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Evaluation of Verteporfin as a Novel Antifibrotic Agent in a Rabbit Model of Glaucoma Filtration Surgery

A Pilot Study

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Purpose: Verteporfin is a benzoporphyrin derivative which is Food and Drug Administration-approved for treatment of choroidal neovascularization in conjunction with photodynamic therapy. It has been shown to prevent fibrosis and scar formation in several organs and represents a promising novel antifibrotic agent for glaucoma surgery. The goal of this study is to determine the effect of verteporfin on wound healing after glaucoma filtration surgery.

Design: Preclinical study using a rabbit model of glaucoma filtration surgery.

Subjects: Eight New Zealand white rabbits underwent glaucoma filtration surgery in both eyes.

Methods: Eyes were randomized into 4 study groups to receive a postoperative subconjunctival injection of 1 mg/mL verteporfin (n = 4), 0.4 mg/mL mitomycin C (MMC; n = 4), 0.4 mg/mL MMC + 1 mg/mL verteporfin (n = 4), or balanced salt solution (BSS) control (n = 4). Bleb survival, vascularity, and morphology were graded using a standard scale over a 30-day period, and intraocular pressure (IOP) was monitored. At 30 days postoperative or surgical failure, histology was performed to evaluate for inflammation, local toxicity, and scarring.

Main Outcome Measures: The primary outcome measure was bleb survival. Secondary outcome measures were IOP, bleb morphology, and bleb histology.

Results: Compared to BSS control blebs, verteporfin-treated blebs demonstrated a trend toward increased surgical survival (mean 9.8 vs. 7.3 days, log rank $P = 0.08$). Mitomycin C-treated blebs survived significantly longer than verteporfin-treated blebs (log rank $P = 0.009$), with all but 1 MMC-treated bleb still surviving at postoperative day 30. There were no significant differences in survival between blebs treated with combination verteporfin + MMC and MMC alone. Mitomycin C-treated blebs were less vascular than verteporfin-treated blebs (mean vascularity score 0.3 ± 0.5 for MMC vs. 1.0 ± 0.0 for verteporfin, $P < 0.01$). Bleb histology did not reveal any significant toxicity in verteporfin-treated eyes. There were no significant differences in inflammation or scarring across groups.

Conclusions: Although verteporfin remained inferior to MMC with regard to surgical survival, there was a trend toward increased survival compared with BSS control and it had an excellent safety profile. Further studies with variations in verteporfin dosage and/or application frequency are needed to assess whether this may be a useful adjunct to glaucoma surgery.

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Glaucoma is the leading cause of irreversible blindness worldwide, with reduction of intraocular pressure (IOP) the only known modifiable factor proven to slow disease progression.[1] Intraocular pressure reduction can be achieved using medication, laser, or glaucoma surgery. Bleb-forming glaucoma surgeries, which include both traditional trabeculectomy and newer minimally invasive glaucoma surgeries such as XEN Gel Stent (Abbvie, Inc) and Preserflo MicroShunt (Santen) lower IOP by creating an accessory aqueous outflow channel to the subconjunctival space. Surgical success depends upon postoperative wound healing and scar modulation. Excessive postoperative scarring of the conjunctiva and the outflow channel results in bleb failure and poor IOP control.[2,3] Mitomycin C (MMC) remains the most commonly utilized antifibrotic agent for bleb-forming glaucoma procedures.[4–6] Mitomycin C is an alkylating agent that causes apoptosis of conjunctival fibroblasts responsible for scar formation.[7] Although MMC is effective at improving success rates, surgical failure can still approach 50% at 5 years

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postoperatively.[8–10] Moreover, because MMC is cytotoxic, its use is associated with numerous sight-threatening postoperative complications including hypotony, bleb leak, and endophthalmitis.[11–13] Novel safe and effective antifibrotic agents are needed to improve surgical outcomes for bleb-forming glaucoma procedures.

Verteporfin, a benzoporphyrin derivative, has been shown to prevent fibrosis in several organs.[14–18] A recent study in mice skin wounds found that verteporfin injection prevented scar formation and promoted regeneration of healthy tissue architecture by inhibiting the yes-associated protein, which plays a role in inducing transformation of fibroblasts into a profibrotic phenotype.[19] Within ophthalmology, verteporfin (trade name Visudyne—formulated as liposomal verteporfin, Bausch and Lomb) is already Food and Drug Administration-approved as an intravenous injection for choroidal neovascularization when used in conjunction with photodynamic therapy.[20] Although this is unrelated to the mechanism of action by which verteporfin prevents scarring, its established safety profile and use in ophthalmology makes it a promising candidate as a novel antifibrotic agent for glaucoma surgery. We therefore aimed to determine the effect of verteporfin on wound healing after glaucoma filtration surgery in a rabbit model of glaucoma filtration surgery, and compared the antiscarring effect of verteporfin against that of MMC and a combination therapy of verteporfin and MMC. This pilot study provides new insights into the potential and limitations of using verteporfin as an antifibrotic agent for ophthalmology.

Methods

Study Design and Surgery

All animal research was conducted in compliance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research and was approved by the Institutional Animal Care and Use Committee at Stanford University. Eight female New Zealand White rabbits 10 to 12 weeks old weighing 1.8 to 2.2 kg underwent glaucoma filtration surgery performed by a single glaucoma surgeon (W.W.L.) using a previously established model that creates a bleb and uses a tube shunt sclerostomy.[21,22] After anesthesia with 0.25 mg/kg midazolam intramuscularly (IM), 5 to 10 mg/kg ketamine IM, 0.025 to 0.05 mg/kg dexmedetomidine IM, and 0.03 mg/kg buprenorphine IM, a superior fornix-based conjunctival dissection was completed and a 25-gauge intravenous catheter was inserted into the anterior chamber approximately 1 mm posterior to the limbus. The distal end of the tube crossed the pupillary margin to avoid tube-iris capture. The tube was secured to the sclera with 10-0 nylon suture, and the conjunctiva was closed with 10-0 nylon suture. Both eyes of each rabbit underwent filtration surgery. At the end of the surgery, eyes of each rabbit received a subconjunctival injection adjacent to the bleb. Eyes (independent of animal) were randomized into 4 groups for treatment with (1) balanced salt solution (BSS) control (n = 4), (2) 1 mg/mL verteporfin (n = 4), (3) 0.4 mg/mL MMC (Accord Healthcare) (n = 4), or (4) a combination therapy of 0.4 mg/mL MMC and 1 mg/mL verteporfin (n = 4). Four eyes were included in each group based on similar numbers in previous studies.[23] Eyes received a 0.1 mL injection, except for eyes in group (4), which received 0.2 mL total. Visudyne (Bausch and Lomb) was used for verteporfin injections. Dosage for verteporfin in Visudyne was determined from preliminary studies which had demonstrated no evidence of toxicity (such as corneal edema, corneal epithelial defects, anterior chamber inflammation, and cataracts) with both low (0.1 mg/mL) and high dose (1 mg/mL) adjunctive topical verteporfin in the same rabbit model of glaucoma filtration surgery. Sedation was reversed with atipamezole IM (volume of dexmedetomidine used in 1:1 ratio) and if needed, 0.01 mg/kg flumazenil IM. Postoperatively, eyes received 0.5% moxifloxacin eye drops daily for 1 week and 1% prednisolone eye drops daily for 3 weeks.

Postoperative Evaluation

Bleb morphology, vascularity and survival were graded twice-weekly over 30 days postoperative. Bleb height and vascularity were scored on a 0 to 3 scale using the Indiana Bleb Appearance Grading Scale.[24] Bleb survival was defined as the presence of an elevated subconjunctival fluid pocket at the surgical site. Anterior segment photographs were obtained during these assessments. Two independent investigators (M.T.S. and R.M.C.) objectively graded each bleb for survival, morphology, and vascularity based on clinical exam and anterior segment photography. Intraocular pressure was checked using an iCare tonometer (TONOVET Plus, iCare Finland) at baseline, and at each postoperative visit. A mean reading of 3 IOP recordings was documented per time point. The primary outcome measure was bleb survival. Secondary outcome measures were IOP, bleb morphology, and bleb histology.

Histology

At surgical failure or 30 days postoperative, whichever occurred later for the longer-surviving bleb in each animal, animals were euthanized and underwent enucleation. Eyes were immediately fixed in 10% formalin, dehydrated and embedded in paraffin for microtome sectioning. Sections representing the area of the bleb were stained with hematoxylin and eosin to evaluate for degree of inflammation based on the presence of inflammatory cells, and tissue morphology surrounding the glaucoma surgical site. Masson's trichrome staining was used to assess for collagen deposition. Inflammation and collagen deposition were graded on a 0 to 3 scale in a masked fashion by an ophthalmic pathologist (J.H.L.). Approximately 8 to 10 sections were analyzed per eye.

Statistics

Descriptive statistics were performed using mean and standard deviation for continuous measures. Surgical failure according to treatment groups were compared graphically using Kaplan-Meier methods and statistically using log-rank tests. Linear mixed effect models with Tukey's multiple comparisons tests were used to analyze bleb morphology and IOP across different time points. Final bleb vascularity was determined at surgical failure or 30 days postoperative, whichever occurred later. Differences in final bleb vascularity and histopathological parameters across treatment groups were compared using analysis of variance with Sidak's multiple comparisons test. Statistical analyses were conducted using Stata version 16 and Prism. P values < 0.05 were considered statistically significant.

Results

Animal Model

All 16 eyes from 8 rabbits underwent uncomplicated glaucoma filtration surgery. All eyes had transient hyphema postoperatively which resolved by the first week

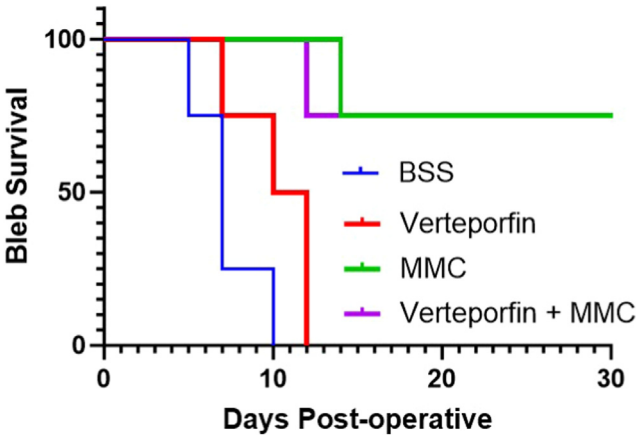

Figure 1. Kaplan Meier curves demonstrating bleb survival across treatment groups. Eyes were treated with balanced salt solution (BSS; n = 4), 1 mg/mL verteporfin (n = 4), 0.4 mg/mL mitomycin C (MMC; n = 4), or a combination therapy of 1 mg/mL verteporfin and 0.4 mg/mL MMC (n = 4). The verteporfin group trended toward increased bleb survival compared with the BSS group ($P = 0.08$). The MMC group survived longer than the verteporfin group ($P = 0.009$).

postoperative. One eye had transient corneal edema due to inadvertent tube contact with the endothelium intraoperatively which resolved by 1 week postoperative without additional intervention. No treatment eyes demonstrated any adverse reaction to topical verteporfin or MMC, such as anterior chamber inflammation, corneal decompensation, or cataract formation.

Bleb Survival

Kaplan-Meier survival curves are depicted in Figure 1. Compared to the BSS group, the verteporfin group demonstrated a trend toward increased surgical survival (log rank $P = 0.08$). Mean survival was 9.8 days for verteporfin group and 7.3 days for BSS control group. All blebs in the verteporfin and BSS groups failed within 12 days of surgery. In contrast, most blebs in the MMC group and the verteporfin + MMC group survived until postoperative day 30. The mean survival in the MMC group and the verteporfin + MMC group was 25.5 days. Compared to the verteporfin group, the MMC group had significantly longer survival (log rank $P = 0.009$). There were no significant differences in survival between verteporfin + MMC and MMC alone groups (log rank $P = 0.9$).

Bleb Morphology and Vascularity

Bleb height, extent, and morphology are important indicators for bleb function, as subconjunctival scarring can cause bleb flattening. Figure 2 demonstrates the typical appearances of the blebs in each group at postoperative week 1. We found that bleb height and extent scores were identical throughout the treatment period. Verteporfin-treated blebs were lower, fleshy-looking and diffuse, while MMC-treated blebs were larger and more cystic (Fig 2A–D). The MMC group had significantly higher bleb heights at week 1 onward compared with the verteporfin ($P = 0.005$) and BSS groups ($P = 0.01$) (Fig 2E). There

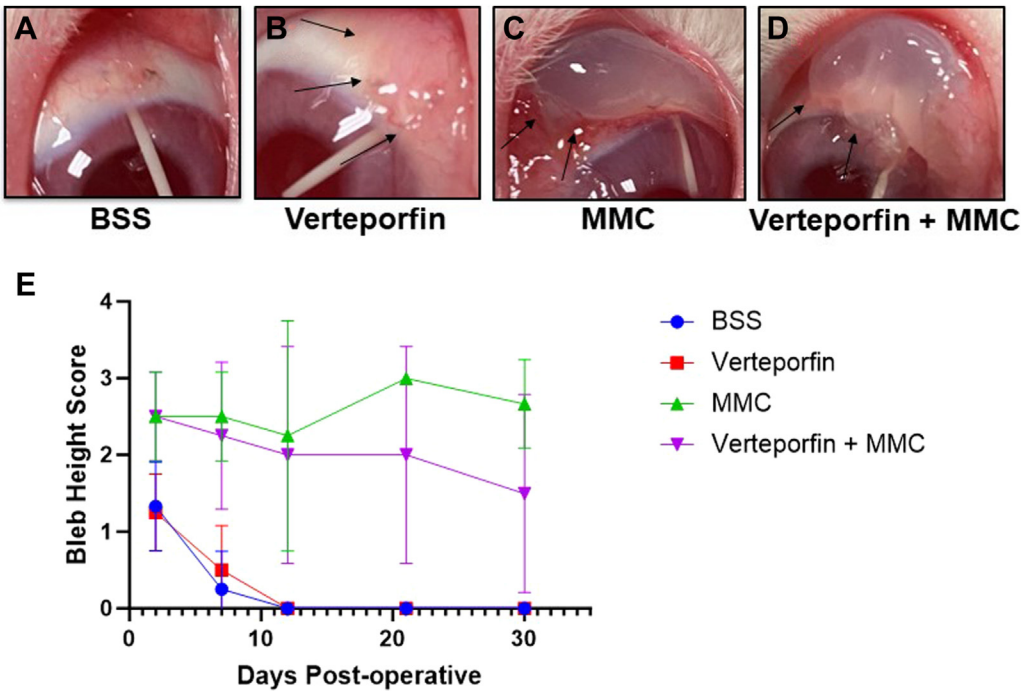

Figure 2. Bleb morphology across treatment groups. Top, photos of bleb appearance at day 7 after glaucoma filtration surgery. One representative eye is shown per group. Eyes were treated with balanced salt solution (BSS; **A**), 1 mg/mL verteporfin (**B**), 0.4 mg/mL mitomycin C (MMC; **C**), or a combination therapy of 1 mg/mL verteporfin and 0.4 mg/mL MMC (**D**). Arrows show the bleb boundaries. **E,** Bleb height scores across treatment groups over time. The MMC and verteporfin + MMC groups had significantly higher bleb heights at week 1 onward compared with the verteporfin ($P = 0.005$) and BSS groups ($P = 0.01$).

Table 1. Bleb Vascularity Across Treatment Groups

	Vascularity Score (Mean ± SD)
BSS (n = 4)	1.0 ± 0.0
Verteporfin (n = 4)	1.0 ± 0.0
MMC (n = 4)	0.3 ± 0.5
Verteporfin + MMC (n = 4)	0.0 ± 0.0

BSS = balanced salt solution; MMC = mitomycin C; SD = standard deviation.

were no other differences in bleb height or extent between the groups ($P > 0.05$).

Cytotoxic antifibrotic agents like MMC can cause nonperfused, avascular areas in treated tissues. Avascularity is characteristic of cystic blebs with thin walls that are prone to leakage and infection. Both MMC and verteporfin + MMC groups produced avascular blebs (mean vascularity score 0.3 ± 0.5 for MMC, 0.0 ± 0.0 for verteporfin + MMC; Table 1). There were no avascular blebs in the verteporfin and BSS groups (mean vascularity score 1.0 ± 0.0 for both groups; Table 1). The MMC alone and verteporfin + MMC treated blebs showed less vascularity than verteporfin or BSS treated blebs ($P < 0.01$).

IOP Measurements

Analysis of mean IOP in the surgical eyes showed no statistically significant differences among the verteporfin, MMC, verteporfin + MMC, and BSS treatment groups during the study period ($P = 0.20$; Fig 3).

Bleb Histology

Histology was performed to assess for tissue inflammation and scarring across treatment groups. Hematoxylin and eosin staining showed that blebs across groups showed a similar level of inflammation ($P > 0.5$; Table 2). A mild inflammatory infiltrate was typically seen around the bleb site in all groups (Fig 4). Masson's trichrome staining showed no differences in collagen deposition and scarring between the groups ($P > 0.5$; Table 2), with most blebs showing moderate collagen content (Fig 4).

Discussion

Here we find that although adjunctive subconjunctival 1 mg/mL verteporfin use was associated with a trend toward increased bleb survival compared with BSS control and further showed an excellent safety profile, it remained inferior to MMC for bleb survival in a rabbit glaucoma filtration surgery model. The addition of verteporfin to MMC did not affect surgical survival or histological changes in the short-term, although longer-term studies would be beneficial to better evaluate the impact on bleb morphology.

Mitomycin C has remained the gold standard antifibrotic agent for use during glaucoma filtration surgery since the early 1990s when it was found to inhibit subconjunctival and scleral fibroblasts in vivo.[13,25] Another antimetabolite commonly used as an antifibrotic adjunct is 5-fluorouracil.[26] While significantly improving surgical survival, antimetabolite use is associated with various complications including hypotony, bleb avascularity, and bleb leak due to cytotoxicity.[6,27] Our study also finds that MMC-treated blebs were avascular, in contrast to verteporfin- or BSS-treated blebs. Numerous alternative agents have since been investigated in glaucoma filtration surgery models,[4,23,28–30] although with varying degrees of surgical success and none which have translated into mainstream clinical practice. Verteporfin represents a promising novel agent not just because of its demonstrable antifibrotic properties in vivo,[14,15,19] but also given it is already Food and Drug Administration-approved for ocular use, albeit through a different route of administration (intravenous injection). We did not observe any adverse effects of verteporfin treatment in this study. Other preclinical studies delivering verteporfin systemically and locally as an

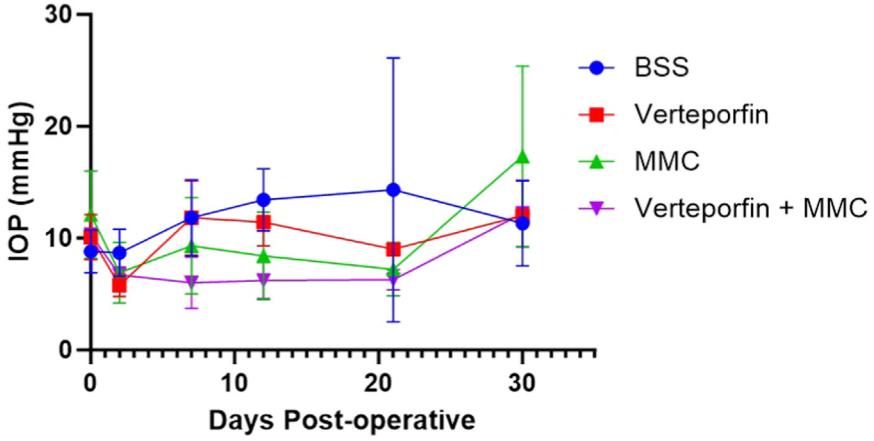

Figure 3. Intraocular pressure (IOP) across treatment groups. There were no significant differences in IOP between groups ($P = 0.2$). BSS = balanced salt solution; MMC = mitomycin C.

Table 2. Bleb Histology Across Treatment Groups

	Inflammation (Mean ± SD)	Collagen Deposition (Mean ± SD)
BSS (n = 3)	1.3 ± 0.6	1.3 ± 0.6
Verteporfin (n = 4)	1.0 ± 0.0	1.3 ± 1.3
MMC (n = 4)	0.3 ± 0.5	0.5 ± 0.6
Verteporfin + MMC (n = 4)	0.8 ± 1.0	1.5 ± 1.0

BSS = balanced salt solution; MMC = mitomycin C; SD = standard deviation.

antifibrotic also showed that verteporfin was not cytotoxic at similar doses in a variety of cell lines and tissues.[19,31]

This study used Visudyne, the Food and Drug Administration-approved formulation of verteporfin, which was injected subconjunctivally 1 time at the end of surgery. Further work is required to better optimize dosage and/or delivery. In similar rabbit models of glaucoma filtration surgery, some studies delivered the antifibrotic agent under investigation repeatedly (> 7 administrations) over the course of weeks, with promising initial results.[32,33] Another study combining valproic acid, an investigational antifibrotic, with low dose MMC (0.1 mg/mL) showed low toxicity and favorable scarring modulation.[23] Given our modest results with a single subconjunctival injection, it is possible that repeated injections or sustained release formulations of verteporfin, perhaps in combination with low dose MMC, may allow optimal bleb survival and morphology without some of the undesirable consequences on bleb morphology commonly seen with currently used doses of MMC. Future studies incorporating different treatment protocols would be of value.

Verteporfin exerts an antifibrotic effect by inhibiting yes-associated protein, a process that does not rely on photoactivation. This agent has been shown to inhibit the expression of profibrotic genes and collagen production.[17,19,31] We did not find any differences in inflammation and collagen deposition across treatment groups in this study, possibly due to short follow-up. Examining the gene expression changes of conjunctival fibroblasts after verteporfin treatment would be of interest in a future study. In addition, although we did not find any evidence of corneal toxicity on clinical examination, future studies investigating the effects of verteporfin on the corneal endothelium and ocular surface would be important due to potential exposure.

None of the treatments caused any significant changes in the IOP throughout the study period. This observation is

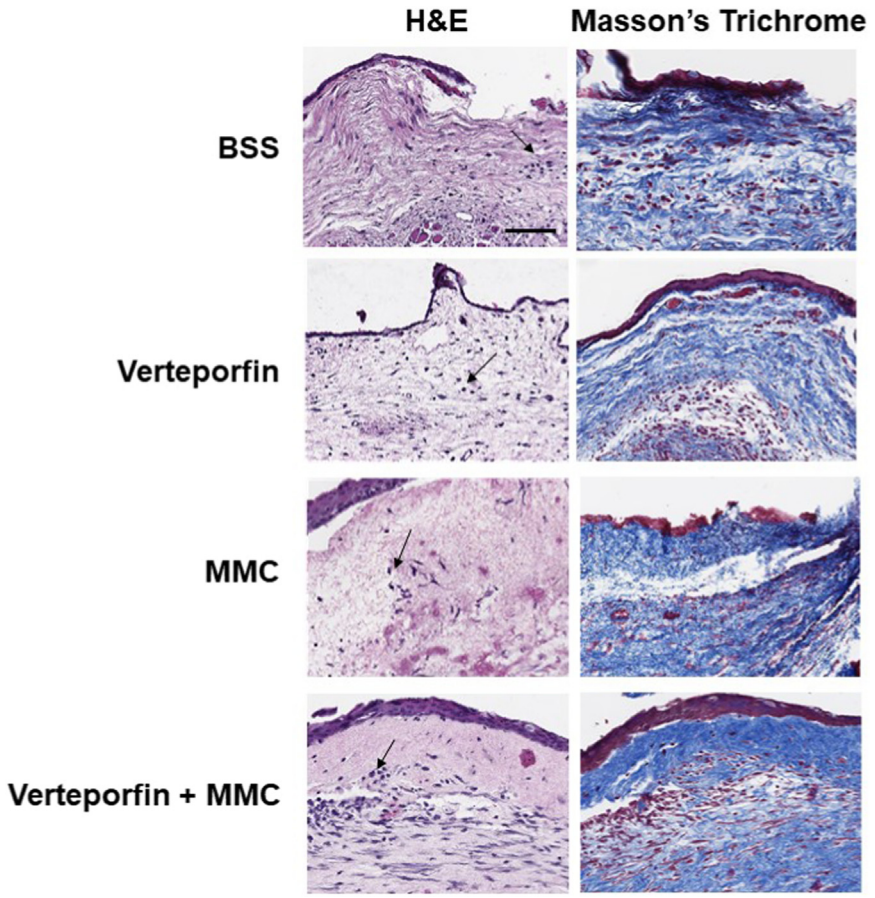

Figure 4. Histology of failed blebs across treatment groups. Hematoxylin and eosin (H&E) staining (left) showing inflammatory cells (arrows) in a background of collagen fibers and fibroblasts. Masson's trichrome (right) shows collagen fiber deposition (stained in blue) in the conjunctiva stromal matrix. Scale bar: 100 μm. BSS = balanced salt solution; MMC = mitomycin C.

consistent with that reported by other groups in similar studies.[4,32] Since normotensive rabbits were used, IOP is not thought to be a reliable indicator of filtration in this model.[4] Hence, bleb survival rather than IOP was used as a primary outcome measure. A glaucoma model may be needed to investigate any IOP lowering effect of verteporfin in hypertensive eyes.

Our study is limited by a small sample size and short follow-up. Interanimal variation in healing profile may have also played a role in variability between study groups. While we used an established model of glaucoma filtration surgery, rabbits are known to exhibit strong fibrotic responses resulting in quick failure of glaucoma blebs within days to weeks.[34,35] It is possible verteporfin may have different effects in species with less aggressive wound healing. Additional larger scale studies with a variety of timed end-points investigating a range of doses would be of value to better assess the potential role of verteporfin in glaucoma surgery.

While MMC is by far the most common antifibrotic agent used adjunctively at the time of glaucoma filtration surgery, this drug is not ideal for early postoperative wound modulation in cases where the intraoperative antifibrotic effect is insufficient. Verteporfin, if shown to be safe and less toxic than MMC, may be another useful tool in improving surgical outcomes when used intraoperatively and/or postoperatively for both bleb morphology and IOP control, similar to how 5-fluorouracil is used today. Further studies of verteporfin in bleb modulation are warranted.

Footnotes and Disclosures

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HUMAN SUBJECTS: No human subjects were included in this study.

ANIMAL SUBJECTS: Animal subjects were included in this study. All animal research was conducted in compliance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research and was approved by the Institutional Animal Care and Use Committee at Stanford University.

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Abbreviations and Acronyms:
BSS = balanced salt solution; **IM** = intramuscularly; **IOP** = intraocular pressure; **MMC** = mitomycin C.

Keywords:
Antifibrotic, Verteporfin, Glaucoma filtration surgery, Scarring, Rabbit model.

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