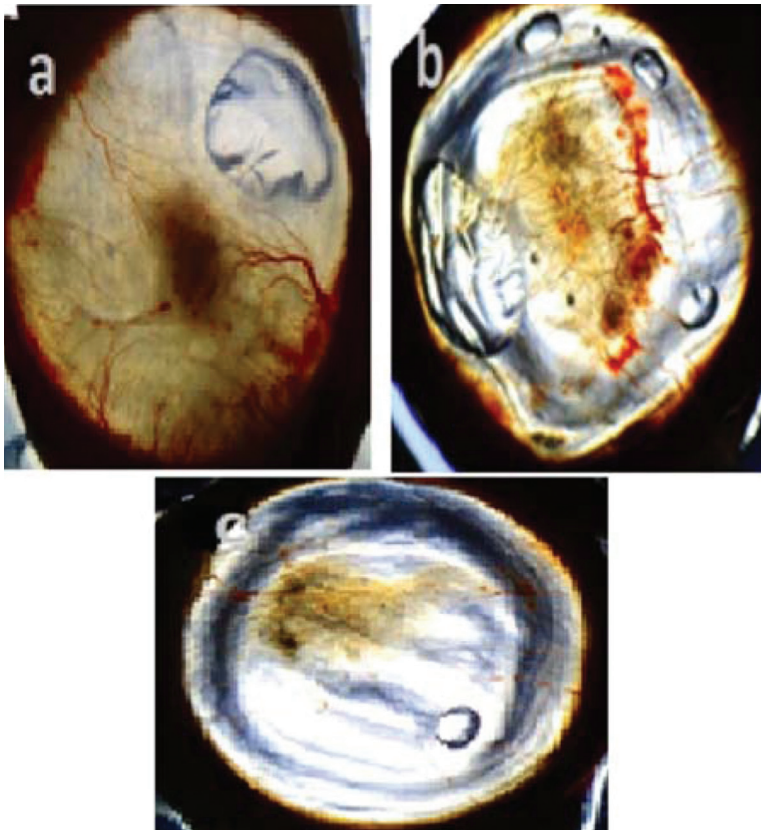

Fig 5. The digital photographs of the rabbit corneas taken on the 14th day of the experiment **a-** Control Group, **b-** Bevacizumab Group, **c-** Ranibizumab Group

Şekil 5. Tavşan korneasından çalışmanın 14. gününde alınmış dijital fotoğraflar **a-** Kontrol Grup, **b-** Bevacizumab Grup, **c-** Ranibizumab Grup

DISCUSSION

This experimental study was designed to evaluate and compare the effects of anti-VEGF agents, ranibizumab

As the optimal vision can only be achieved with an avascular and transparent cornea, to totally prevention or minimizing is an essential step in corneal neovascularization. Corneal avascularity requires low levels of angiogenic factors

and the high levels of anti-angiogenic factors. The angiogenic factors include basic and acidic fibroblast growth factor (FGF), VEGF, angiogenin, transforming growth factor, interferon, tumor necrosis factor-α and platelet derived growth factor [24]. Anti-angiogenetic factors include interferon-α, thrombospondin-1, angiostatin, endostatin, and pigment epithelium-derived factor. The imbalance between the angiogenic and anti-angiogenic factors lead to corneal neovascularization and scar formation. Steroids, methotrexate, heparin, thalidomide, artepillin, C-caffeic acid phenetylester (CAPE), and anti-VEGF agents are proposed as inhibitors of CNV [24].

Bevacizumab is a full-length, recombinant humanized monoclonal immunoglobulin G1 (IgG1) that binds to and inhibits the activity of VEGF-A, thereby inhibiting angiogenesis [8]. A related compound, Ranibizumab (Lucentis), is a high affinity recombinant monoclonal antibody derived from the same parent murine antibody as bevacizumab and also neutralizes all isoforms of VEGF-A [6].

It has been reported that; no retinal damage was observed and the histopathologic studies yielded similar after repeated intravitreal injections of bevacizumab and ranibizumab in rabbit eyes [25]. Ranibizumab has a high, but 40-fold reduced, affinity towards rabbit VEGF in comparison with human VEGF. But bevacizumab binds rabbit VEGF with a 7.27-fold lower affinity than for human VEGF [26,27].

There are main differences between these 2 anti-VEGF agents. First difference is their molecular weights; ranibizumab is a 48-39-kDa Fab fragment, whereas bevacizumab is a complete 149-kDa antibody [28]. As second difference, ranibizumab is not glycosylated, resulting in a 140-fold higher binding affinity of a single site compared to bevacizumab [15,29].

Other differences are the development in different cell lines: bevacizumab is developed in Chinese hamster ovary mammalian cell expression system; while ranibizumab is produced by an *Escherichia coli* bacterial expression system; and the formulation for intraocular use: ranibizumab was formulated for intraocular use, while bevacizumab was formulated for intravenous use [30]. In addition, the pharmacokinetics of these 2 agents are different: the vitreous half-life of 1.25 mg intravitreal bevacizumab is 4.32 days, while ranibizumab (0.5 mg) has a vitreous half-life of 2.9 days with minimal systemic exposure in a rabbit eye [31,32].

In another study Christoforidis et al. were defined that there was no significant escape of bevacizumab and ranibizumab from the vitreous cavity after intravitreal injection [33]. This period in primates is 6.9 days for bevacizumab and 3.5 days for ranibizumab. Peak serum concentration after intravitreal injection is 3000 ng/ml in bevacizumab and 0.3 ng/ml in ranibizumab. Systemic intravenous bevacizumab treatment in primates leads to a half-life of 21 days. On the other hand, half-life of ranibizumab applied in the same way is less than 1 day [34]. To achieve detectable accumulation of bevacizumab in the vitreous of the injected rabbit, the dosing interval should be shorter than four half-lives [27].

This fact leads us to set the dosage regimen subconjunctivally on days 1 and 7 of the study to minimize the effect of the difference in pharmacokinetics between these 2 agents.

It has been demonstrated that, at clinical doses, ranibizumab and bevacizumab are equally potent in neutralizing VEGF in a porcine retina-retina pigment epithelium-choroid organ culture and retina pigment epithelium cell culture[35]. Another in vitro study showed that bevacizumab and ranibizumab targeted several of the steps required during the angiogenic process, namely endothelial cell proliferation, migration and assembly into capillary-like structures [36]. Akova et al.[37] (escrs.org/vienna2011/programme/poster presentation) studied to determine and compare the effects of subconjunctival ranibizumab, bevacizumab and pegaptanib injections on CNV in a rat model. Bevacizumab showed the highest inhibitory effect on corneal neovascular vessels between three anti-angiogenic agents. The anti-neovascular effect of pegaptanib was higher than ranibizumab. It was observed that ranibizumab was effective in the inhibition of corneal NV seconder to alkali burn. Chan et al.[38] were observed that in different corneal NV rabbit models; subconjunctival injection of bevacizumab was effective in inhibiting corneal NV in several rabbit models. Also in their study Sener et al.[39] observed that bevacizumab, ranibizumab, pegaptanib, and trastuzumab were found effective for the inhibition of corneal NV. In this study, it was determined that the most effective agent was bevacizumab. Stevenson et al.[40] emphasized that ranibizumab and bevacizumab are safe and effective treatments for corneal NV and their results suggest that ranibizumab may be modestly superior to bevacizumab in terms of both onset of action and degree of efficacy. In contrast to Stevenson's study Dursun et al.[41] observed that both subconjunctival bevacizumab and ranibizumab treatments may be effective methods in reducing corneal NV; furthermore, bevacizumab is more effective than ranibizumab in the inhibition of corneal NV. In their study Christoforidis et al.[42] were defined that there was no statictically significant difference in terms of effect on peripheral wound healing between intravitreal injected ranibizumab and bevacizumab.

These studies indicate that bevacizumab is more effective to inhibit CNV than ranibizumab. In contrary to previously reported results, we have noted that ranibizumab was more effective. This fact was related to the repeat injection frequency and the non- glycosilated molecular structure, which made it 140 times more specific than bevacizumab. Early studies on bevacizumab explained that bevacizumab was not capable of neutralizing mouse and rat VEGF-A. Lu et al.[43] studied efficacy and reliability of intravitreal injection of bevacizumab, ranibizumab, and

pegaptanib in choroidal NV treatment in a rat model and they indicated that these three anti-VEGF agents had no efficacy in stopping leakages in choroidal neovascularization. However, efficacy of these agents for choroidal neovascularization in humans has been demonstrated [44]. Based on these results, we think that the affinity of anti-VEGF agents for VEGF-A in muridae family may really below. This situation may explain the difference between the earlier studies' results and our results.

In conclusion, bevacizumab and ranibizumab were found effective for the inhibition of corneal neovascularization, whereas ranibizumab was found more effective to reduce the percentage of corneal neovascularization than bevacizumab. As ranibizumab was produced specifically for human ophthalmic use, we think that further studies are needed to detect the minimal effective dose, injection repeat frequencies, and the way of the drug administration for ranibizumab to inhibit corneal neovascularization.

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