Effects of topical and subconjunctival use of bevacizumab on corneal neovascularization in rabbits' eyes

Efeitos do uso tópico e subconjuntival do bevacizumabe na neovascularização corneana de olhos de coelhos

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ABSTRACT

Purpose: To evaluate and compare the effects of topical application and subconjunctival injection of bevacizumab on corneal neovascularization (CNV) in rabbits' eyes after chemical burning of the cornea.

Methods: The animals were randomly distributed into four groups of five animals. In one group, the drug was instilled, while in another, it was administered by subconjunctival injection. The two procedures using bevacizumab were compared with instillation and subconjunctival injection of saline solution (S). Neovascularization was evaluated according to the size of the invasion area of new blood vessels and through computerized analysis of this area. The data were analyzed using the Kruskal-Wallis test followed by Dunn's test for two-by-two comparison of the groups, to assess the external examination of CNV. Analysis of variance was used to assess the area of CNV. P<0.05 was considered statistically significant.

Results: Assessing both the external examination and the invasion area of neovessels on the 5th and 10th days, there was a clear difference between the groups. The group to which saline solution had been applied showed higher scores for CNV, as well as increases in the invasion area of neovessels. Two-by-two comparison of groups revealed no significant differences. However, an analysis of the factors involved (injection vs. instillation and bevacizumab vs. saline solution) showed that injection did not differ from instillation, but that bevacizumab differed from saline solution.

Conclusion: Bevacizumab showed an inhibitory effect on CNV in rabbits' eyes after chemical burning of the cornea. There was no difference between the topical or subconjunctival administration of bevacizumab in the inhibition of CNV.

Keywords: Cornea; Corneal neovascularization; Bevacizumab; Injections; Angiogenesis inhibitors; Vascular endothelial growth factor; Animals; Rabbits

RESUMO

Objetivos: *Avaliar e comparar o efeito do uso tópico e da injeção subconjuntival do bevacizumabe na neovascularização corneana de olhos de coelhos após queimadura química.*

Métodos: *Os animais foram distribuídos de forma aleatória em quatro grupos de cinco animais. Em um grupo de coelhos a droga foi instilada, enquanto em outro foi aplicada injeção subconjuntival, sendo os dois procedimentos comparados com a instilação e injeção subconjuntival de soro fisiológico 0,9% (SF) e entre si. A neovascularização foi avaliada conforme o tamanho da área de invasão dos neovasos e com análise computadorizada da mesma. Na análise de dados aplicou-se o teste de Kruskal-Wallis seguido do teste de Dunn com p<0,05 para comparação dos grupos dois a dois na análise do exame externo da neovascularização corneana. Na análise da área de neovascularização corneana, aplicou-se o teste F de análise de variância. A significância estatística foi definida como valor de p<0.05.*

Resultados: *A avaliação do exame externo e da área de invasão de neovasos, no 5º e 10º dia, mostrou nítida diferença entre os grupos. Com o uso de soro fisiológico houve maior graduação na escala de neovascularização corneana e também na área de invasão dos nevasos, o que demonstrou o efeito inibitório do bevacizumabe. Nas comparações dos grupos dois a dois não foram detectadas diferenças significativas, porém, ao analisar os fatores envolvidos (procedimentos: injeção ou instilação, e as drogas: bevacizumabe ou soro fisiológico), verificou-se que a injeção não diferiu da instilação, mas o bevacizumabe diferiu do soro fisiológico.*

Conclusão: *O bevacizumabe apresentou efeito inibitório na neovascularização corneana de olhos de coelhos após queimadura química, tanto por via tópica como por via subconjuntival e não houve diferença entre a via tópica e a via subconjuntival de administração do bevacizumabe na inibição da neovascularização corneana.*

Descritores: *Córnea; Neovascularização de córnea; Bevacizumab; Injeções; Inibidores da angiogênese; Fator A de crescimento do endotélio vascular; Animais; Coelhos*

INTRODUCTION

Studies of bevacizumab for topical use in the eye are opportune given that inhibition of the growth of neovessels on the ocular surface is desirable in numerous situations. Corneal neovascularization (CNV) is a response of the cornea to injury or inflammation. The presence of neovessels leads to the loss of transparency of the cornea, altering vision and making it necessary to take measures to reduce the inflammation and impede the growth of neovessels[1-3]. CNV depends on the balance between vascular growth factors and inhibitory vascular growth factors present in the vascular endothelium[4]. After corneal trauma, cell proliferation occurs in the vascular endothelium, which depends on the vascular endothelial growth factor (VEGF)[5-6].

Bevacizumab is defined as an antineoplastic agent; it is a humanized monoclonal antibody that inhibits angiogenesis, acting as specific inhibitor of VEGF[7-9]. It was approved by the Food and Drug Administration in 2004 for use in metastatic colon cancer administered via the systemic route[4,8,10]. This drug is also administered via the intraocular route, and in off-label use, age-related macular degene-

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ration, central retinal vein occlusion, and diabetic retinopathy[7,10-13]. The effects of the use of bevacizumab on the ocular surface in conjunctival and corneal diseases still remains to be evaluated through controlled clinical trials.

The aim of this study was to evaluate and compare the effect of topical application and subconjunctival injection of bevacizumab on CNV of rabbits' eyes after chemical burning of the cornea.

METHODS

After approval by the Ethics Committee in Animal Experimentation of the Universidade Estadual de Londrina (UEL), the study utilized 20 male white New Zealand rabbits (*Oryctolagus cuniculus*), each weighing about 2.5 kg. The animals were randomly distributed into four groups of five animals each: A, B, C, and D. In one group, the drug was instilled, while in another, it was administered by subconjunctival injection (A and B). The two procedures were compared with instillation and subconjunctival injection of saline solution (S) (C and D) and also among them. The animals were treated in accordance with the guidelines recommended by the Brazilian College of Animal Experimentation[14] and the Canadian Council on Animal Care[15].

For chemical burning and injections, the animals underwent general anesthesia with intramuscular ketamine (40 mg/kg) and xylazine (3 mg/kg), followed by instillation of anesthetic eye drops of 0.5% proxymetacaine hydrochloride[2,16,17]. Analgesia was effected with oral tramadol at a dose of 2 to 4 mg/kg, twice within the first 24 h[18].

Corneal burning was performed with a surgical microscope, using Whatman No. 50 filter paper cut into circles 3 mm in diameter and soaked in 1 N sodium hydroxide. The paper was applied eccentrically to the cornea approximately 1 mm from the limbus in the upper part of the rabbits' right eyes for 60 s. Immediately after the burning, the eye was irrigated with 20 mL of saline solution[5,16,19].

Group B received a single subconjunctival injection of the drug (in the upper portion of the bulbar conjunctiva) immediately after corneal burning, at a dose of 2.5 mg of bevacizumab (0.1 mL of 100 mg/4 mL solution for intravenous infusion)[17,20] (Avastin®, Genentech Inc., South San Francisco, CA, USA; packaged by F. Hoffmann-La Roche Ltd., Kaiseraugst, Switzerland, and imported and distributed in Brazil by Roche Chemicals and Pharmaceuticals S.A., Rio de Janeiro, RJ). In group A, bevacizumab was instilled at a dose of approximately 1 mg (one drop, approximately 0.04 mL of the same solution for intravenous infusion) into the conjunctival sac, immediately after corneal burning and twice daily for 10 days[17]. The other two groups, C and D, were challenged with saline solution using the same quantity and forms of administration, topically and subconjuntival, respectively[2,16,17,19].

Blinded examination of CNV was conducted by a single examiner (lecturer in the Ophthalmology Department of UEL). The rabbits' eyes were examined by light microscopy utilizing a surgical microscope (DF Vasconcellos, model MC-M58, with optic fibers). Evaluation, data, and photographic recording occurred on the 5th and 10th days after initiation of the experiment[1,3,16,17]. On the 10th day, the animals were sacrificed. CNV was classified as shown in table 1[1,21].

Table 1. Scoring of CNV in rabbits' eyes

Cornea	Neovascularization score
Without vessels in the cornea	0
Vessels <1 mm in the cornea	1+
Vessels of 1-1.9 mm in the cornea	2+
Vessels of 2-2.9 mm in the cornea	3+
Vessels ≥3 mm in the cornea	4+

Source: adapted Deutsch, Hughes[21].

Photographic recording was made with a Canon® Digital Rebel XT camera with a macro lens of 100 mm and MR-14 ring flash. Analysis of the corneal images was performed using the public-domain program ImageJ 1.38x (Java 1.6.0_02, Wayne Rasband, National Institutes of Health, USA)[2,17]. The invasion area of neovascular vessels of each cornea was measured on photographs as the percentage of the total corneal area. The area of neovascularization was delimited using a computer and measured in pixels. Its ratio to the entire corneal area was determined. Three measurements were made, resulting in a final mean[2,17].

The Kruskal-Wallis test was conducted because the criteria for using analysis of variance (ANOVA) were not met. This was followed by Dunn's test, with $p<0.05$ for two-by-two comparison of the groups, in the analysis of the external assessment of CNV. To analyze the area of CNV, the ANOVA F-test was used. Statistical significance was defined as $p<0.05$.

RESULTS

On day 1 after the burn, all eyes demonstrated diffuse redness of the conjunctiva, vessels that were not easily visible in the upper portion, total corneal opacity at the burned site (uniform burning in all eyes), absence of vessels in the cornea, iris with congestion and slow reaction to light, and very intense corneal staining in the fluorescein eye test.

Table 2 presents the results of the external examination and determination of the size of the invasion area of new blood vessels of the cornea, shown as percentages. In both the evaluation of the external examination and the invasion area of new blood vessels,

Table 2. Results of the evaluation of the external examination and of the neovascularization area of rabbits' eyes on the 5th and 10th days of observation (n=20)

	External examination		Area of neovessels	
Group A - instillation BVZ				
Rabbit 16	1+	2+	1.9%	1.4%
Rabbit 17	1+	2+	1.2%	1.5%
Rabbit 18	-	-	-	-
Rabbit 19	3+	3+	5.6%	5.0%
Rabbit 20	0	0	0.0%	0.0%
Group B - injection BVZ				
Rabbit 6	1+	2+	2.2%	1.8%
Rabbit 7	-	-	-	-
Rabbit 8	1+	2+	1.3%	2.9%
Rabbit 9	2+	3+	5.4%	7.4%
Rabbit 10	1+	2+	1.4%	3.2%
Group C - instillation saline				
Rabbit 11	3+	4+	3.1%	8.8%
Rabbit 12	3+	4+	5.7%	8.4%
Rabbit 13	3+	4+	6.7%	10.0%
Rabbit 14	3+	4+	6.3%	8.7%
Rabbit 15	3+	4+	5.4%	8.2%
Group D - injection saline	Day 5	Day 10	Day 5	Day 10
Rabbit 1	2+	4+	4.3%	12.7%
Rabbit 2	2+	4+	5.0%	9.1%
Rabbit 3	2+	4+	7.9%	12.3%
Rabbit 4	2+	4+	4.9%	6.6%
Rabbit 5	2+	4+	5.1%	8.6%

BVZ= bevacizumab; (-) = died.

on the 5th and 10th days, there was a clear difference between the groups: with the use of saline solution, there was a higher score of CNV, as well as a larger area of neovessels formed (Figures 1-4).

Two rabbits died (rabbits 7 and 18) on the first day of the experiment and were not included in the calculations. According to the veterinarian in charge, the cause of death was acute respiratory failure due to pneumonia.

In the two-by-two comparisons of the groups, no significant differences were detected. However, in analyzing the factors involved (procedures: injection vs. instillation, and drugs: bevacizumab vs. saline solution), the group B (injection group) did not differ from the instillation group A ($p>0.05$), while the bevacizumab group differed from the saline solution group ($p<0.05$) (Table 3).

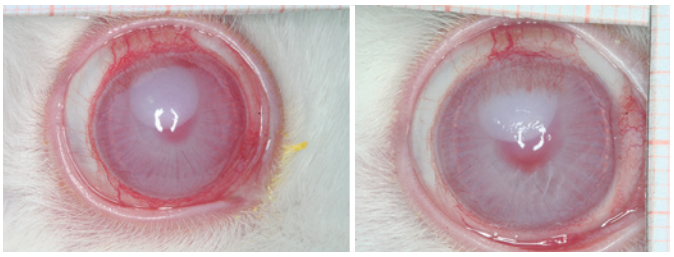

Figure 1. Eye of rabbit 3 on the 5th and 10th days after corneal burning and injection of saline solution, showing neovascularization.

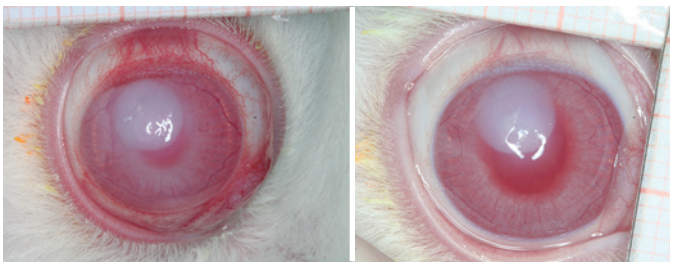

Figure 2. Eye of rabbit 6 on the 5th and 10th days after corneal burning and injection of bevacizumab, showing the effects of the drug.

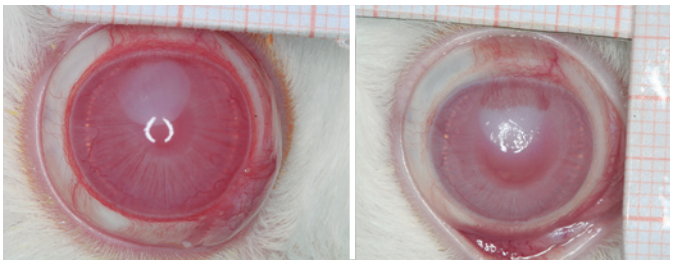

Figure 3. Eye of rabbit 11 on the 5th and 10th days after corneal burning and instillation of saline solution, showing neovascularization.

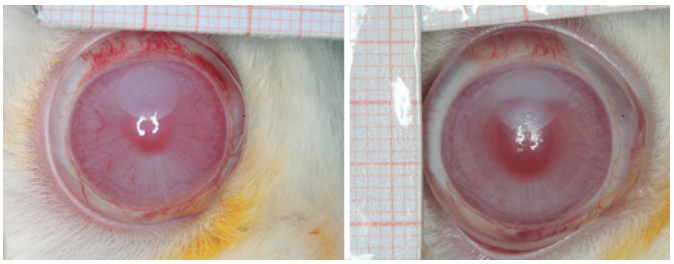

Figure 4. Eye of rabbit 20 on the 5th and 10th days after corneal burning and instillation of bevacizumab, showing effects of the drug (no neovascularization in the cornea).

In the external examination, the mean neovascularization score for the injection and instillation (B and A) of bevacizumab was 1.25 on day 5 of observation. When saline solution was used, the means were 2.00 (D, injection) and 3.00 (C, instillation). On day 10, the mean values for injection (B) and instillation (A) of the study drug were 2.25 and 1.75, respectively, and the mean value for the saline solution was 4.00 (C and D, injection and instillation). The p-values in the Kruskal-Wallis test on days 5 and 10 of observation were 0.0099 and 0.0013, respectively.

On day 5 of observation, the mean percentages of neovessel invasion area in relation to the total corneal area with the injection and instillation of bevacizumab (Groups B and A, respectively) were 2.58 and 2.18, respectively. When saline (S) was used, the mean values were 5.44 in both administration methods (Groups C and D). On day 10, the results were 3.83 and 1.98 (injection and instillation of bevacizumab, groups B and A), while with S, these values were 9.86 for injection and 8.82 for instillation (D and C respectively). The p-value for the analysis of variance test was 0.0220 on day 5 and 0.0001 on day 10 of observation.

DISCUSSION

Bevacizumab showed an inhibitory effect on CNV when comparing the experimental group with the control group with saline. However, when comparing the instillation and injection groups using bevacizumab, there was no statistically significant difference. This finding may help in the selection of the topical route for drug administration, since it is easy and non-invasive, thereby resulting in fewer complications[22], albeit being more expensive.

CNV is part of the reparative process after a corneal injury, and manifestation of inflammation and infiltrating leukocytes plays an important role[3,16]. Vascularizing corneal injuries is associated with inflammatory reactions, and migration and proliferation of capillary endothelium may occur in relation to the activities of various types of cells. Histologic examination in untreated corneal burn in rabbits showed the stroma to be infiltrated with polymorphonuclear leucocytes, most abundant during the first 3 days[21]. CNV depends on the balance between vascular growth factors and inhibitory vascular growth factors, present in the vascular endothelium[4]. After corneal trauma, cell proliferation occurs in the vascular endothelium, which depends on VEGF [5,6].

The exact tissue distribution of bevacizumab in the cornea is unknown. One mechanism to be considered is that the available bevacizumab solution contains polysorbate 20 (1.6 mg polysorbate 20 per 4 mL solvent carrier). In higher concentrations, this agent acts as a detergent and must be considered to enhance tissue penetration[22]. Regarding the mechanism of action of the subconjunctival injection of bevacizumab, it can be observed that this occurs in the conjunctiva itself, since the corneal blood vessels derive from the pericorneal vascular plexus. On the corneal surface, there is an invisible borderline between the corneal and conjunctival epithelium, although there is a functional difference. In corneal injury, there is an invasion onto the cornea of conjunctival-type epithelium containing goblet cells and blood vessels (neovascularization)[5].

Anti-VEGF drugs can decrease the formation of neovessels in the cornea, as various studies have shown. In a study of bevacizumab given topically (two times a day for seven days) in rats' eyes after corneal burning, the drug was effective in inhibiting neovascularization of the cornea[17]. Other authors administered bevacizumab using the subconjunctival route in a study of rats, and results suggested that this drug is capable of inhibiting corneal angiogenesis[23]. A literature review concluded that the subconjunctival route must be appropriate to focal, profound, and peripheral CNV, and that the topical route is appropriate for superficial, diffuse, and central CNV[24].

Table 3. Results of the evaluation of the external examination and of the neovascularization area of rabbits' eyes on the 5th and 10th days of observation

	n	Mean	SD	Minimum	25%	Median	75%	Maximum	*P*
Examination day 5									
Injection of BVZ	4	1.25	0.50	1.0	1.0	1.0	1.5	2.0	0.0099*
Injection of saline	5	2.00	0.00	2.0	2.0	2.0	2.0	2.0	
Instillation of BVZ	4	1.25	1.26	0.0	0.5	1.0	2.0	3.0	
Instillation of saline	5	3.00	0.00	3.0	3.0	3.0	3.0	3.0	
Examination day 10									
Injection of BVZ	4	2.25	0.50	2.0	2.0	2.0	2.5	3.0	0.0013*
Injection of saline	5	4.00	0.00	4.0	4.0	4.0	4.0	4.0	
Instillation of BVZ	4	1.75	1.26	0.0	1.0	2.0	2.5	3.0	
Instillation of saline	5	4.00	0.00	4.0	4.0	4.0	4.0	4.0	
Area day 5									
Injection of BVZ	4	2.58	1.93	1.3	1.4	1.8	3.8	5.4	0.0220†
Injection of saline	5	5.44	1.41	4.3	4.9	5.0	5.1	7.9	
Instillation of BVZ	4	2.18	2.41	0.0	0.6	1.6	3.8	5.6	
Instillation of saline	5	5.44	1.40	3.1	5.4	5.7	6.3	6.7	
Area day 10									
Injection of BVZ	4	3.83	2.46	1.8	2.4	3.1	5.3	7.4	0.0001†
Injection of saline	5	9.86	2.59	6.6	8.6	9.1	12.3	12.7	
Instillation of BVZ	4	1.98	2.13	0.0	0.7	1.5	3.3	5.0	
Instillation of saline	5	8.82	0.70	8.2	8.4	8.7	8.8	10.0	

*= *P* value for Kruskal-Wallis test; †= *P* value for F test of analysis of variance; BVZ= bevacizumab.

Bevacizumab utilized topically in rabbits' eyes demonstrated a beneficial effect on CNV and opacity[21]. Another study using subconjunctival injection of 2.5 mg bevacizumab (in two applications, at 6 h and 12 h, totaling 5 mg) showed a significant effect on the neovascularization of rabbits' eyes after burning with sodium hydroxide[25]. These data coincide with those of the present study.

In this study and others previously published[17,25], the inhibition of CNV was not accomplished. One explanation could be that bevacizumab acts specifically on VEGF and that other factors are also implicated in CNV, such as basic fibroblast growth factor (bFGF)[5,6], platelet-derived growth factor (PDGF), and angiopoietins 1 and 2[6,26]. Other possibilities are that bevacizumab, a humanized antibody, is not species-specific for rabbits[22] and is therefore less potent in rabbits. Therefore, the dose may have been insufficient for total inhibition of CNV[17,25].

Regarding the inhibition of VEGF by bevacizumab, a combination with other drugs might lead to better results[25]. The concomitant inhibition of VEGF-A and PDGF-B is more effective than only blocking VEGF-A[6]. The concept of combined therapy is becoming increasingly interesting to the ophthalmological scientific community, and the co-administration of two antiangiogenic agents is promising in the treatment of ocular diseases, as well as oncology and immunology[27].

In an experimental study with sumarin (a heparin analog that binds to heparin-binding proteins, including growth factors such as VEGF, PDGF, and bFGF), it was observed that subconjunctival sumarin reduced CNV in rabbits' eyes after corneal suturing. In other respects, the effect of sumarin is less potent than bevacizumab during the early period of treatment. Thus, a combination of bevacizumab and sumarin may have more potential and more prolonged antiangiogenic effects, making it possible to reduce the frequency of subconjunctival bevacizumab administration and the risk of serious complications. More studies are needed to elucidate this effect[28].

A new drug, VEGF-Trap, has been tested on ocular neovascularization. VEGF-Trap is a specific blocker that binds and inactivates VEGF in the blood and in extravascular and extracellular spaces through the formation of a stable immune complex, which has optimal pharmacokinetic properties and a high affinity for VEGF-A[29].

Another modality investigated has been the regulatory factors of angiogenesis, known as master regulators, so-called because of their crucial role in the initiation of the chain of angiogenic events, in which they can act simultaneously in the modulation of the activity of various angiogenic mediators. Examples are the transcription factor induced by hypoxia (HIF-α) and protein kinase CK2. These master regulators are promising targets for new ocular treatments[27].

Although bevacizumab seems to be safer than conventional therapies, such as steroids, cyclosporine, and mitomycin C[24], it is important to consider the collateral ocular and systemic effects of anti-angiogenic therapy[27]. VEGF is a survival factor for all endothelial cells, and the loss of VEGF can result in an inability of the normal vasculature to proliferate in response to trauma and to maintain a normal endothelial morphology, including fenestration in the ocular choriocapillary system and even in the renal glomerulus[27]. Well-developed clinical studies are needed to determine the best anti-VEGF agent, to optimize the dose and schedule[13], and to show the efficacy and safety of anti-VEGF for the long-term treatment of CNV[24,30].

The merit of this study resides in its comparison of the effects of topical and subconjunctival routes of administration of bevacizumab on corneal CNV in the rabbit eye after chemical burning. Bevacizumab showed an inhibitory effect on CNV in the eyes of rabbits following chemical burning, independent of the route utilized (topical or subconjunctival).

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