Topical Bevacizumab as Adjunctive Therapy for Bleb Survival after Trabeculectomy in the Rabbit Model

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ABSTRACT

Objective: To determine the effect of topically administered bevacizumab on bleb survival and histology after trabeculectomy in rabbit eyes.

Methods: This is an experimental interventional comparative animal study. Sixteen rabbit eyes underwent trabeculectomy, 8 of which were enhanced with intraoperative mitomycin-C. Eyes were randomized to receive either topical balanced salt solution (BSS) or topical bevacizumab at a concentration of 12.5 mg/mL. Intraocular pressure, bleb dimensions and vascularity grading were measured. IOP was recorded as a ratio of IOP of the experimental operated eye divided by the IOP of the contralateral control eye (IOP_{ratio}) as a function of time. Bleb morphology was recorded as a percentage of the maximum estimated bleb volume (% bleb) as a function of time. Bleb failure occurred if $IOP_{ratio} \geq 0.8$, or if % bleb=0. The eyes were then submitted for histopathological analysis after bleb failure has occurred.

Results: In plain trabeculectomy, the mean bleb survival in terms of IOP were 6.3 and 9.2 days in the BSS and topical bevacizumab groups respectively (ρ=0.25). In mitomycin-C-enhanced trabeculectomy, the mean bleb survival was 16 and 18.2 days respectively (ρ=0.40). In plain trabeculectomy, mean bleb survival in terms of bleb morphology were 8 and 12.2 days for the BSS and bevacizumab groups respectively (ρ=0.08). In enhanced trabeculectomy, mean bleb survival were 19.5 and 20 days respectively (ρ=0.99). Mean vascularity grading were 2 and 1.9 for the BSS groups, and 1.6 and 1.4 for the bevacizumab groups.

Conclusion: Topical bevacizumab as adjunctive therapy after trabeculectomy, whether plain or enhanced with mitomycin-C, showed a trend towards prolonged bleb survival, even though the results of this study were not statistically significant.

Keywords: Bevacizumab, trabeculectomy, filtering bleb, bleb scarring, mitomycin-C.

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Glaucoma is the second leading cause of blindness worldwide.[1] At present, intraocular pressure (IOP) is the only modifiable risk factor in the treatment of glaucoma. It has been found that patients with glaucoma require normal to low IOPs to prevent progression of the disease.[2] Current treatment modalities to lower IOP include medical therapy, laser treatment, and glaucoma filtering surgery (trabeculectomy). Trabeculectomy is the procedure of choice when medical and laser therapy fail to lower IOP to optimum levels. This procedure lowers IOP by creating a fistula in which aqueous flows from the posterior chamber, through a peripheral iridectomy into the anterior chamber, then through a sclerostomy and out into the subconjunctival space, forming a bleb. Survival of this bleb, and thus its IOP-lowering function, is dependent on the wound healing and the degree of postoperative scarring[3].

Ocular wound healing involves overlapping phases of hemostasis, inflammation, angiogenesis, fibroblast migration, and tissue remodeling[4]. Surgical damage to the ocular tissues induces the activation of the clotting and complement systems, as well as stimulating the release of growth factors such as transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF). These in turn stimulate the migration and proliferation of inflammatory cells. The inflammatory cells then activate and stimulate the migration and proliferation of fibroblasts. This is followed by blood vessel endothelial migration and proliferation as modulated by the continued release of VEGF. After resolution of healing, what is left is a fibrous conjunctival scar[5]. Modulating these phases will reduce scarring and fibrosis and prolong bleb survival.

Anti-metabolites, such as mitomycin-C, have been found to reduce postoperative fibrosis and improve the success rate of trabeculectomy by targeting the DNA replication of fibroblasts, inhibiting their proliferation, and thereby limiting scarring.[6,7,8] However, the use of mitomycin-C has been shown to increase susceptibility to long term complications, such as bleb leak, blebitis, and endophthalmitis.[9] It is because of these long term complications that other methods of wound modulation are being developed.

An expanding amount of evidence points to the secreted protein, vascular endothelial growth factor (VEGF), as an important mediator in the process of angiogenesis. VEGF has been found to be a potent stimulator of endothelial cell growth in vitro and neovascularization in vivo.[10,11] Inhibition of VEGF has been shown to prevent the formation, halt progression, and even cause the regression of ocular neovascularization[12].

VEGF is a homodimeric glycoprotein specific for endothelial cells. It has been shown to be a critical regulator of vasculogenesis, angiogenesis, and vascular permeability[13]. An increase in VEGF is associated with proliferation and migration of endothelial cells, and breakdown of basement membrane via activation of metalloproteinase production, which facilitates the invasion of new blood vessels into the surrounding tissue stroma. VEGF also serves as a chemotactic agent for monocytes, with VEGF receptors on all inflammatory cells[12].

Li and associates showed that VEGF levels increased in the aqueous humor of glaucoma patients undergoing trabeculectomy[14]. They found that VEGF could stimulate the growth of human Tenon's capsule fibroblasts and hypothesized that inhibition of VEGF could improve the success rate of filtering surgery.

Bevacizumab (Avastin, Genentech) is a full-length, humanized, murine monoclonal antibody directed against all the biologically active forms of VEGF-A.[15] Bevacizumab has been approved by the FDA for the treatment of colorectal cancer. It is currently being used off-label, for the treatment of neovascular age-related macular degeneration (AMD). The success of bevacizumab in the treatment of retinal and choroidal neovascularization prompted investigators to study its possible applications in anterior segment neovascularization. Topical administration of bevacizumab has been shown to improve corneal neovascularization in several animal studies[15, 16]. Qin et al found that bevacizumab, at concentrations of 5 mg/mL and above, induced a dose-dependent reduction of viable Tenon's fibroblast cells in vitro[17]. Bevacizumab reduced Tenon's fibroblast cell numbers by inducing apoptosis. Rate of reduction was found to be comparable with 5-FU in vitro. The safety of bevacizumab for ocular use has been the subject of several studies. Yoeruek and colleagues investigated the toxicity of bevacizumab on cultures of human corneal cells and concluded that it was nontoxic to the cornea in vitro[18]. It was thus deemed safe for both intravitreal and topical use in humans.

Kahook et al described success in needle bleb revision of encapsulated filtering bleb with bevacizumab. After needling and injection of 1 mg, the bleb was noted to be more diffuse with a decrease in vascularity[19]. Other studies compared the outcomes of bevacizumab-augmented trabeculectomy with 5-FU-augmented trabeculectomy and found comparable mean IOPs[20]. Intracameral bevacizumab during trabeculectomy in patients at high risk of bleb failure showed a mean IOP drop of 11.5 mmHg at 3 months postoperatively[21]. These studies showed the beneficial use of bevacizumab for the treatment not only of retinal and choroidal neovascularization but also of anterior segment neovascularization, specifically wound modulation for a successful trabeculectomy.

This study determined the effect on bleb survival of topically administered bevacizumab, alone or as an adjunct to mitomycin-C, after trabeculectomy in rabbit eyes.

METHODOLOGY

An experimental interventional comparative animal study was conducted using 16 albino rabbits weighing between 1-1.5 kg. Rabbits were then randomly allocated to 4 groups. Group 1 underwent plain trabeculectomy and received topical balanced salt solution (BSS) and served as the negative control. Group 2 underwent plain trabeculectomy and received topical bevacizumab. Groups 3 and 4 underwent mitomycin-C-enhanced trabeculectomy and received topical BSS and bevacizumab, respectively. Group 3 served as the positive control. All rabbits were handled in accordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Baseline IOPs of both eyes of each rabbit were measured with a Medtronic Tonopen (currently Avia Tonopen, Reichert Corp., CA, USA). General anesthesia was induced with an intramuscular injection of ketamine at a dose of 50 mg/kg. Topical 5% betadine and proparacaine were instilled. An 8-0 Vicryl corneal traction suture was placed and a limbal-based conjunctival flap was made. A limbal groove was created with blade #15 and extended to clear cornea, after which a paracentesis was made with a 15-degree stab knife. For eyes undergoing mitomycin-C-enhanced trabeculectomy, a cotton tip saturated with 0.4 mg mitomycin-C was placed underneath the conjunctival flap for 2 minutes and washed with 20 cc BSS. The anterior chamber was entered through the limbal groove with a 15-degree stab knife. A sclerostomy was created using a 1.5 mm scleral punch. Peripheral iridectomy was done, and the chamber reformed with BSS. The conjunctival incision was repaired with running 8-0 Vicryl sutures. BSS was injected through the paracentesis to inflate the bleb. Eight right eyes underwent plain trabeculectomy and 8 left eyes underwent mitomycin-C-enhanced trabeculectomy. Contralateral eyes served as controls for IOP.

Rabbits were randomized to receive either topical BSS or topical 12.5 mg/mL bevacizumab, given four times daily, receiving a total of 3.33 mg daily. Postoperatively, atropine once daily and gatifloxacin 4 times daily were instilled. Topical steroids were not included in the regimen so as not to interfere with the wound modulation effects of the test drug. Observers were masked with regards to treatment assignments.

IOPs of both postoperative and control eyes were measured with a tonopen and recorded. Bleb height, width, and length were measured with a caliper. Digital pictures were also taken. Bleb vascularity was graded as 0 = avascular, 1 = normal (based on vascularity of non-operated contralateral eye), 2 = hyperemic, 3 = very hyperemic (Figure 1). Observations were done on days 1,3,5,7, then every 3 days thereafter until bleb failure was detected or after 21 days postoperatively. To minimize variance between animals due to baseline differences, IOP was recorded as a ratio of the IOP of the experimental postoperative eye divided by the IOP of the contralateral control eye ($IOP_{ratio} = IOP_{postop} / IOP_{control}$) and bleb morphology was recorded as a percentage of the maximum estimated bleb volume (% bleb = estimated bleb volume / maximum bleb volume). Bleb failure was said to have occurred if $IOP_{ratio} \geq 0.8$, or if % bleb = 0.

One randomly selected rabbit from each group was sacrificed at day 21. The eyes were enucleated, preserving the superior conjunctiva and fixed in 10% buffered formaline solution. Sections containing the bleb were processed in paraffin and stained with hematoxylin-eosin. Fibroblast cell counts were done at sites within the bleb areas.

Mean IOP, mean IOP_{ratio}, mean estimated bleb volume, and mean % bleb were plotted as a function of time. Mann-Whitney U test was used to compare

groups across each period of observation. Mean bleb survival times were recorded. One-way analysis of variance (ANOVA) was done to compare rates of bleb failure between treatment groups. Correlation analysis was performed using Pearson's Product Moment.

RESULTS

One rabbit died 1 week postoperatively and was dropped from the analyses. A total of 15 eyes out of 16 were analyzed. Baseline mean IOP ratios and % bleb volume achieved better homogeneity when compared with the mean IOP and bleb volume.

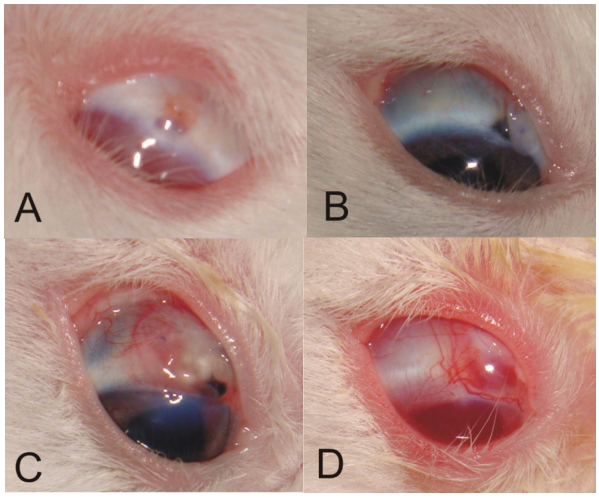

Figure 1. Vascularity grading: (A) 0 = avascular, (B) 1 = normal vascularity, (C) 2 = hyperemic, (D) 3 = very hyperemic.

Table 1. Baseline parameters.

Parameter	BSS (n=3)	Bevacizumab (n=4)	Mitomycin + BSS (n=4)	Mitomycin +Bevacizumab (n=4)	ρ-value
Mean IOP (mmHg)	16.7 ± 1.1	15.5 ± 2.9	14.2 ± 0.5	17.8 ± 0.6	0.08
Mean IOP ratio	0.98 ± 0.03	0.97 ± 0.04	1.0 ± 0.06	1.0 ± 0.0	0.56
Mean estimated bleb volume (mm^3)	18.3 ± 10.7	20.0 ± 11.0	33.0 ± 19.1	15.5 ± 5.2	0.23
Mean % bleb volume	96.7 ± 5.7	100 ± 0.0	94.4 ± 6.4	90.0 ± 11.5	0.32

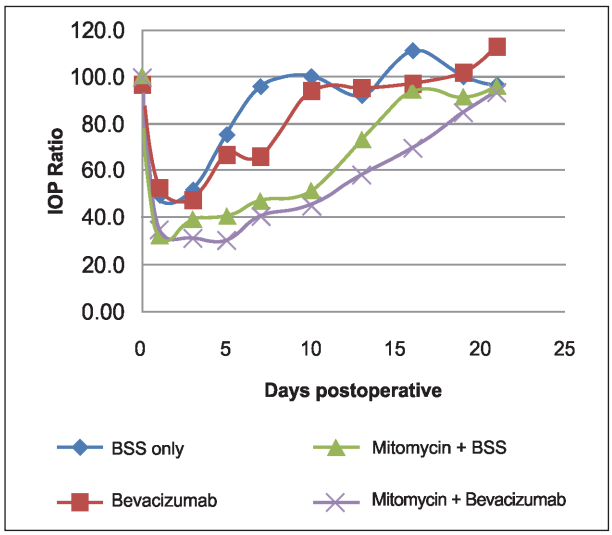

Figure 2. Mean IOP ratio vs. time.

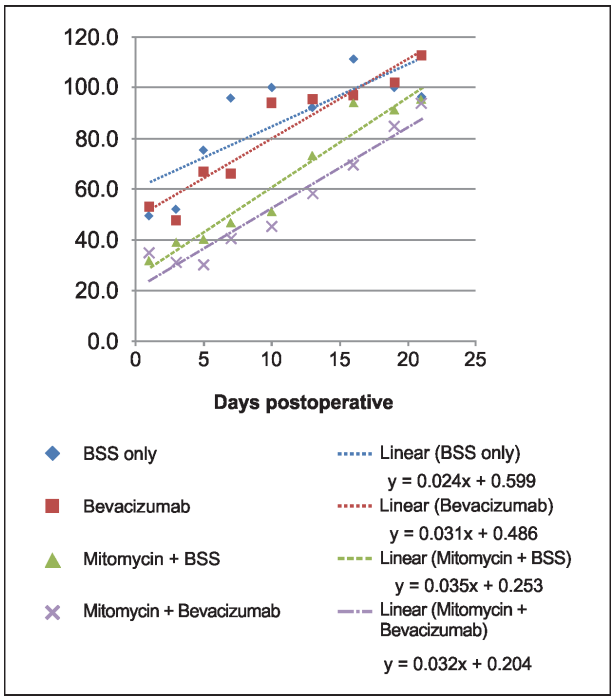

Figure 3. Linear regression plots of mean IOP ratio vs. time.

The mean IOP ratio as a function of time (Figure 2) and the linear regression plots (Figure 3) showed an increase in IOP ratio after baseline. One-way ANOVA of the slopes of the linear regression plots showed no statistical significant differences (ρ=0.64) among the groups.

In eyes that underwent plain trabeculectomy, mean bleb survival in terms of IOP ratio (Table 2) were 6.3 (±1.2) and 9.2 (±1.5) days in the BSS and topical bevacizumab groups (ρ=0.25) respectively. In eyes that underwent mitomycin-C-enhanced

trabeculectomy, mean bleb survival were 16 and 18.2 days (ρ=0.40) respectively. Significant differences were found between the following groups: BSS and mitomycin + BSS (ρ=0.00), BSS and mitomycin + bevacizumab (ρ=0.00), bevacizumab and mitomycin + BSS (ρ=0.002), bevacizumab and mitomycin + bevacizumab (ρ=0.00).

Table 2. Bleb survival time in terms of IOP.

Group	N	Mean bleb survival time (days)
BSS	3	6.3 ± 1.1
Bevacizumab	4	9.2 ± 1.5
Mitomycin + BSS	4	16.0 ± 2.4
Mitomycin + Bevacizumab	4	18.2 ± 1.5

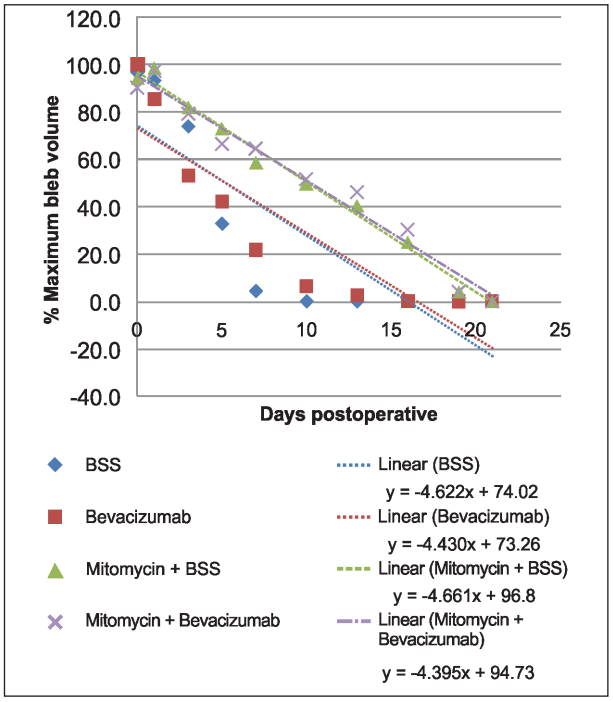

Figure 4. Mean % maximum estimated bleb volume vs time.

Table 3. Bleb survival time in terms of bleb morphology.

Group	N	Mean bleb survival time (days)
BSS	3	8.0 ± 1.7
Bevacizumab	4	12.2 ± 2.9
Mitomycin + BSS	4	19.5 ± 1.0
Mitomycin + Bevacizumab	4	20.0 ± 1.1

The mean maximum extended bleb volume was plotted as a function of time (Figure 4) and the slopes of the linear regression plots showed no statistically significant differences (ρ=0.74).

In eyes that underwent plain trabeculectomy, mean bleb survival in terms of bleb morphology (Table 3) were 8 (±1.7) and 12.2 (±2.9) days for the BSS and bevacizumab groups (ρ=0.08) respectively. In eyes that underwent mitomycin-C-enhanced trabeculectomy, mean bleb survival were 19.5 (±1.0) and 20 (±1.1) days (ρ=0.99) respectively. Significant differences were shown between the following groups: BSS and mitomycin + BSS (ρ=0.00), BSS and mitomycin + bevacizumab (ρ=0.00), bevacizumab and mitomycin + BSS (ρ=0.002), bevacizumab and mitomycin + bevacizumab (ρ=0.001).

There were no significant differences in mean bleb vascularity among the groups (Table 4). However, there was a trend favoring less vascularization for the bevacizumab groups.

The relationships between mean IOP ratio slope, % bleb slope, bleb vascularity, and bleb survival time are shown in Table 5. The only significant correlation was found between bleb survival time based on IOP and survival time based on % bleb ($\rho < 0.01$).

Table 4. Mean bleb vascularity.

Group	N	Vascularity Grading
BSS	3	2.0 ± 0.4
Bevacizumab	4	1.6 ± 0.1
Mitomycin + BSS	4	1.9 ± 0.0
Mitomycin + Bevacizumab	4	1.4 ± 0.1

Table 5. Correlations among IOP ratio, % bleb, vascularity, and bleb survival time.

		Vascularity	IOP ratio slope	% bleb slope	Bleb survival time (based on IOP)	Bleb survival time (based on % bleb)
Vascularity	Pearson correlation	1.0	0.13	-0.39	-0.39	-0.32
	ρ value	0	0.64	0.15	0.15	0.24
	N	15	15	15	15	15
IOP ratio slope	Pearson correlation	0.13	1.0	-0.44	-0.15	0.01
	ρ value	0.64	0	0.11	0.59	0.97
	N	15	15	15	15	15
% bleb slope	Pearson correlation	-0.39	-0.44	1.0	-0.02	-0.03
	ρ value	0.15	0.11	0	0.96	0.91
	N	15	15	15	15	15
Bleb survival time (based on IOP)	Pearson correlation	-0.32	-0.15	-0.02	1.0	0.90*
	ρ value	0.24	0.59	0.96	0	0.0
	N	15	15	15	15	15
Bleb survival time (based on %bleb)	Pearson correlation	-0.32	0.01	-0.03	0.90*	1.0
	ρ value	0.24	0.97	0.91	0.0	0
	N	15	15	15	15	15

*Correlation significant at 0.01 (2-tailed).

Table 6. Fibroblast counts.

Group	Fibroblast Count (per 2 high power fields)
BSS	92
Bevacizumab	61
Mitomycin + BSS	11
Mitomycin + Bevacizumab	5

Fibroblast counts were taken per two-high power field of a section taken at the bleb site. Mitomycin with bevacizumab had the lowest fibroblast counts (Table 6).

DISCUSSION

Wound healing occurs in several overlapping phases.[4] After tissue trauma, the wound healing process begins via increased vascular permeability, which gives blood elements such as fibroblasts, inflammatory cells, cytokines, and macrophages, access to the wound. This is followed by fibroblastic proliferation. These fibroblasts then produce collagen which serves as a scaffold for angiogenesis. New vessel formation, as facilitated by VEGF, starts as capillary buds which extend into the collagen matrix. These new vessels provide nutrients for the fibroblasts, which produce more collagen. The end result is scarring.

Targeting more than one phase in the wound healing process would theoretically maximize efficacy of therapy. Mitomycin-C inhibits the DNA replication of the fibroblast cells, targeting fibroblast migration and scar formation. Bevacizumab targets the angiogenesis and chemotactic properties of VEGF during wound healing. This two-prong approach is designed to attack the inflammatory process at two separate but co-dependent sites: fibroblast proliferation and angiogenesis.

Our results showed a trend towards lower IOPs, larger blebs, less vascularity, and lower fibroblast counts in eyes treated with topical bevacizumab as compared with those treated with BSS. Mean bleb survival time was also longer in the bevacizumab groups. However, when compared with the respective negative (plain trabeculectomy + BSS) and positive controls (Mitomycin-C-enhanced trabeculectomy + BSS), the differences were not statistically significant. Statistical significance was noted between eyes that underwent enhanced trabectulectomy and those that underwent plain trabeculectomy, consistent with results of other studies on mitomycin-C. From this, we can infer that better bleb survival rates could be obtained using topical bevacizumab as an adjunct to mitomycin-C-enhanced trabeculectomy as opposed to plain trabeculectomy alone.

Correlational analyses in this study suggested that increased vascularity would result in lower bleb survival rates, consistent with other studies. Although not statistically significant, there was a trend toward positive correlation between vascularity and rate of postoperative rise in IOP, as well as a negative correlation with postoperative decrease in bleb size.

In summary, topical application of bevacizumab has been shown to decrease bleb vascularization in rabbit eyes. This trend implied that topical bevacizumab may have a contributory role in wound modulation after trabeculectomy. Larger sample sizes, higher dose or more frequent application, or a different route of administration such as subconjunctival or intracameral injection, may yield better statistical results.

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