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RESEARCH ARTICLE

Inhibition of Corneal Neovascularization by Ranibizumab (Lucentis): An Experimental Study in Rabbit Cornea

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Summary

The purpose of this study was to evaluate the effects of the use of the subconjunctival injection of ranibizumab (Lucentis) on angiogenesis in the rabbit cornea. Corneas of 12 New Zealand Rabbits were cauterized with silver nitrate crystal. Animals were divided in two groups: control group (GC) that received 0.02 ml of 0.9% saline solution; group ranibizumab (GR) that received 0.5 mg of ranibizumab subconjunctivally at the 24th h after the lesion was formed. Animals corneas were extracted on the 14th day under general anesthesia. The newly formed vessels digital photographs were obtained and analyzed in a computerized system (google sketch-up program). In the control group, neovascularization covered 64.66%±20.81 (mean±standard deviation [SD]) of the corneal surface, compared with 34.17%±4.53 (mean±SD) in the GR group. When vascular density is compared between treated groups, statistical differences were observed (P<0.002). The results showed an inhibition of angiogenesis when the control group was compared with ranibizumab treated groups. These results suggest that subconjunctival injection of ranibizumab is able to inhibit corneal angiogenesis.

Keywords: *Ranibizumab, Rabbit cornea, Neovascularization, Angiogenesis*

Kornea Neovaskülarizasyonunun Ranibizumab (Lucentis) ile Baskılanması: Tavşan Korneasında Deneysel Çalışma

Özet

Bu çalışma, tavşan kornea anjiogenezisi üzerine ranibizumab'ın (Lucentis) subkonjonktival enjeksiyon kullanımının etkilerini değerlendirmek amacıyla yapıldı. Araştırmada 12 Yeni Zelanda tavşanı kullanılarak korneaları gümüş nitrat kristal ile koterize edildi. Hayvanlar iki gruba ayrıldı. Lezyon oluşturulduktan sonraki 24. saatte kontrol grubuna (GK) 0.02 ml %0.9 tuz solüsyonu, ranibizumab grubuna (GR) ise 0.5 mg ranibizumab subkonjonktival olarak enjekte edildi. Hayvanlar 14. günde korneaları genel anestezi altında alındı. Yeni oluşan damarların dijital fotoğrafları alınarak bilgisayar sistemi ile (google sketch-up programı) incelenmesi yapıldı. Kontrol grubunda neovaskülarizasyonun 64.66%±20.81 (ortalama±standart sapma [SD]) korneal yüzeyi kaplaması, GR grubundaki 34.17%±4.53 (ortalama±standart sapma [SD]) neovaskülarizasyonun korneal yüzeyi kaplaması ile kıyaslandı. Damar yoğunluğu tedavi grupları arasında karşılaştırıldığında, istatistiksel olarak farklılık (P<0.002) belirlendi. Sonuç olarak, kontrol grubu ve ranibizumab ile tedavi edilen grup karşılaştırıldığında anjiyogenezin inhibe edildiği saptandı. Bu sonuçlar ranibizumabın subkonjonktival enjeksiyonu ile kornea anjiogenezisinin inhibe edilmesinin mümkün olduğunu göstermektedir.

Anahtar sözcükler: *Ranibizumab, Tavşan korneası, Neovaskülarizasyon, Anjiogenezis*

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INTRODUCTION

Corneal neovascularization represents an important cause of diminished corneal clarity and subsequent reduction of vision [1]. Various models have been proposed to explain corneal clarity and avascularity and also to elucidate corneal neovascularization processes, which may be associated with various inflammatory, infectious, degenerative and traumatic corneal disorders, reaction to corneal transplantation and extended lens wear [1-3]. In addition, corneal neovascularization represents a major reason for corneal graft rejection [4,5]. Various risk factors that are associated with an increased likelihood of corneal neovascularization after penetrating keratoplasty have been proposed [4,6]. Corneal neovascularization leads to scar formation, lipid deposition, immune rejection of corneal grafts and, therefore, significant visual impairment [7]. It represents a major public health concern worldwide, it is the common pathway to blindness from diseases such as trachoma and oncocerciasis, whereas in the US, 4% of the population has corneal neovascularization [1,5].

Various chemical compounds and drugs, such as steroids [8], methotrexate [9], heparin [10] and thalidomide [11] have been proposed as inhibitors of corneal neovascularization. Steroids remain the first choice in clinical practice, because neovascularization is assumed to be secondary to some degree of inflammation. If this is correct, inhibition of inflammation by steroids should also inhibit subsequent neovascularization. However, when inflammation is not the cause of angiogenesis, such as in diseases associated with deficiency of limbal cells or corneal neovascularization secondary to corneal hypoxia, anti-inflammatory corticosteroids have little or no effect on capillary growth. The side effects of steroids, such as glaucoma and cataract formation, should not be underestimated. For the above reasons, effective alternatives are required.

Vascular endothelial growth factor (VEGF) has been proven to be a major inducer of corneal neovascularization, both in experimental models and in the human cornea [12-14]. VEGF and its tyrosine kinase receptors, flt-1 and flk-1/KDR are key mediators in a variety of angiogenesis models. Adamis and Shima have emphasized that VEGF is both necessary and sufficient for the occurrence of pathologic ocular neovascularization in multiple ocular tissues [15].

Corneal epithelial and endothelial cells, vascular endothelial cells of limbal vessels, and fibroblasts and macrophages in scar tissue have all been found to excrete VEGF, especially in inflamed and vascularized corneas [16-18]. Therefore, VEGF antagonists that act to inhibit its expression [19] may reduce or even prevent neovascularization. This would be of great clinical usefulness, for example, in minimizing corneal graft failure [20] or improving rehabilitation after alkali burns. It also may benefit diseases that involve deficiency of limbal stem cells and even contact lens-associated corneal neovascularization.

Ranibizumab is the Fab fraction of the whole antibody and it also neutralizes all the forms of VEGF-A, it is a non-glycosilated molecule, which makes it 140 times more specific than other anti-VEGF's [21,22]. Ranibizumab was developed for local ocular anti-angiogenic therapy, to assure better penetration through the retina than obtained with a larger full-sized antibody after an intravitreal injection. In animal models, ranibizumab has been demonstrated to penetrate the retina and reach the subretinal space following intravitreal injection [23]. It has been shown to reduce retinal and choroidal neovascularization as well as leakage from established vessels effectively [24]. It was found that ranibizumab had a high affinity toward rabbit VEGF but lower by 40-fold compared with its affinity to human VEGF [25].

The aim of this study was to evaluate the effects of the use of the subconjunctival injection of ranibizumab 0.5 mg on angiogenesis in the rabbit cornea that cauterized with silver nitrate crystal.

MATERIAL and METHODS

This study involved 12 healthy New Zealand albinos white male rabbits, aged 10-12 months, weighing around 3-3.5 kg and were obtained from the Experimental Animal Care Center, Faculty of Veterinary Medicine, Kafkas University (Kars, Turkey). The study was conducted in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Ethical Committee for Medical Experimental Research and Application Centre of Kafkas University (KAÜ-HADYEK/2011-002). Rabbits were kept under standard conditions (20±1°C, 12-h light/12-h dark cycles) and were randomly divided into 2 equal groups as control group (GC) and Ranibizumab group (GR) (n=6) respectively.

General anesthesia was induced by intramuscular injection of ketamine HCl (Ketalar®, Pfizer) (5 mg/kg body weight) and xylazine HCl (Rampun®, Bayer) (2 mg/kg body weight), as previously described [26]. In addition to the general anesthesia, topical anesthesia was performed into the eyes of all animals with 0.5% propocaine HCl (Alcaine®, Alcon) 1 min after the topical anesthesia was performed, (75% silver nitrate 25% potassium nitrate) alkalic burn was formed by steeping the silver nitrate sticks over cornea during 2 min. No complications as necrosis or perforation was seen. Alcali-induced corneal neovascularization model was performed as decscribed by Roberta et al.[27] with some modifications. The eyes were then carefully rinsed with approximately 10 ml of saline solution.

At the first day of the application, by starting treatment a single dose of topical antimicrobial pomade (Tobrased®, Bilim İlaç, İstanbul - Türkiye) and drops (Tobrased®, Bilim

İlaç, İstanbul-Türkiye) during a week subconjunctivally anti VEGF agents ranibizumab 0.5 mg (Lucentis®, Genentech/ Novartis) was performed as single dose into the eyes of the rabbits in GR group.

Subconjunctival serum was inoculated into the eyes of the rabbits that form the control group. At the 14th day of the study, by performing sevoflurane (Sevorane Likit®, Abbott) anesthesia with mask induction to the subjects under general anesthesia 2.5% (1.5 MAC), their corneas were were extracted with 360 degree incision. After their corneas were taken, the operation was finished by being tapped with sclera and conjunctiva 6/0 polyglactin 910 suture (Vicryl Ethicon®, Johnson&Johnson) and performed antimicrobial pomade.

During a week after the operation, 3x1 antimicrobial drops was used. By being photographed their corneas at digital media, the ratio of neovascularizational zone to all corneal zones was calculated via google sketch-up program. Statistical analyses of the data was performed by t-test.

RESULTS

In the ranibizumab - treated eyes, the vascular density of new blood vessels was lower than in control eyes.

In the control group, neovascularization covered 64.66%±20.81 (mean±standard deviation [SD]) of the corneal surface, compared with 34.17%±4.53 (mean±SD) in the GR group (*Fig. 1, 2* and *3*). When vascular density is

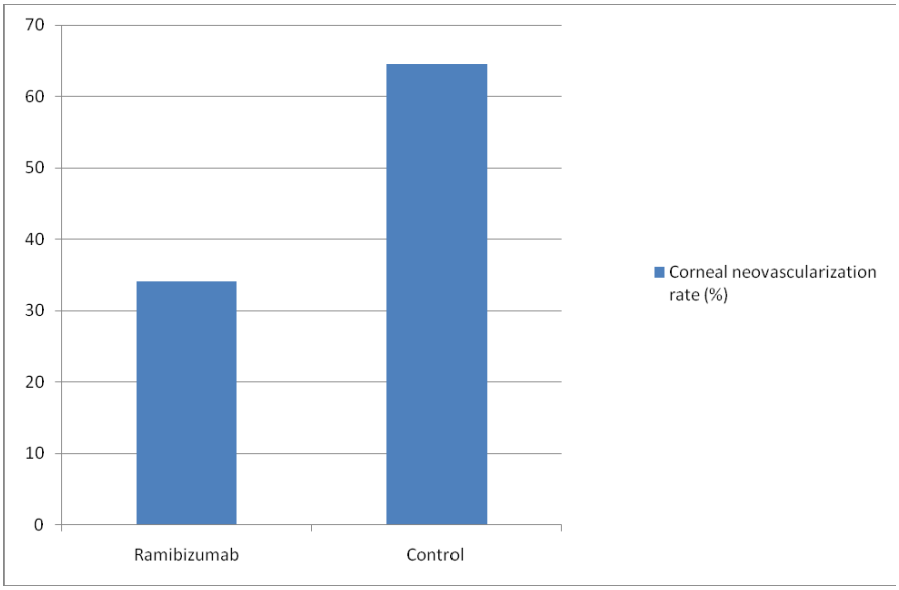

Fig 1. Corneal neovascularization rate (%) in ranibizumab treated and control groups

Şekil 1. Ranibizumab uygulanan ve Kontrol gruplarında korneal neovaskülarizasyon oranı (%)

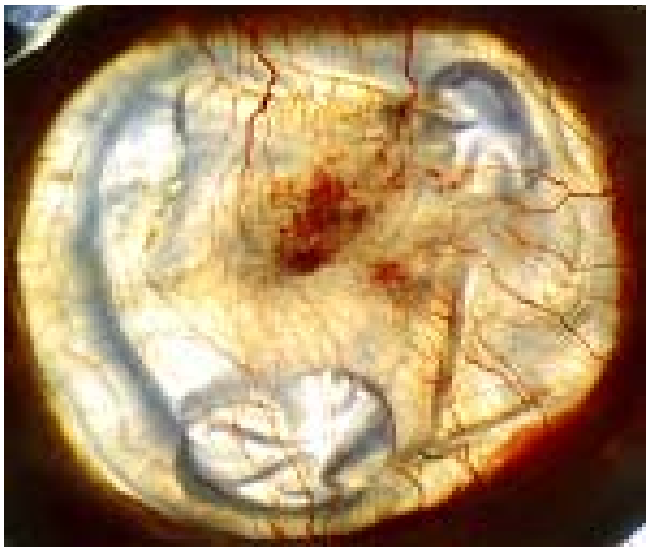

Fig 2. A rabbit cornea's macroscopic view at 14th day in control group

Şekil 2. Kontrol grubunda ki bir tavşanın korneasının 14. gündeki makroskobik görünüşü

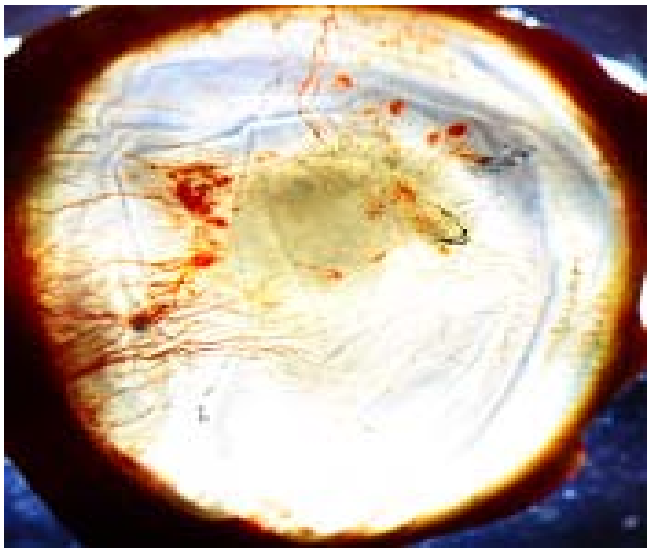

Fig 3. A rabbit cornea's macroscopic view 14th day in ranibizumab group

Şekil 3. Ranibizumab grubundan bir tavşanın korneasının 14. gündeki makroskobik görünüşü

compared between treated groups, statistical differences were observed (P<0.002). No adverse effects related to Ranibizumab injection were observed in all treated animals.

DISCUSSION

Although there are lots of studies upon the control of the corneal neovascularization about bevacizumab, no sufficient number of studies about ranibizumab were noticed. The aim of our study was to evaluate the effects of the use of the subconjunctival injection of ranibizumab on angiogenesis in the rabbit cornea that cauterized with silver nitrate crystal.

To maintain the transparency of the cornea, it is very important to maintain vascularity, and for this, the appropriate homeostasis of vascular growth factors should be maintained. When the balance of angiogenic factors such as fibroblast growth factor (FGF) and VEGF, and anjiogenic supressors such as angiostatin, endostatin and pigment epithelium derived factor (PEDF) are disrupted by diseases, neovascularization develops [1].

For the treatment of corneal neovasculature, various drug therapies, laser photocoagulation and surgical therapy have been attempted but established therapeutic methods are not currently available. Recently, photodynamic treatment using verteporfin also has been used for the treatment of corneal neovascularization. Its short term effect has been reported to be very positive, but its long term effect has not been proven [28,29].

As surgical therapy on reconstruction of damaged corneas, transplantation of autologous limbal epithelial cells cultured on amniotic membrane has been attempted and positive effects were reported [30].

As drug terapy, various drugs have been identified as inhibitors in experimental and clinical corneal neovascularization, including steroids [8,31], non-steroidal anti-inflammatory drugs [32], heparin [10], cyclosporın A [33], methotrexate [9] and thalidomide [11]. Steroids have been the mainstay of treatment for corneal neovascularization and corneal graft rejection in clinical practice. Steroids, however, are not always effective and chronic use may cause glaucoma, as well as precipitate infection or cataract formation.

The prominent role of VEGF in the pathophysiology of corneal neovascularization has been demonstrated in experimental models of corneal neovascularization [12,34], in experimental herpes simplex keratitis [35] and in studies from human corneal buttons [14,36]. Additionally, VEGF antagonism, whether at the protein or mRNA level, has been shown to reduce corneal neovascularization and improve corneal graft survival in experimental animals [20,37].

It was demonstrated that a single subconjuctival injection of a VEGF trap can promote a dose-dependent regression of newly formed vessels in a suture-induced model of corneal neovascularization [38]. In the initial clinical phase 1 study with neovascular AMD patients, 0.5 mg of ranibizumab was identified as the maximum tolerated single intravitreal dose, with intraocular inflammation the dose-limiting toxicity [23]. The rabbits corneas were extracted at the 14th day of the study. To achieve detectable accumulation of bevacizumab in the vitreous of the injected rabbit, the dosing interval should be shorter than four half-lives [39]. Because the half-life of bevacizumab in the rabbit vitreous is 4.32 days [40], reinjection is needed in the rabbits every 14th day. The reported vitreous half-life of ranibizumab in monkeys is 3 days [41] and is yet to be determined in rabbits. We tried to do the dosing interval similar to the bevacizumab.

In the ranibizumab-treated eyes the vascular density of new blood vessels was lower than in control eyes. In the control group (GC), neovascularization covered 64.66%±20.81 (mean±SD) of the corneal surface, compared with 34.17%±4.53 (mean±SD) in the ranibizumab group (GR). When vascular density is compared between treated groups statistical differences were observed (P<0.002).

Although our results were highly significant, inhibition of corneal neovascularization was far from complete. There are several possible reasons for this. Firstly, as insufficient dose and/or diffusion and absorption of *ranibizumab* through the conjunctiva with partial inhibition of VEGF activity. Secondly, it is clear that cytokines other than VEGF (eg, transforming growth factor α and β1, and fibroblast growth factor) can induce corneal neovascularization [1,36].

The subconjuctival injection seems to be a good option to inhibit corneal neovascularization. This delivery method is easy and simple to be performed and has minimal related complications. The possible systemic absorption and extra ocular side effects need to be thought and addressed adequately to avoid potential complications.

In conclusion, this study showed that subconjuntival injection of Ranibizumab is effective in limiting corneal neovascularization in this rabbit experimental model (*Fig. 2* and *3*). No adverse effects on the cornea were noted in our study. More research is needed to define the ideal concentration and time of administration to achieve the best clinical outcome.

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