Intravitreal Bevacizumab as Adjunctive Therapy for Bleb Survival in Trabeculectomy in Rabbit Eyes

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ABSTRACT

Objective: To determine the effect of intravitreally administered bevacizumab, alone or as adjunct to mitomycin-C, after trabeculectomy on bleb survival and histology in rabbit eyes.

Methods: An experimental, interventional, comparative, animal study consisting of 16 rabbit eyes underwent trabeculectomy, 8 of which were enhanced with intra-operative mitomycin-C. Eyes were randomized to receive intravitreal bevacizumab at a concentration of 12.5 mg/mL. Intraocular pressure (IOP), bleb dimensions, and vascularity grading were obtained. IOP was recorded as a ratio of the 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) over time. Bleb failure occurred if $IOP_{ratio} \geq 0.8$, or if % Bleb =0. Eyes were enucleated and submitted for histopathological analysis.

Results: In terms of IOP, mean bleb survival of the plain trabeculectomy group was 7.00(±0.00) days compared to 11.00 (±1.00) days in the intravitreal bevacizumab group (p=0.02). In mitomycin-C-enhanced trabeculectomy eyes, the mean bleb survival was 15.25 (±0.75) days compared to 19.00 days in the intravitreal bevacizumab group (p=0.002). In terms of bleb morphology, bleb survival were 9 (±1.00) and 13 (±0.00) days for the plain trabeculectomy and intravitreal bevacizumab groups respectively (p=0.02); and 18.25 (±0.75) and 20.00 (±0.58) days for the trabeculectomy with mitomycin and intravitreal bevacizumab groups respectively (p=0.11). Mean vascularity grading were 1.67 (± 0.33) and 1.33 (± 0.33) for the plain trabeculectomy and bevacizumab groups and 1.50 (±0.59) and 1.25 (±0.25) for the mitomycin and bevacizumab groups respectively (p=0.72). Histologic analysis showed less fibroblast count for eyes treated with bevacizumab.

Conclusion: Intraoperative intravitreal bevacizumab as adjunctive therapy after trabeculectomy, whether plain or enhanced with mitomycin-C, was associated with improved bleb survival rates in the rabbit model.

Keywords: Trabeculectomy, Wound healing, Vascular endothelial growth factor, Bevacizumab, Bleb failure

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Trabeculectomy is a commonly performed glaucoma procedure when medications and laser surgery fail to adequately control the intraocular pressure. The basic goal behind trabeculectomy is to create a small hole, or fistula, in the anterior chamber of the eye to allow extraocular subconjunctival drainage of the aqueous fluid, hence lowering intraocular pressure. As the fluid flows through the new drainage opening, the tenon's and conjunctiva over the sclerostomy rises to form a little blister or bubble, called a bleb.[1-3]

The success of surgical intervention in glaucoma primarily depends on the functional integrity of the filtering bleb. Filtration or bleb failure due to excessive postoperative scarring or fibroplasia remains the major problem after glaucoma surgery.[3] Surgical damage to ocular tissues causes activation of clotting and complement systems and consequently the release of endothelial growth factors is initiated. Proliferation and migration of inflammatory cells and fibroblasts promote formation of a conjunctival scar.[4,5] Several interventions have, therefore, been studied to lessen the degree of postoperative scarring after trabeculectomy, thus enhancing the functional integrity and prolonging the survival of the filtering bleb.

The introduction of mitomycin C (MMC) and 5-fluorouracil (5-FU) as adjuncts at the time of surgery or in the early postoperative phase represented major steps towards overcoming the natural healing process. Unfortunately, the improved success at lowering the intraocular pressure (IOP) achieved with MMC and 5-FU came at the expense of increased complications, such as ischemic blebs, leaking blebs, and higher rates of endophthalmitis.[3,4,6] At present, approximately 10-15% of trabeculectomies fail in the first few months due to excessive conjunctival scarring even with use of adjunctive therapies like MMC and 5-FU.[4,7]

Alternatives to these antimetabolite agents that may prove superior or able to supplant these agents in glaucoma procedures have been explored. Recent studies showed that vascular endothelial growth factor (VEGF) plays a role in the formation of conjunctival scarring post-trabeculectomy. VEGF is a hypoxia-inducible cytokine intimately involved in the formation of vessels throughout the body. It is also involved in inflammation and cellular proliferation.[8]

The class of drugs that inhibits VEGF, known as anti-VEGF drugs, includes ranibizumab and its parent molecule, bevacizumab. Ranibizumab is the anti-VEGF developed for ocular use in the treatment of wet age-related macular degeneration. However, its prohibitive cost had ophthalmologists trying out its cheaper parent drug, bevacizumab, with numerous studies reflecting similar results between the two drugs. Currently, the National Eye Institute in the United States is conducting a definitive trial on the equivalence of these two drugs.[9]

Bevacizumab was originally used as part of the chemotherapeutic regimen for metastatic cancers of the colon and rectum. It is given intravenously in doses as high as 10 milligrams per kilogram of body weight every 14 days. This dose takes an average of 20 days to be cleared from the body. Off-label, it is injected into the vitreous cavity in doses ranging from 1.25 mg to 2.5 mg. This route of administration has been reported to have beneficial effects in ischemic disease entities like wet age-related macular degeneration, neovascular glaucoma, proliferative diabetic retinopathy, and central retinal vein obstruction without significant intraocular toxicity.[9, 10-18]

The rising popularity of the off-label use of bevacizumab for the eye has prompted studies into its ocular pharmacokinetics. *Nomoto* investigated the pharmacokinetics of bevacizumab in rabbits for three different routes of administration and found that the most effective route in rabbit ocular tissue was the intravitreal route, compared to subconjunctival or topical, by measuring concentrations in the ocular tissues using enzyme-linked immunosorbent assay. Injection into the vitreous cavity has the drug staying longer in the eye (half-life of 4.32 days, maintained in the vitreous for 30 days) compared to injection into the anterior chamber or subconjunctival space (peak at 3 days post-injection).[19]

Using the drug topically as adjunct to trabeculectomy, *Tripon* showed that bleb survival time was higher compared to trabeculectomy with mitomycin-C alone in rabbits (unpublished data). *Memarzadeh* showed that subconjunctival bevacizumab resulted in better survival time in trabeculectomy in rabbits.[20] The use of intravitreal bevacizumab as adjunct to trabeculectomy, however, has not yet been tested. It has been found that intraocular tissue concentrations are ten times higher with delivery into the vitreous cavity compared to subconjunctival or topical delivery.[19] These findings indicate the superiority of the vitreous as a depot for the drug should we need to use it as an adjunct to aid in bleb formation and remodelling.

To date, case series and clinical trials have already been conducted using bevacizumab as adjunct to trabeculectomy. Several studies[21-22] demonstrated that subconjunctival bevacizumab had better outcomes when used as an adjunct to trabeculectomy for glaucoma. In neovascular glaucoma, intravitreal bevacizumab resulted in decrease in neovascularization and lowering of IOP but not in trabeculectomy outcomes,[15-18] while others showed improved short term trabeculectomy results.[13-14] In primary glaucoma, the combination of intravitreal ranibizumab and topical MMC at the time of trabeculectomy resulted in more diffuse blebs with less vascularity when compared to use of topical MMC alone. With the lack of studies demonstrating long term safety of intravitreal bevacizumab for non-neovascular diseases, an animal study was chosen prior to a pilot study on humans for intravitreal bevacizumab in trabeculectomy for primary glaucoma.

This study determined the effect on bleb survival and histology of intravitreally injected bevacizumab, alone or as adjunct to MMC, after trabeculectomy in rabbit eyes.

METHODOLOGY

An experimental, interventional, comparative, animal study was conducted using 16 rabbits weighing between 1-1.2 kilograms. Rabbits were randomly allocated to 4 groups: Group A underwent plain trabeculectomy and served as the negative control; Group B underwent plain trabeculectomy and received intravitreal bevacizumab at a concentration of 1.25 mg/0.05 mL (bevacizumab group); Group C underwent MMC-enhanced trabeculectomy. and served as the positive control; Group D underwent MMC-enhanced trabeculectomy and received intravitreal bevacizumab at a concentration of 1.25 mg/0.05 mL. All rabbits were handled in accordance with the ARVO (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 Perkins' tonometer. Three measurements were taken per eye by a single observer. General anesthesia was induced with an intramuscular injection of ketamine at a dose of 50 mg/kg. Standard limbal-based trabeculectomy was performed by a single surgeon. Topical 5% betadine and proparacaine were instilled. A partial thickness 8-0 vicryl suture was passed through the superior peripheral cornea and the eye was infraducted. A limbal-based conjuctival flap was created and gently dissected up to the limbus. A limbal groove was created with blade #15 and extended to clear cornea. A paracentesis was done with a 15 degree stab knife. For eyes undergoing MMC-enhanced trabeculectomy, a cotton ball saturated with 4% MMC was placed underneath the conjuctival flap for 2 minutes, then washed with 20cc balanced salt solution (BSS). The anterior chamber was entered through the limbal groove with a 15 degree stab knife. A limbal sclerostomy was created using a 1.5mm scleral punch. Peripheral iridectomy was done and the chamber reformed with BSS. The sclera flap was sutured with nylon 10-O and the conjunctival incision was repaired with running 10-O nylon sutures. BSS was injected through the paracentesis to test patency of the sclerostomy and inflate the bleb. For the bevacizumab groups, 0.05 mL of bevacizumab was injected inferotemporally around 9 o'clock, 3mm from the limbus, before removing the retractors. 16 left eyes underwent plain trabeculectomy and MMC-enhanced trabeculectomy. Contralateral right eyes served as controls for IOP.

All postoperative eyes received atropine once daily, moxifloxacin 4 times daily, and prednisolone acetate 4 times daily. Observers were masked with regards to treatment assignments.

A single masked observer measured IOPs of both post operative and control eyes with a Perkins' tonometer. Bleb height, width, and length were measured with a caliper. Three measurements were taken for each eye. Digital pictures were also obtained. Bleb vascularity was graded using the Moorfields Bleb Vascularity Grading System (Figure 1). Observations were done on days 1, 3, 5, 7, then every 3 days thereafter until bleb failure detected or after 21 days postoperatively. To minimize variance between animals due to baseline differences, intraocular pressure (IOP) was recorded as a ratio of the IOP of the experimental postoperative (postop) eye divided by the IOP of the contralateral control eye ($IOP_{ratio} = IOP_{postop} / IOP_{control}$) and bleb morphology (% bleb = estimated bleb volume/maximum bleb volume; where bleb volume = length x width x height) was recorded as a percentage of the estimated bleb volume (taken during follow-up examinations) and maximum bleb volume (bleb volume immediately postop). Bleb failure occurred if IOP_{ratio} was ≥ 0.8, or if % bleb =0.

One randomly selected rabbit from each group was sacrificed at day 21. The eyes were enucleated,

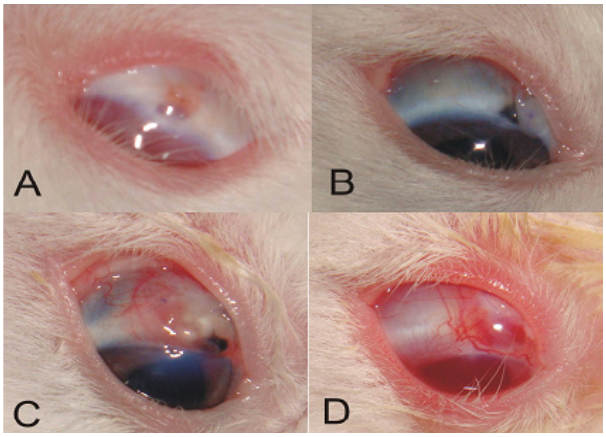

Figure 1. Moorfields Bleb Vascularity Grading: A, 0 = Avascular; B, 1 = Normal vascularity (vascularity of non-operated contralateral eye as reference); C, 2 = Hyperemic; D, 3= Very hyperemic.

preserving the superior conjunctiva, and fixed in 10% buffered formaldehyde solution. Sections containing the bleb were dissected and processed in paraffin and stained with hematoxylin-eosin and PAS stains. Fibroblast cell counts were done at sites within the bleb areas by a masked observer (expert pathologist).

Mean IOP, mean IOP_{ratio}, mean estimated bleb volume, and mean percent (%) bleb volume were plotted as a function of time. Mean bleb survival time in days were recorded. One-way analysis of variance was done to compare rates of bleb failure between treatment groups.

RESULTS

Two rabbits (1 from the plain trabeculectomy and 1 from the trabeculectomy + bevacizumab groups) died within 1 week postoperatively and were excluded from the analyses. A total of 14 eyes out of 16 were analyzed. Baseline parameters (mean IOP, mean IOP_{ratio}, mean estimated bleb volume, and % bleb volume) were similar in the 4 groups (Table 1).

Mean IOP ratio plotted as a function of time showed an increasing pattern for all groups, with the plain trabeculectomy group failing at day 5 and the combined mitomycin and bevacizumab at day 13. Linear regression plots of the increase in IOP ratio after baseline (Figure 3) and one-way ANOVA of the slopes showed statistical significance (p=0.00).

Table 1. Baseline Parameters.

Parameter	Plain Trabeculectomy (N=3)	Trabeculectomy + Bevacizumab (N=3)	Trabeculectomy + Mitomycin (N=4)	Trabeculectomy + Mitomycin + Bevacizumab (N=4)	p-value
Mean IOP (mmHg)	9.89 ± 0.26	9.78 ± 0.22	9.75 ± 0.18	9.75 ± 0.18	0.96
Mean IOP Ratio	1.01 ± 0.02	1.01 ± 0.02	1.01 ± 0.03	0.98 ± 0.02	0.66
Mean Estimated Bleb Volume (mm^3)	25.00 ± 0.00	25.00 ± 0.00	26.88 ± 1.02	26.25 ± 1.25	0.50
Mean % Bleb Volume	83.33 ± 3.33	80.00 ± 0.00	85.50 ± 2.63	82.50 ± 2.50	0.53

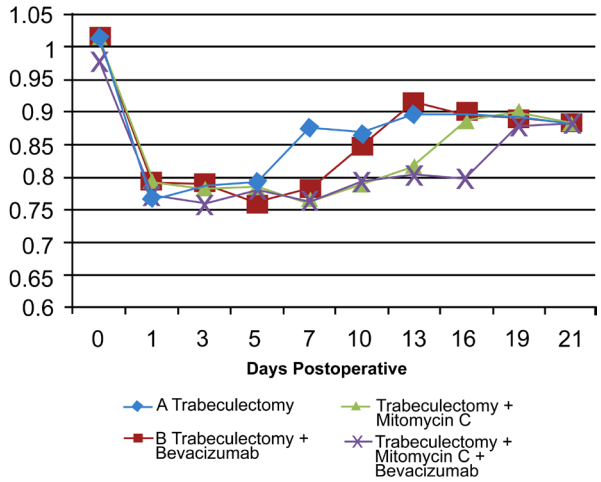

Figure 2. Mean IOP ratio vs. time in the 4 groups.

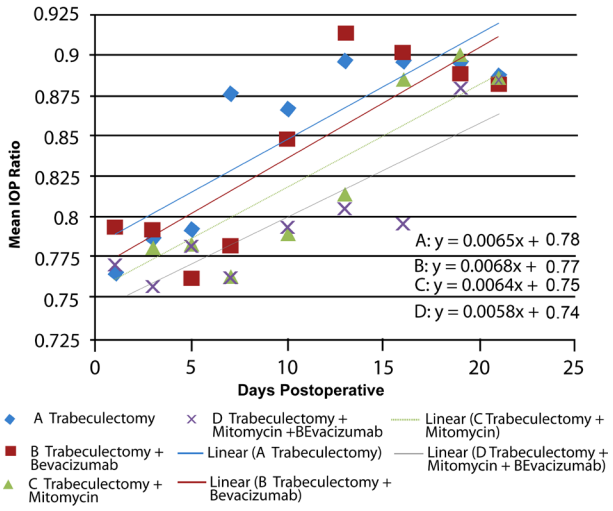

Figure 3. Linear regression of mean IOP ratio vs. time in the 4 groups.

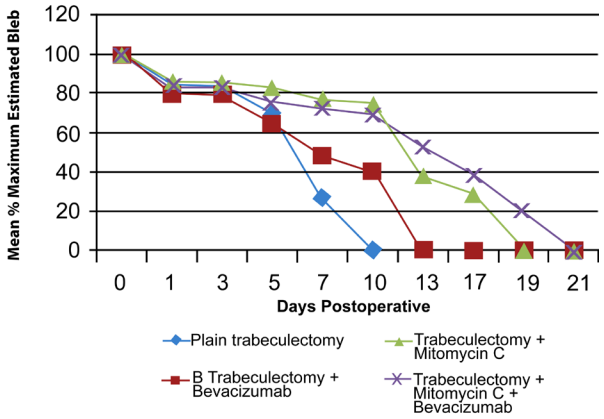

Figure 4. Mean percent maximum estimated bleb vs. time in the 4 groups.

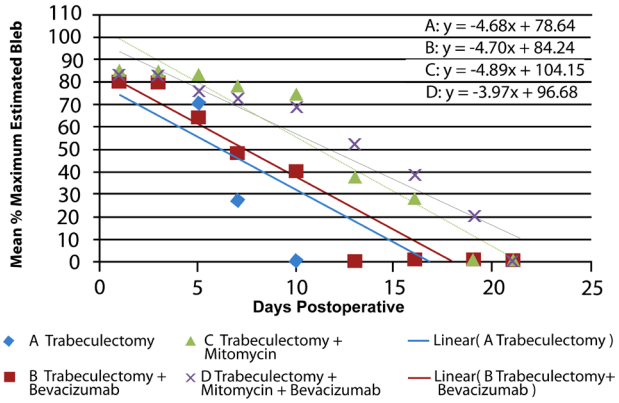

Figure 5. Linear regression plots of mean % maximum estimated bleb vs. time in the 4 groups.

Mean % maximum bleb plotted as a function of time (Figure 4) showed a decreasing pattern for all groups, with the plain trabeculectomy group failing at day 10 and the combined mitomycin and bevacizumab at day 21. Linear regression plots of the decrease in mean maximum bleb volume after baseline (Figure 5) and one-way ANOVA of the slopes showed statistical significance (p=0.00).

In terms of IOP ratio, the mean bleb survival of the plain trabeculectomy group was 7.00 days compared to 11.00 days in the intravitreal bevacizumab group (p=0.02) (Table 2). The mean bleb survival of the plain trabeculectomy + mitomycin group was 15.25 days compared to 19.00 days in the intravitreal bevacizumab group (p=0.002) (Table 2). Significant differences were found between the following groups: plain trabeculectomy and trabeculectomy + mitomycin (p=0.00), plain trabeculectomy and trabeculectomy + mitomycin + bevacizumab (p=0.00), trabeculectomy + bevacizumab and trabeculectomy + mitomycin (p=0.02), and trabeculectomy + bevacizumab and trabeculectomy + mitomycin + bevacizumab (p=0.00).

Table 2. Mean bleb survival time and vascularity grading in the 4 groups.

Group	Mean Bleb Survival time (days) by IOP	Mean Bleb Survival time (days) by Bleb Morphology	Vascularity Grading
Plain (3)	7.00 ± 0.00	9.00 ± 1.00	1.67 ± 0.33
+ Bevacizumab (3)	11.00 ± 1.00	13.00 ± 0.00	1.33 ± 0.33
+ Mitomycin (4)	15.25 ± 0.75	18.25 ± 0.75	1.5 ± 0.29
+ Mitomycin + Bevacizumab (4)	19.00 ± 0.00	20.00 ± 0.58	1.25 ± 0.25

In terms of bleb morphology, that mean bleb survival of the plain trabeculectomy group was 9.00 days compared to 13.00 days in the intravitreal bevacizumab group (p=0.02) (Table 2). The mean bleb survival of the plain trabeculectomy + mitomycin group was 18.25 days and 20.00 days in the intravitreal bevacizumab group (p=0.11) (Table 2). Significant differences were found between the following groups: plain trabeculectomy and trabeculectomy + mitomycin (p=0.00), plain trabeculectomy and trabeculectomy + mitomycin + bevacizumab (p=0.00), trabeculectomy + bevacizumab and trabeculectomy + mitomycin (p=0.002), and trabeculectomy + bevacizumab and trabeculectomy + mitomycin + bevacizumab (p=0.00).

There were no significant differences in the mean bleb vascularity scores among the groups (Table 2). There was, however, less vascularization for the bevacizumab groups (Figure 6).

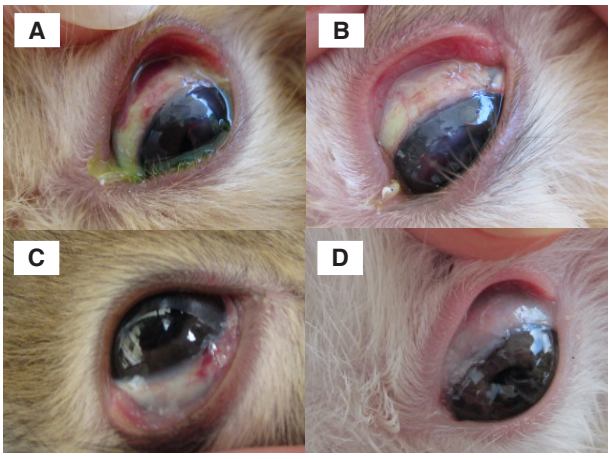

Figure 6. Bleb vascularity in the 4 groups.

A = Plain trabeculectomy
B = Trabeculectomy + Bevacizumab
C= Trabeculectomy + Mitomycin
D= Trabeculectomy + Mitomycin + Bevacvizumab

Table 3. Fibroblast Counts in the 4 groups.

Group (Trabeculectomy)	Fibroblast Count (per 2 high power fields)
Plain	24
+ Bevacizumab	18
+ Mitomycin	13
+ Mitomycin + Bevacizumab	5

Fibroblast counts were taken per two high power fields of a section taken at the bleb site showing less fibroblast counts for the bevacizumab groups (Table 3).

DISCUSSION

Glaucoma filtration surgery remains the definitive surgical management for glaucoma uncontrolled by medical management. The success of glaucoma filtration surgery is dependent on the degree of postoperative wound healing and the amount of scar tissue formation. Wound healing occurs in several phases, beginning with increased vascular permeability after tissue trauma, providing access of fibroblasts and inflammatory cells to the wound. Bleb failure involves vascularization and occurs as fibroblasts proliferate and migrate toward the wound, eventually causing scarring and closure of the fistula tract.[3,4,5]

Mitomycin-C (MMC) and 5-fluorouracil (5-FU), which inhibit the DNA replication of the fibroblast cells, used as adjuncts at the time of surgery or in the early postoperative phase represented major steps towards overcoming the natural healing process. However, they have also been associated with postoperative complications, such as wound leaks and ischemic blebs leading to bleb infection and a 10-15% failure rate. Hence, other agents have been studied.

Vascular endothelial growth factor (VEGF) is a unique mitogen specific to vascular endothelial cells. It is a powerful inducer of angiogenesis and the key growth factor and mediator in the signal transduction for wound healing. VEGF not only has a role in angiogenesis, but also has a direct action on fibroblast activity that may be positively modified directly at the time of filtration surgery.[21-23] Since angiogenesis forms an integral part of wound healing which is an unwanted process in the postoperative period after glaucoma filtering surgery, bevacizumab as antibody against vascular endothelial growth factor can be considered for use in combination with conventional filtering surgery or as adjunctive administration to antimetabolites to help reduce the rate of bleb failure.[23-24]

In this study, eyes treated with intravitreal bevacizumab had lower IOPs, higher bleb volume, longer bleb survival rates, lower bleb vascularity, and lower fibroblast counts. In terms of bleb survival rates by IOP, the treatment groups were statistically different to their respective negative controls (plain trabeculectomy and positive controls (trabeculectomy + mitomycin). In terms of mean bleb survival rates by % bleb volume, the treatment groups were statistically different compared to the negative controls, but insignificant when compared to the positive controls.

Comparing both treatment groups (trabeculectomy + bevacizumab vs. trabeculectomy + mitomycin + bevacizumab) and both control groups (trabeculectomy vs. trabeculectomy + mitomycin), they were statistically different. The findings were also seen in the study by Tripon (unpublished data), and was attributed to bevacizumab and mitomycin targeting different phases in the wound healing process, maximizing efficacy of therapy by targeting both fibroblast proliferation by mitomycin and angiogenesis by bevacizumab.

The primary limitation of this study was the limited number of rabbits per treatment group. A larger sample size may produce more convincing results. The optimum time to inject bevacizumab (whether a few days pre-op, intra-op, or a few days or weeks post op) may also be studied.

In summary, we have shown that intraoperative intravitreal injection of bevacizumab alone after trabeculectomy was associated with longer bleb survival in the rabbit model, and even more so when used as an adjunct to mitomycin-enhanced trabeculectomy. Bevacizumab is a useful agent for improving success rates in trabeculectomy in rabbits within the timeframe described.

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