



The effects of the subconjunctival injection of bevacizumab (Avastin®) on angiogenesis in the rat cornea

LUIZ F.M. BARROS¹ and RUBENS BELFORT-JR²

¹Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo
Av. Prof. Orlando Marques de Paiva, 87, Cidade Universitária, 05508-900 São Paulo, SP, Brasil

²Departamento de Oftalmologia, Universidade Federal de São Paulo
Rua Botucatu, 822, Vila Clementino, 04023-062 São Paulo, SP, Brasil

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ABSTRACT

The purpose of this study was to evaluate the effects of the use of the subconjunctival injection of bevacizumab (Avastin®) on angiogenesis in the rat cornea. Corneas of 20 Wistar male rats were cauterized with silver nitrate crystal. Animals were divided in four groups: control group (GC) that received subconjunctivally 0.02 ml of 0.9% saline solution on the day of the lesion; group G0 that received subconjunctivally 0.02 ml of bevacizumab just after the lesion; group G3 that received bevacizumab on day 3 and group G5 that received bevacizumab on day 5 after lesion. Animals were euthanized on day 7. The newly formed vessels were quantified after China Ink perfusion and photographs were obtained and analyzed in a computerized system (Image Pro-Plus®). In the control group, neovascularization covered $53.56\% \pm 15.11$ (mean \pm SD) of the corneal surface, compared with $35.57\% \pm 18.80$ (mean \pm SD) in the G0 group, $30.60\% \pm 11.82$ (mean \pm SD) in the G3 and $35.86\% \pm 0.07$ (mean \pm SD) in the G5. The results showed an inhibition of angiogenesis when the control group was compared with all treated groups. These results suggest that subconjunctival injection of bevacizumab is able to inhibit corneal angiogenesis independently of the day of treatment.

Key words: bevacizumab, cornea, neovascularization, angiogenesis.

INTRODUCTION

Corneal neovascularization is a sequel of several inflammatory disease of the anterior segment, such as infections, degenerative and traumatic disorders, reaction to corneal transplantation and extended lens wear (Chang et al. 2001, Hosseini and Nejabat 2007).

VEGF (Vascular Endothelial Growth Factor) has been implicated in corneal neovascularization. The implantation of nylon discs impregnated with VEGF or bFGF (Basic Fibroblast Growth Factor) can promote grossly prominent corneal neovascularization extending from the limbus to the implant 7 days after the implantation (Coxon et al. 2002). The application of silver ni-

trate on the cornea induces corneal neovascularization and increases the levels of VEGF-C e VEGFR-3 three days after lesion (Edelman et al. 1999, Mimura et al. 2001). Other experimental models such as alkali burn and krypton laser photocoagulation can also induce neovascularization that is accompanied by increased levels of VEGF (Gan et al. 2004, Kvanta et al. 2000).

Inhibition of VEGF and, thereby, inhibiting angiogenesis can be an effective treatment for a variety of ocular diseases followed by neovascularization. Anti-VEGF antibodies, such as ranibizumab (Lucentis®) and pegaptanib (Macugen®) have been designed with the aiming to control neovascularization in neovascular age-related macular degeneration (AMD) (Heier et al. 2006, Rosenfeld et al. 2005, 2006, Gragoudas et al. 2004), proliferative diabetic retinopathy (PDR) (Macugen Diabetic

*Member Academia Brasileira de Ciências
Correspondence to: Luiz Felipe de Moraes Barros
E-mail: luizfelipemb@yahoo.com.br

Retinopathy Study Group 2006) and corneal neovascularization (Hosseini and Nejabat 2007, Manzano et al. 2006).

Bevacizumab, a humanized monoclonal anti-body to VEGF was designed for intravenous applications and approved for the treatment of colorectal cancer (Kabinavar et al. 2003, Hurwitz et al. 2004, Emmanouilides et al. 2004). Recently bevacizumab has been used, with promising results, as a systemic or intra-vitreous treatment for exudative AMD (Michels et al. 2005, Avery et al. 2006a). Good results were also reported using bevacizumab for the treatment of diabetic retinopathy reducing retina and iris neovascularization (Avery et al. 2006b, Avery 2006). One case of neovascular glaucoma following central vein occlusion was treated with bevacizumab with markedly improvement of IOP and discomfort (Kahook et al. 2006). Bevacizumab seems to be an effective option to inhibit corneal neovascularization. A twice a day topic application of 4mg/ml bevacizumab for 7 days decreased corneal vascularization in 40% (Manzano et al. 2006). Partial regression of new vessels was observed when Avastin was injected in the stroma of five eyes with corneal neovascularization related to corneal graft and limbic stem cell deficiency (Höfling-Lima et al. 2006).

The subconjunctival injection is also a widely used delivery method of drugs in the eye. Liu et al. (2006) demonstrated that a single subconjunctival injection of a VEGF trap can promote a dose-dependent regression of newly formed vessels in a suture-induced model of corneal neovascularization.

The aim of this paper was to study the effect of the subconjunctival injection of bevacizumab on the experimental induced corneal neovascularization in rats after silver nitrate lesion.

MATERIALS AND METHODS

Twenty male Wistar rats, aging 8 to 10 weeks and weighing 250g to 300g were used. Under general anesthesia induced and maintained by isoflurane supplemented with topical anesthesia (0.5% proparacaine hydrochloride) corneas were cauterized with silver nitrate to induce neovascularization. Both corneas of each animal were cauterized by pressing a crystal of silver nitrate, 2 mm far from the limbus, under surgical microscope. The eyes were then carefully rinsed with approximately

10 ml of saline solution. The experiment was performed according to the ARVO animal care regulation and approval of the local animal research ethics committee.

Following cauterization, animals were divided into four groups: Group GC (n=10) received a subconjunctival injection of 0,02 ml of 0,9% saline solution; group GO (n=10) received a subconjunctival injection of 0,02 ml of bevacizumab (Avastin[®]) just after the lesion; group G3 (n=10) received a subconjunctival injection of 0,02 ml of bevacizumab (Avastin[®]) at day 3 after lesion and group G5 (n=10) received a subconjunctival injection of 0,02 ml of bevacizumab (Avastin[®]) at day 5 after lesion. Seven days after lesion, animals were euthanized by hyper inhalation of isoflurane and corneas were perfused with China ink. Eyes were fixed in paraformaldehyde for 24 hours, dehydrated with increasing concentrations of ethanol and diaphanized with benzene. Corneas were dissected and mounted onto slides. Under microscopy photographs were taken and the newly formed vessels were quantified and analyzed by computerized system (Image Pro-Plus[®]). Three standardized areas (1350 × 1020 μm) were examined and an average value was taken from each cornea. The Kruskal-Wallis test followed by the Dunn test with P < 0.05 was used for comparisons.

RESULTS

In the bevacizumab-treated eyes the vascular density of new blood vessels was lower than in control eyes, independently of the injection day. In the control group, neovascularization covered 53.56% ± 15.11 (mean ± standard deviation [SD]) of the corneal surface, compared with 35.57% ± 18.80 (mean ± SD) in the GO group, 30.60% ± 11.82 (mean ± SD) in the G3 and 35.86% ± 0.07 (mean ± SD) in the G5 (Figs. 1 and 2). When vascular density is compared between treated groups no statistical differences were observed. No adverse effects related to bevacizumab injection were observed in all treated animals.

DISCUSSION

Many drugs as Hypericin, Rapamycin and non-steroidal anti-inflammatory drugs with cyclooxygenase inhibitory activity have been experimentally evaluated for corneal neovascularization inhibition (Lavie et al. 2005, Kwon

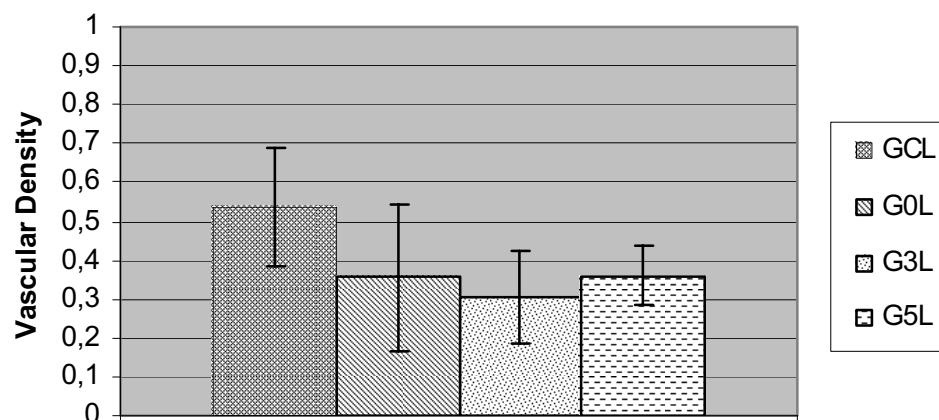


Fig. 1 – Comparison of vascular density (mean \pm SD) between control group and bevacizumab treated groups. A significant reduction of vascular density was observed in all treated groups ($p < 0.05$).

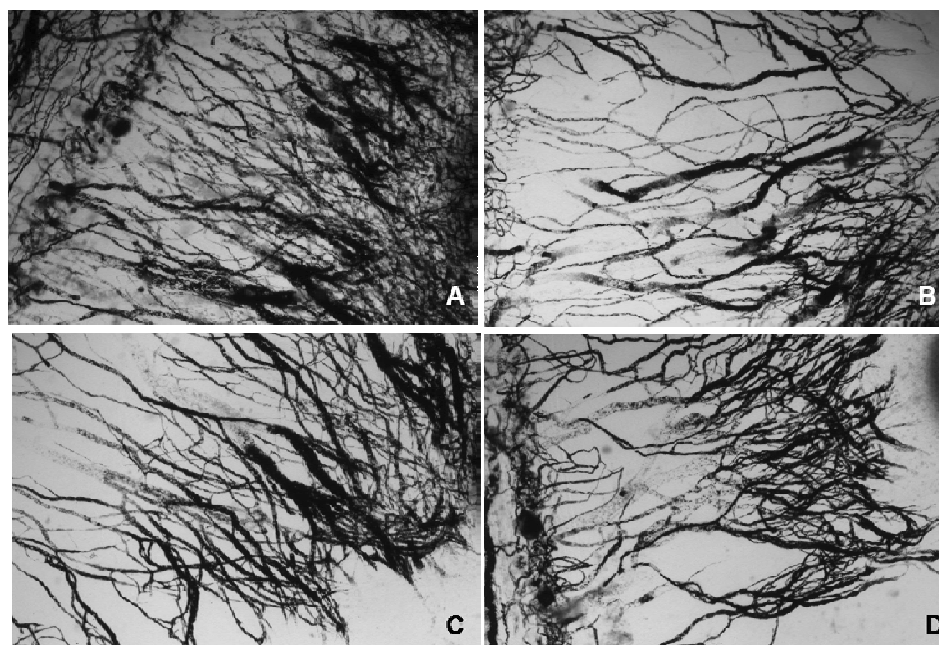


Fig. 2 – Images of the corneal newly formed vessels on day 7 after silver nitrate cauterization. A) Control group [GC]; B) injection at day of lesion [G0]; C) injection at the third day after lesion [G3]; D) injection at the fifth day after lesion [G5].

and Kim 2006, Castro et al. 2004). Among these drugs, steroids seem to be the best therapy to inhibit corneal neovascularization (Riazi-Esfahani et al. 2006) and remain the mainstay of therapy to prevent corneal graft rejection (Randleman and Stulting 2006).

Our results suggest that bevacizumab can inhibit corneal neovascularization in this rat model in all tested

groups. Although our data showed statistical significance ($p < 0,05$), the inhibition of corneal neovascularization was far from complete. Some possibilities can explain this incomplete inhibition, as insufficient dose and/or diffusion and absorption of bevacizumab through the conjunctiva with partial inhibition of VEGF activity. Besides that, other cytokines, as FGF (Fibroblast Growth Factor)

contributes to the angiogenic process and cannot be inhibited by Bevacizumab (Gaudric et al. 1992).

Bevacizumab has been successfully used systemically and intravitreally in humans with exsudative age related macular degeneration, and diabetic retinopathy controlling retina, choroidal and iris neovascularization with minimal or no adverse effects (Michels et al. 2005, Avery et al. 2006b, Avery 2006, Feiner et al. 2006). Topical application of bevacizumab (4mg/ml) was effective to control experimentally induced corneal neovascularization in rats (Manzano et al. 2006). Two intrastromal injections of 0.05 ml of Avastin[®] with a month interval were also effective to promote regression of newly formed vessels in all studied patients (Höfling-Lima et al. 2006).

It is interesting to observe that these findings demonstrate the effect of the bevacizumab inhibition in a short time period. There are clinical data that reveal that bevacizumab intravitreal and intrastromal application can be transitory and persist only for few weeks. The observation of the effects of bevacizumab longer than our study could be done to investigate how long the inhibition of neovascularization can persist.

The subconjunctival 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 thought to be adequately addressed to avoid potential complications.

We have shown that subconjunctival injection of bevacizumab (Avastin[®]) is effective in controlling corneal neovascularization in this experimental animal model. Controlled clinical trials must be performed to demonstrate the efficacy of bevacizumab in the treatment of corneal neovascularization or corneal graft rejection.

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RESUMO

O objetivo deste estudo foi avaliar os efeitos da aplicação subconjuntival de bevacizumab (Avastin[®]) na angiogênese corneal em ratos. Vinte ratos Wistar, machos, foram submetidos a cauterização química com cristal de nitrato de prata. Os animais foram divididos em 4 grupos: O grupo controle (GC), recebeu injeção de 0,02 ml de solução fisiológica pela via subconjuntival no momento da lesão. O grupo G0 recebeu 0,02 ml de bevacizumab (Avastin[®]) imediatamente depois da lesão. O grupo G3 recebeu 0,02 ml de bevacizumab no terceiro dia após a lesão. O grupo G5 recebeu 0,02 ml de bevacizumab no quinto dia após a lesão. Os animais foram eutanasiados 7 dias após a cauterização. Os vasos neoformados foram quantificados após preenchimento do leito vascular com Tinta da China e imagens foram obtidas e analisadas em sistema computadorizado (Image Pro-Plus[®]). No grupo controle a neovascularização ocupou $53,56\% \pm 15,11$ (média \pm DP) da superfície corneal comparando a $35,57\% \pm 18,80$ no grupo G0, $30,60\% \pm 11,82$ (média \pm DP) no G3 e $35,86\% \pm 0,07$ (média \pm DP) no G5. Os resultados mostram uma inibição da angiogênese quando se compara GC com os grupos tratados. Os resultados sugerem que a injeção subconjuntival de Bevacizumab é capaz de inibir a angiogênese corneal independentemente do dia de aplicação.

Palavras-chave: bevacizumab, córnea, neovascularização, angiogênese.

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