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VEGF-C and 5-Fluorouracil Improve Bleb Survival in a Rabbit Glaucoma Surgery Trabeculectomy Model

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Purpose. To evaluate VEGF-C–induced lymphoproliferation in conjunction with 5-fluorouracil (5-FU) antimetabolite treatment in a rabbit glaucoma filtration surgery (GFS) model.

Methods. Thirty-two rabbits underwent GFS and were assigned to four groups ($n = 8$ each) defined by subconjunctival drug treatment: (a) VEGF-C combined with 5-FU, (b) 5-FU, (c) VEGF-C, (d) and control. Bleb survival, bleb measurements, and IOP were evaluated over 30 days. At the end, histology and anterior segment OCT were performed on some eyes. mRNA was isolated from the remaining eyes for RT-PCR evaluation of vessel-specific markers (lymphatics, podoplanin and LYVE-1; and blood vessels, CD31).

Results. Qualitatively and quantitatively, VEGF-C combined with 5-FU resulted in blebs which were posteriorly longer and wider than the other conditions: vs. 5-FU ($P = 0.043$ for longer, $P = 0.046$ for wider), vs. VEGF-C ($P < 0.001$, $P < 0.001$) and vs. control ($P < 0.001$, $P < 0.001$). After 30 days, the VEGF-C combined with 5-FU condition resulted in longer bleb survival compared with 5-FU ($P = 0.025$), VEGF-C ($P < 0.001$), and control ($P < 0.001$). Only the VEGF-C combined with 5-FU condition showed a negative correlation between IOP and time that was statistically significant ($r = -0.533$; $P = 0.034$). Anterior segment OCT and histology demonstrated larger blebs for the VEGF-C combined with 5-FU condition. Only conditions including VEGF-C led to increased expression of lymphatic markers (LYVE-1, $P < 0.001$–0.008 and podoplanin, $P = 0.002$–0.011). Expression of CD31 was not different between the groups ($P = 0.978$).

Conclusions. Adding VEGF-C lymphoproliferation to standard antimetabolite treatment improved rabbit GFS success and may suggest a future strategy to improve human GFSs.

Keywords: glaucoma surgery, trabeculectomy, lymphatics, animal model, intraocular pressure

Glaucoma filtering surgeries (GFSs) are common glaucoma surgical treatments used to reduce IOP.[1] GFSs are often reserved for the most advanced glaucoma cases given the potential for greater IOP reduction.[1] GFSs include traditional bleb-forming glaucoma surgeries (such as trabeculectomies and glaucoma drainage devices) and new subconjunctival minimally invasive glaucoma surgeries (such as the Xen gel stent [Allergan] and the Preserflo microshunt [Santen, Osaka, Japan]).[1–4] The goal of GFSs is to create a communication between the anterior chamber and the subconjunctival space, resulting in a filtering bleb, to lower the IOP.[4,5]

For GFSs to be successful, the aqueous humor must freely flow both into and out of the filtering bleb. Prior subconjunctival surgical research focused on aqueous inflow by improving surgical tools and by creating stents (such as in glaucoma drainage devices, the Xen gel stent, and the Preserflo microshunt) to ensure a patent pathway between the anterior chamber and the subconjunctival space.[4–9] Considerable research has also targeted minimizing fibrosis during subconjunctival surgery by using antimetabolites such as mitomycin C or 5-fluorouracil (5-FU).[10–12] Antimetabolites are used both as a part of initial surgery and if needed during subsequent surgical revisions. These drugs aim to limit the proliferation of scar forming fibro-proliferative cells to minimize bleb scarring.[10–14] Despite advances in bleb inflow strategies and fibrosis management, surgical failure with increasing IOP still occurs after GFSs,[15,16] and this

finding suggests that other factors such as aqueous outflow from a bleb must also be understood and potentially improved as well.

Less research has been performed regarding subconjunctival or glaucoma filtering bleb outflow. Previous work clearly identified the existence of bleb outflow pathways in nonhuman primates eyes, and a lymphatic identity was hypothesized.[4,17] Recent investigations confirmed this lymphatic (as opposed to blood vessel) hypothesis using structural and molecular evaluation in enucleated porcine and human eyes in the laboratory.[18–20] Subconjunctival outflow studies in human patients receiving subconjunctival anesthesia before intravitreal treatment for retinal diseases further confirmed these results by showing that outflow pathways draining the subconjunctival space contained structural lymphatic hallmarks.[21] These results are consistent with human studies showing that there are no lymphatics found in failed trabeculectomy[22] and glaucoma drainage device[23] blebs. Moreover, a greater IOP reduction is seen after trabeculectomy in blebs associated with a higher number of lymphatic-like outflow pathways.[24]

Given the potential role of lymphatics, drugs or biologics that potentially alter lymphatics could impact GFS success. VEGF-C belongs to a family of VEGFs and is a key mediator of endogenous lymphatic angiogenesis through its binding to the VEGF receptor 3.[25] Given VEGF-C's purpose, our previous research demonstrated that subconjunctival delivery of VEGF-C in rabbit and mouse eyes significantly increased the number of subconjunctival lymphatic vessels without altering subconjunctival blood vessels.[26] However, it is unknown whether this VEGF-C–induced proliferation of subconjunctival lymphatics can lead to improved aqueous outflow from GFSs and consequently greater GFS success.

Therefore, in this study, we test the impact of VEGF-C lymphoproliferation by combining VEGF-C treatment with a commonly used GFS adjunctive antimetabolite, 5-FU, in a rabbit GFS model. We hypothesize that, with greater lymphatic presence to support subconjunctival outflow, GFSs will be more successful.

Methods

Rabbit Trabeculectomy Studies

Thirty-two male New Zealand White rabbits (Xi Lingjiao Co., Jinan, China; aged 12–14 weeks; 2–3 kg average body weight) were acquired and treated according to Institutional Animal Care protocol SDSYKYJS.No20180036 and the Statement of ARVO. Before trabeculectomy, rabbits were anesthetized using intravenous sodium pentobarbital (30 mg/kg), and their eyelids were gently opened using forceps after topical anesthesia using ropivacaine hydrochloride (Alcon, Fort Woth, TX, USA). For all minor procedures (such as conjunctival injection, conjunctival measurements, and imaging) a lower dose of sodium pentobarbital (15 mg/kg) with additive topical surface anesthesia was used.

Standard rabbit GFS surgical technique was used on all animals by the same surgeon (LZ). In all cases, the right eye was the operative eye. Rabbits were given intravenous sodium pentobarbital (30 mg/kg), prepped and draped in a sterile fashion, and placed on a heating pad. To avoid the third eyelid during follow-up imaging, the trabeculectomy was positioned between 10:00 and 1:00 o'clock. The conjunctiva and Tenon's capsule were opened via a fornix-based approach using Wescott scissors. A 3 × 3 mm square one-half thickness scleral flap was created until the trabeculum was visible. Trabeculectomy boundaries of approximately 2 × 1 mm were created using a 15° blade. The trabeculum was then excised and a peripheral iridectomy was made. The scleral flap was sutured to the sclera at its corners using two 10-0 nylon sutures (Mani, Utsunomiya City, Japan). The conjunctiva was then closed using 10-0 nylon sutures. 0.3% tobramycin (Tobrex, Alcon, Istanbul, Turkey) and 0.1% dexamethasone ointment (Maxidex, Alcon, Istanbul, Turkey) as well as 0.5% levofloxacin eye drops (Santen, Shanghai, China) were applied to the eye at the end of surgery. Animals was recovered on a warm heating pad and returned to their cages. Animals were followed daily postoperatively. No unanticipated postoperative complications or animal deaths were noted.

Rabbit Trabeculectomy Experimental Conditions

The experimental rabbits were numbered from 1 to 32 and were randomly separated into four groups ($n = 8$) based upon subconjunctival injection protocols for 5-FU (5 mg, 25 mg/mL; Jinyao, Tianjin, China), VEGF-C (0.05 mg/mL; Biovision 4633; Milpitas, CA, USA), both 5-FU and VEGF-C, or normal saline (Solarbio IN9000; Beijing, China) (Fig. 1). Subconjunctival injections for all conditions were performed on postoperative days 2, 4, 6, 8, 10, 12, and 14. Multiple injections, up to 7, match prior reports evaluating 5-FU and other agents that can impact GFSs in rabbits.[27–29] All injections were performed using a 31G needle, and the location was immediately posterior to the bleb for a volume of 30 µL. For the 5-FU combined with VEGF-C condition, if the drugs were injected simultaneously, they would mix and dilute each other. Thus, in this case, the drugs were injected on separate occasions on each day (approximately >3 h apart).

Rabbit Bleb Assessment

All bleb assessments were made by two masked graders blinded to the rabbit condition. IOP of the experimental eye was measured on postoperative days 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 using an applanation tonometer (Tonopen XL Medtronic Solan Company of Jacksonville, FL, USA) (Fig. 1). This was done under topical anesthesia (ropivacaine) between 5 PM and 7 PM, before general anesthesia on days where the rabbit also received subconjunctival injections Three readings were taken and averaged.

Bleb measurements (length and width) were acquired using calipers every other day (starting on day 2) after surgery and recorded until postoperative day 30 (Fig. 1). Length was defined as the maximum distance of a line (perpendicular to the limbus) between the limbus to the posterior edge of the elevated bleb. Width was defined as the maximum distance of a line (parallel to the limbus) over the elevated bleb. Photographs of the conjunctival blebs were taken on the 10th, 20th, and 30th postoperative days using a camera attached to the surgical microscope (OPMI Lumera I; Zeiss, Jena, Germany). Photographs were taken before subconjunctival injections on postoperative day 10. Bleb survival was defined as the appearance of a scarred and flat bleb with clear visualization of the scleral flap on two consecutive assessments with the record of failure marked on the second day.[30,31]

On the 30th postoperative day, some rabbits were anesthetized, and anterior-segment optical coherence tomog-

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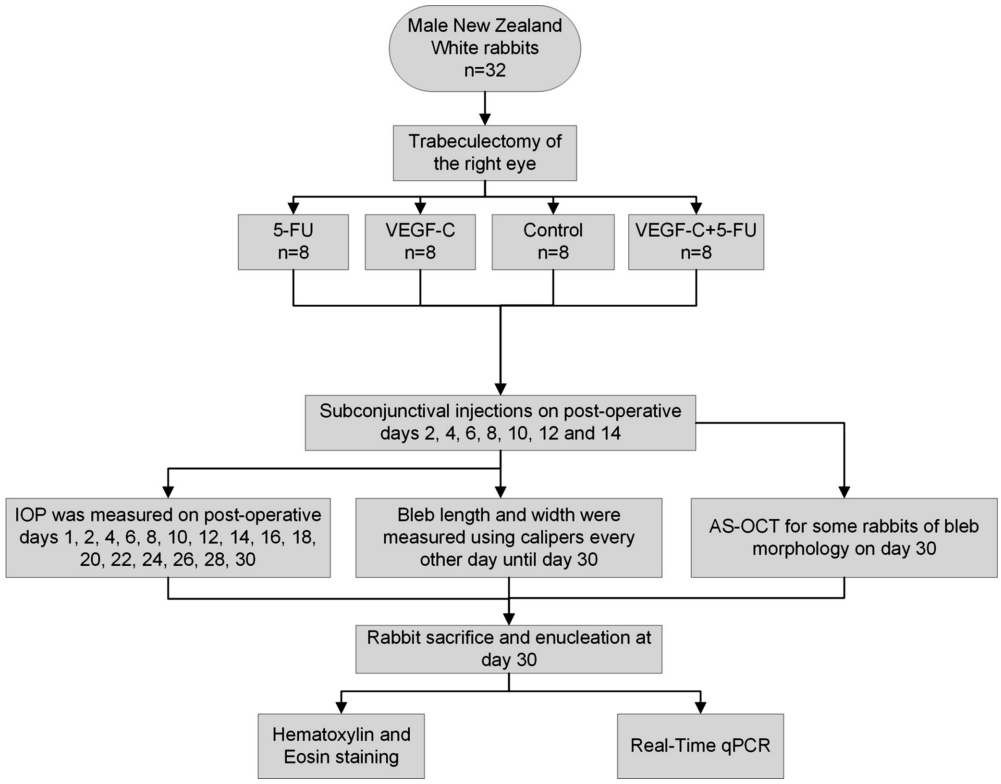

Figure 1. Methodological flow chart.

raphy was performed (AS-OCT) (RTVue 100-2 OCT, Inc., Fremont, CA, USA) to record the bleb morphology using the corneal line scan function (Fig. 1). These images were exported as TIFFS, and maximum bleb height was measured using imageJ/Fiji platform (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij). As the bleb arose above the scleral plane, deeper structures could be difficult to delineate. Thus, the scleral edge was identified near the corneal–scleral junction where there was no bleb, and a line was extended from this point posterior. Maximum bleb height was defined as the maximum distance from this line to the apex of the bleb. Measurements were made by a grader blinded to the condition (XZ; UCSD). In-built scale bars (4 µm/pixel) allowed conversion to microns.

Hematoxylin and Eosin Staining

On postoperative day 30, one rabbit in each group was euthanized with a lethal intravenous overdose injection of sodium pentobarbital, and the right eye was enucleated for histology (Fig. 1). The eye was removed with the upper lid left intact and attached to the globe to preserve the architecture of the superior fornix and conjunctival tissue around the surgical site. After enucleation, the tissue was rinsed with phosphate buffered saline, fixed in 4% paraformaldehyde (Solarbio P1110; Beijing, China) for 24 hours, and then embedded in paraffin wax. After fixation, sequential 4-µm sections of the surgical site were cut onto Superfrost plus slides and stained with hematoxylin and eosin, dehydrated, rinsed in xylenes, and mounted using a coverslip. Slides were viewed, and pictures were taken (Nikon E800; Nikon Inc., Melville, NY, USA).

Real-time qPCR Analysis

On postoperative day 30, the remaining seven animals from each group were euthanized using a lethal intravenous overdose injection of sodium pentobarbital for a PCR study (Fig. 1). The conjunctiva, Tenon's capsule, and all tissue above the sclera of the right eye were removed from the surgical site. The tissue was frozen in liquid nitrogen.

Total RNA was also extracted from frozen tissues using a Nucleospin RNA Kit (BD Biosciences, Palo Alto, CA, USA) and pooled. Isolated RNA was reverse transcribed into cDNA using the Primescript First-Strand cDNA Synthesis kit (Vazyme, Nanjing, China). Quantitative PCR was performed using SYBR Green reagents (Vazyme). The results were analyzed using the Sequence Detection System software (Applied Biosystems, Waltham, MA, USA). For relative quantization, 2-ΔΔCt was calculated and used as a relative index of expression level. RT-qPCR was conducted using the following primers according to the manufacturer's protocol:

Podoplanin forward, 5′-CTCAGAGCCTCACGGTGTCAA-3′; Podoplanin reverse, 5′-CGACTCGTAGCCGCATTTG-3′; LYVE-1 forward, 5′-GCATGGAAGTTTGGCTTTGAG-3′; LYVE-1 reverse, 5′-GGCCCCAAATGTTCTGCTAA-3′; CD31 forward, 5′-CACGGTCGTGTCGCAGAAT-3′; CD31 reverse, 5′-ATCCTGGGCTTGGAGAGCAT-3′; GAPDH forward, 5′-AGCAAGAGCACAAGAGGAAG-3′; GAPDH reverse, and 5′- GGTTGAGCACAGGGTACTTT-3′. GAPDH served as an internal control.

Statistical Analyses

Statistical analyses were performed using SPSS v22.0 (IBM, Chicago, IL, USA). Continuous data were expressed using

mean ± SD. Comparisons between multiple groups were performed using one-way ANOVA or repeated measures ANOVA, and Tukey's *t* test was chosen for pairwise comparison's. For IOP, the data was plotted over time, and a best-fit line was drawn to determine the r coefficient as previously described.[32] Evaluating bleb characteristics and IOP for each condition on each day, 70% to 89% of the datasets were normal by Shapiro–Wilk testing. Pearson's method was used for correlation analysis between IOP and time. Kaplan–Meier survival estimates were obtained for each treatment group. The log-rank test was used to compare survival differences between groups. The levels of statistical significance were: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Results

All 32 animals completed the study. On the first day after surgery, hyphema was detected in three animals, but the hyphema self-resolved within 24 hours. No other complications or cases of endophthalmitis were observed.

Qualitative Bleb Assessment

Trabeculectomy GFS blebs were qualitatively assessed on postoperative days 10, 20, and 30. Early on (on postoperative day 10), well-formed blebs were evident in all four groups (Figs. 2A–D). Specifically, the blebs were elevated, diffuse, and the outline of the scleral flaps were not observable. Qualitatively, there was the suggestion that the blebs of the 5-FU group (Fig. 2A) and the VEGF-C combined with 5-FU group (Fig. 2C) were more extensive than those in the VEGF-C (Fig. 2B) and control (Fig. 2D) groups. On postoperative day 20, the extent of the blebs were noted to reduce (Figs. 2E–H). In the control (Fig. 2H) and VEGF-C (Fig. 2F) groups, the blebs seemed to be smaller, flatter, and the outlines of the scleral flaps became more prominent and observable. This finding was in contrast with the blebs in the 5-FU group (Fig. 2E) and the VEGF-C combined with 5-FU group (Fig. 2G), where the blebs still seemed to be morphologically elevated, such that the outlines of the scleral flaps were not seen. On postoperative day 30, blebs in the VEGF-C combined with 5-FU group (Fig. 2K) still showed some elevation, whereas the blebs in the remaining groups further shrank with details of the scleral flaps clearly seen (Figs. 2I, 2J, and 2L).

Table 1. Bleb Survival Across Different Treatment Groups

Pairwise Log-Rank Comparison Test	χ^2 Test	*P* Value
VEGF-C + 5-FU vs. 5-FU	5.018	0.025
VEGF-C + 5-FU vs. VEGF-C	16.654	<0.001
VEGF-C + 5-FU vs. control	16.897	<0.001
5-FU vs. VEGF-C	10.452	0.001
5-FU vs. control	15.024	<0.001
VEGF-C vs. control	4.801	0.028

Bleb survival was assessed by a Kaplan-Meier analysis. VEGF-C combined with 5-FU significantly improved the survival time of the filtration bleb compared with the other three conditions (Fig. 2M). Statistically, a significant difference in survival was seen among the four comparison groups (log-rank = 38.97; $P < 0.001$). In pairwise comparisons, a statistically significant difference was detected between the VEGF-C combined with 5-FU group vs. the 5-FU group ($P = 0.025$), vs. the VEGF-C group ($P < 0.001$), and vs. the control group ($P < 0.001$) (Table 1). A statistically significant difference was also detected between the 5-FU group vs. the VEGF-C group ($P = 0.001$) and vs. the control group ($P < 0.001$) (Table 1). Last, a statistically significant difference was detected between the VEGF-C group vs. the control group ($P = 0.028$) (Table 1). Data from specific days supported these results. On day 20, the VEGF-C combined with 5-FU group and the 5-FU group showed 100% bleb survival, the VEGF-C group showed 87.5% bleb survival, and the control group showed 25% bleb survival (Fig. 2M). On day 30, the VEGF-C combined with 5-FU group showed 100% bleb survival, and the 5-FU group only showed 50% bleb survival. In contrast, the VEGF-C and control groups showed zero bleb survival (Fig. 2M). The mean number of survival days were 27.75 ± 2.73, 21.75 ± 2.54, 15.5 ± 4.97 for the 5-FU, VEGF-C, and control groups, respectively (Fig. 2M).

Quantitative Bleb Assessment and IOP

To support the qualitative results, bleb characteristics were quantitatively measured. The length and width of the blebs were compared among the groups (Figs. 3A, 3B). For length, an overall difference was found between the groups ($P < 0.001$; repeated-measures ANOVA between-subjects test). Averaging length over all 30 days and rabbits, compar-

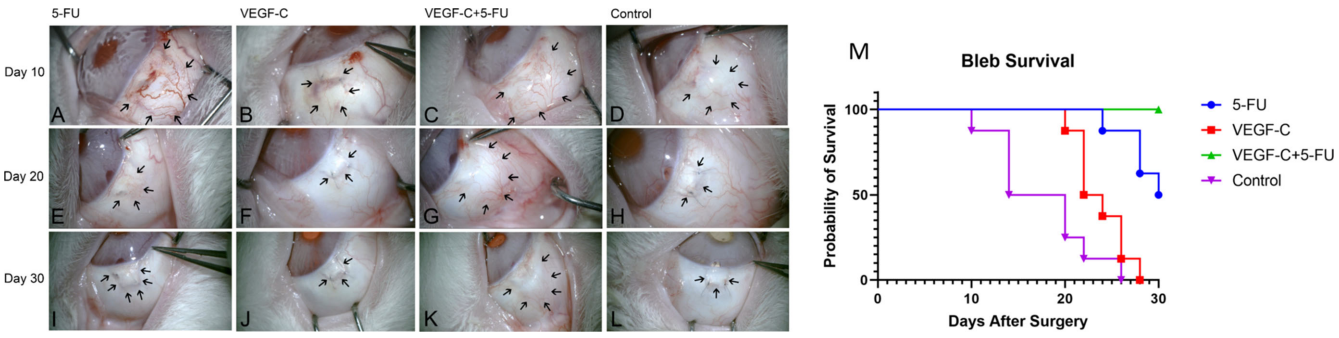

Figure 2. Qualitative bleb assessment. Bleb appearances on postoperative day 10 (**A–D**), day 20 (**E–H**), and day 30 (**I–L**) for each treatment group. Qualitatively, the VEGF-C combined with 5-FU group (**C**, **G**, **K**) maintained better morphology with bulging, diffuse, fleshy blebs compared with the 5-FU (**A**, **E**, **I**), VEGF-C (**B**, **F**, **J**) and control (**D**, **H**, **L**) groups. (**A–L**) *Black arrows* denote the bleb margins. (**M**) Survival curves of filtration blebs. VEGF-C combined with 5-FU treatment group showed prolonged bleb survival.

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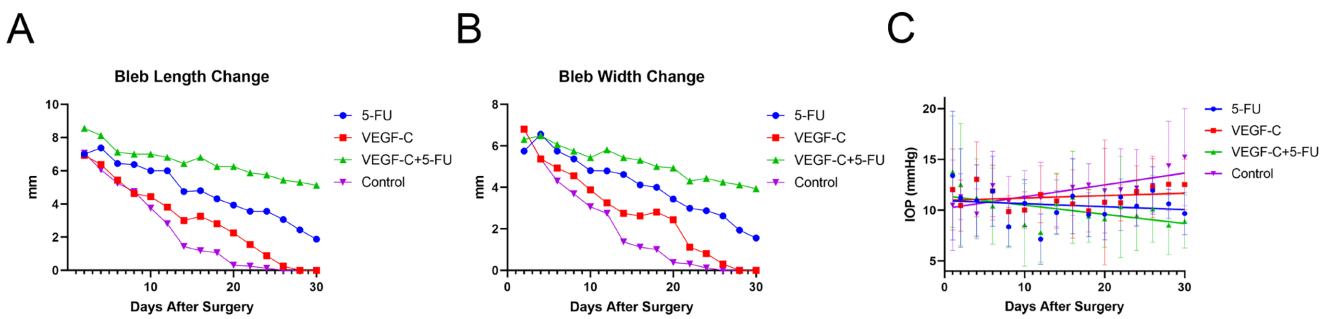

Figure 3. Quantitative bleb assessment and IOP. (**A**) Bleb length, (**B**) bleb width, and (**C**) IOP for the four treatment groups. VEGF-C combined with 5-FU treatment resulted in significantly (**A**) longer and (**B**) wider blebs compared with the other three treatments. (**C**) VEGF-C combined with 5-FU treatment showed the only negative trending IOP that was statistically significant.

Table 2. Comparison of Bleb Characteristics Across Different Treatment Groups

Comparisons (n = 32)	Mean Difference (95% CI)	P Value
Bleb length (mm)		
VEGF-C + 5-FU vs. 5-FU	1.76 (0.04 to 3.47)	0.043
VEGF-C + 5-FU vs. VEGF-C	3.48 (1.77 to 5.20)	<0.001
VEGF-C + 5-FU vs. control	4.25 (2.54 to 5.97)	<0.001
5-FU vs. VEGF-C	1.73 (0.02 to 3.44)	0.047
5-FU vs. control	2.50 (0.79 to 4.21)	0.002
VEGF-C vs. control	0.77 (−0.9 to 2.48)	0.613
Bleb width (mm)		
VEGF-C + 5-FU vs. 5-FU	1.08 (0.2 to 2.13)	0.046
VEGF-C + 5-FU vs. VEGF-C	2.43 (1.38 to 3.49)	<0.001
VEGF-C + 5-FU vs. control	3.16 (1.0 to 4.22)	<0.001
5-FU vs. VEGF-C	1.36 (0.3 to 2.42)	0.008
5-FU vs. control	2.09 (1.03 to 3.15)	<0.001
VEGF-C vs. control	0.73 (−0.33 to 1.79)	0.259

*Adjusted with Tukey's Correction

isons showed that the VEGF-C combined with 5-FU blebs were statistically significantly longer compared with the 5-FU (P = 0.043), VEGF-C (P < 0.001), and control (P < 0.001) blebs (Table 2). The 5-FU blebs were statistically significantly longer than VEGF-C (P = 0.047) and control (P = 0.002) blebs (Table 2). No difference was noted between the VEGF-C and control (P = 0.613) blebs (Table 2).

For bleb width, an overall difference was found between the groups (P < 0.001; repeated-measures ANOVA between-subjects test). Averaging width over all 30 days and rabbits, comparisons showed that the VEGF-C combined with 5-FU blebs were statistically significantly wider than the 5-FU (P = 0.046), VEGF-C (P < 0.001), and control (P < 0.001) blebs (Table 2). The 5-FU blebs were statistically significantly wider than VEGF-C (P = 0.008) and control (P < 0.001) blebs (Table 2). No difference was noted between the VEGF-C and control (P = 0.259) blebs (Table 2).

Analysis of mean IOP in the surgical eyes revealed no differences between the treatment groups on postoperative day 30 (P = 0.176; repeated-measures ANOVA between-subjects test). However, to evaluate IOP over time, the trends of IOPs were studied for each of the four groups (Fig. 3C). Both the VEGF-C combined with 5-FU (r = −0.533) and 5-FU (r = −0.185) groups showed a negative correlation between IOP and time (Fig. 3C). Pearson's correlation test showed that the negative correlation was statistically significant for the VEGF-C combined with 5-FU group (P = 0.034) but not the 5-FU group (P = 0.492) (Table 3). Then, both the VEGF-C (r = 0.217) and control (r = 0.785) groups showed a positive correlation between IOP and time (Fig. 3C). Pearson's correlation test showed that this positive correlation was statistically significant for the control group (P < 0.001), but not the VEGF-C group (P = 0.421) (Table 3).

Table 3. IOP Across Time for Different Treatment Groups

Comparison	Pearson's r	P Value
VEGF-C + 5-FU vs. time	−0.533	0.034
5-FU vs. time	−0.185	0.492
VEGF-C vs. time	0.217	0.421
Control vs. time	0.785	<0.001

AS-OCT and Histopathological Features

Additional trabeculectomy bleb assessments were made from some animals using in vivo AS-OCT imaging and histology from postoperative day 30 eyes. Qualitatively, histology (Figs. 4A–D) and AS-OCT (Figs. 4E–H) showed that surgical blebs were taller in the VEGF-C combined with 5-FU group (Figs. 4C, 4G). The control group showed no appreciable bleb (Figs. 4D/H). The 5-FU (Figs. 4A, 4E) and VEGF-C (Figs. 4B, 4F) conditions were in between. Quantitatively, maximum bleb height was measured using AS-OCT (VEGF-C combined with 5-FU [504 ± 145 microns; n = 3]; 5-FU [514 ± 72 microns; n = 3], VEGF-C [281 ± 42 microns; n = 2]; and control [221 ± 112 microns; n = 2]). The only statistically significant comparisons were 5-FU vs. control (P = 0.035) and 5-FU vs. VEGF-C (P = 0.028). The VEGF-C plus 5-FU condition compared with control trended toward a statistically significant difference (P = 0.1).

Expression of Lymphatic and Blood Vessel Markers

Our previous research demonstrated that subconjunctival injection of VEGF-C promoted the proliferation of conjunctival lymphatic vessels without affecting blood vessels.[26] Therefore, the results of this study should replicate prior work, and we performed RT-qPCR mRNA analysis (podoplanin and LYVE-1 for lymphatics and CD31 for blood vessels) using filtration bleb tissue collected on postoperative day 30 (Table 4).

For LYVE-1, an overall difference in mRNA expression was noted among the groups (P < 0.001). In pairwise comparisons, the VEGF-C combined with 5-FU group

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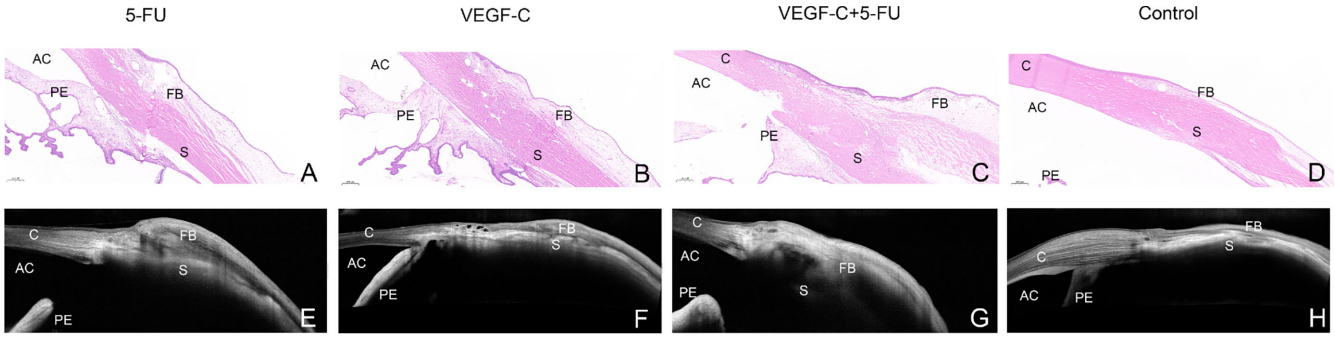

Figure 4. AS-OCT and hematoxylin and eosin staining of filtration blebs. (**A–D**) Hematoxylin and eosin staining and (**E–H**) bleb AS-OCT images at day 30. (**E–H**) The lower right corner inserts shows where the OCT was performed using the corneal line scan protocol. AC, anterior chamber; C, cornea; FB, filtering bleb; PE, iris pigment epithelium; S, sclera. Scale bars, 200 microns.

Table 4. RT-qPCR Comparison Table

Comparisons	Mean Difference (95% CI)	*P* Value
LYVE-1		
VEGF-C + 5-FU vs. 5FU	1.79 (1.13 to 2.45)	<0.001
VEGF-C + 5-FU vs. VEGF-C	0.39 (−0.27 to 1.05)	0.297
VEGF-C + 5-FU vs. control	1.33 (0.67 to 1.99)	<0.001
5-FU vs. control	−0.46 (−1.12 to 0.21)	0.200
5-FU vs. VEGF-C	−1.40 (−2.06 to −0.74)	<0.001
VEGF-C vs. control	0.94 (0.28 to 1.60)	0.008
Podoplanin		
VEGF-C + 5-FU vs. 5-FU	0.90 (0.39 to 1.41)	0.002
VEGF-C + 5-FU vs. VEGF-C	0.21 (−0.30 to 0.72)	0.587
VEGF-C + 5-FU vs. control	0.93 (0.42 to 1.44)	0.002
5-FU vs. control	0.03 (−0.48 to 0.54)	0.997
5-FU vs. VEGF-C	−0.69 (−1.20 to −0.18)	0.012
VEGF-C vs. control	0.72 (0.21 to 1.23)	0.008
CD31		
VEGF-C + 5-FU vs. 5-FU	−0.01 (−0.87 to 0.86)	>0.999
VEGF-C + 5-FU vs. VEGF-C	0.04 (−0.82 to 0.91)	0.998
VEGF-C + 5-FU vs. control	−0.07 (−0.93 to 0.79)	0.993
5-FU vs. control	−0.07 (−0.93 to 0.80)	0.995
5-FU vs. VEGF-C	0.05 (−0.81 to 0.91)	0.998
VEGF-C vs. control	−0.12 (−0.98 to 0.75)	0.972

*Adjusted with Tukey's Correction.

showed increased LYVE-1 mRNA levels (2.33 ± 0.06 relative expression [RE]) compared with the 5-FU group (0.54 ± 0.30 RE; $P < 0.001$) and control group (1.00 ± 0.00 RE; $P < 0.001$), but no difference was seen compared with the VEGF-C group (1.94 ± 0.28 RE; $P = 0.297$) (Fig. 5A). The VEGF-C group also showed increased LYVE-1 mRNA levels compared with the 5-FU group ($P < 0.001$) and control group ($P = 0.008$) (Fig. 3A). No difference was noted between the 5-FU group and control groups ($P = 0.200$) (Fig. 5A).

To reproduce this finding using a different lymphatic marker, an overall difference in mRNA expression was noted among the groups ($P < 0.001$) for podoplanin. In pairwise comparisons, the VEGF-C combined with 5-FU group showed increased podoplanin mRNA levels (1.93 ± 0.31 RE) compared with the 5-FU group (1.03 ± 0.08 RE; $P = 0.002$) and control group (1.00 ± 0.00 RE; $P = 0.002$), but no difference was seen compared with the VEGF-C group (1.72 ± 0.02 RE; $P = 0.587$) (Fig. 5B). The VEGF-C group also showed increased podoplanin mRNA expression compared with 5-FU group ($P = 0.012$) and control group ($P = 0.008$) (Fig. 5B). No difference was noted between the 5-FU group and control group ($P = 0.997$) (Fig. 5B).

For CD31, there was not an overall statistically significant difference among the four groups ($P = 0.978$) (Fig. 5C).

Discussion

Among glaucoma surgeries, GFSs typically result in the greatest potential IOP reduction.[1,3] However, GFSs can fail and be associated with complications.[4] The success of filtration surgery is related to the function of the filtration bleb,

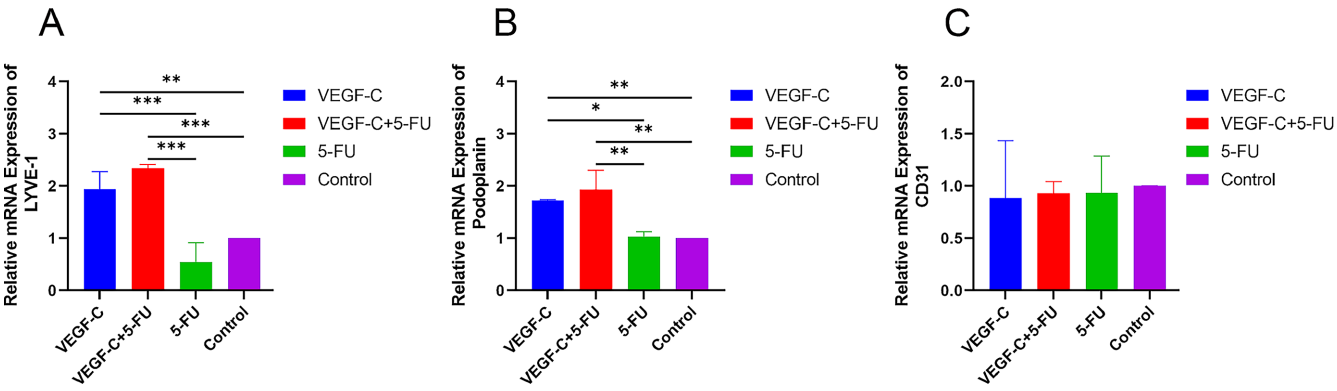

Figure 5. Gene expression of lymphatic and blood vessel markers. Gene expression was assessed across the various conditions evaluating lymphatic (**A**; LYVE-1) and (**B**; podoplanin), as well as blood vessel endothelial (**C**; CD31) markers from blebs harvested on postoperative day 30. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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and bleb biology is complex and poorly understood. To prevent GFS failure and scarring, a wide range of antifibrotic agents are used during and after GFSs, such as in trabeculectomy.[10,12–14] The most commonly used antifibrotic agents are 5-FU and mitomycin C.[10,12,14] Simultaneously, Guo et al.[17] hypothesized that subconjunctival lymphatics play an important role in the drainage of GFS blebs. We followed this work by showing that subconjunctival lymphatic presence can be increased by agents such as VEGF-C.[26] Therefore, in this study, we tested the impact of VEGF-C added to 5-FU on glaucoma surgery in a rabbit GFS trabeculectomy model. We chose 5-FU as the antimetabolite as multiple prior reports had studied 5-FU in conjunction with biologics with established protocols.[27–29] Gene expression analysis in this study showed that VEGF-C successfully promoted lymphatics without a change in blood vessels. This replicated prior findings by our group[26] and others.[33] Then, the VEGF-C combined with 5-FU condition resulted in larger blebs and significantly prolonged bleb survival associated with IOP behavior that decreased over time.

The overall result is that combining VEGF-C with 5-FU is a promising strategy to improve GFS results. First, it is important to point out that the combination group (VEGF-C plus 5-FU) actually received a greater number of subconjunctival injections compared with all other conditions to avoid the effect of drug dilution. Because injections are a form a trauma that could elicit a wound healing response, the combination group had a theoretical bias toward a worse outcome. Yet, the VEGF-C added to 5-FU condition performed the best.

Each adjunctive drug also has its own unique purpose. 5-FU is an antimetabolite used to prevent scarring and failure. VEGF-C enhances lymphatic growth into the bleb to potentially promote bleb outflow. Individually, 5-FU alone had a positive impact on rabbit GFS in this study as reported previously by other investigators[27,29] and consistent with experience in human trabeculectomy surgeries.[10,12] In contrast, VEGF-C alone had a muted effect on rabbit GFS, and this effect may be related to the concept that, if a bleb scars, it does not matter if lymphatics are present to assist in bleb outflow.

Further, the additive benefit of VEGF-C is likely complex. Here we tested the idea of performing GFS and then introducing the adjunctive VEGF-C in the short term (approximately 2 weeks) after surgery. Because VEGF-C structural lymphoproliferation and functional maturation takes time,[34] it is unclear whether VEGF-C should have been administered some time before GFS to preestablish a subconjunctival lymphatic network. Alternatively, it is possible that, for humans, the initial surgery should be performed in the absence of any added growth factor. Then, VEGF-C could be considered just in cases of bleb revision for failed blebs considerably after initial surgery. These considerations emphasize how achieving lymphoproliferation using different strategies may lead to different results. For example, an adenoviral strategy to achieve stable VEGF-C expression has been reported, shown to promote lymphatic growth in the rabbit eye, and may represent an approach to achieve a more stable and longer-lasting lymphoproliferative effect[33] without needing multiple subconjunctival injections. An additional approach is to use sustained drug release methods which are being studied.[35–37]

The IOP must also be carefully interpreted in rabbit GFS studies. First, no difference in IOP was noted on the final day of the study across conditions. However, the IOP changed over time for each condition. It is important to point out that the rabbit GFS model does not exactly match the human condition or human surgery. As rabbit GFSs are particularly prone to failure, rabbit GFSs are typically studied by evaluating bleb survival over approximately 28 to 30 days.[27–29,38]. Potential life-long and robust IOP decreases, which can be seen after trabeculectomy in some human patients with glaucoma, is not seen in rabbits. Similarly, publications investigating animal GFSs either do not record IOP,[29,31] record but do not report IOP,[27,30] report absolute IOP,[28,38] or report IOP compared with the unoperated contralateral eye.[28,39] It is not even uncommon to report improved GFSs blebs without showing any IOP reduction.[27,29–31] In our case, we did not collect contralateral eye IOP or preoperative IOP, but we transparently report that absolute IOP was not different in the operative eye across conditions on postoperative day 30. Future studies need to have these datapoints included. However, because considerable IOP variability exists, we focused on condition-specific longitudinal IOP analysis. We emphasize that the IOP trends showed that the VEGF-C combined with the 5-FU group showed a larger negative IOP correlation with time ($r = -0.533$; statistically significant), the 5-FU group showed a smaller negative IOP correlation with time ($r = -0.185$; not statistically significant), the VEGF-C group showed a smaller positive IOP correlation with time ($r = 0.217$; not statistically significant), and the control group showed a greater positive IOP correlation with time ($r = 0.785$; statistically significant). Notably and in support of biological plausibility, the IOP results matched the relative results from bleb structural assessment (e.g., VEGF-C plus 5-FU being better than 5-FU alone being better than VEGF-C alone being better than control).

There are a number of limitations in this study. First, the sample size of this study is small. Compared with the published literature,[27–30,38] this study is powered to study bleb characteristics, but may not be large enough for evaluating IOP. This is another reason why we reported our IOP results over time for each condition. Second, more objective measures of bleb assessment are needed. Measuring bleb width and length using calipers is relatively easy, but measuring height is challenging. We recognized this after study initiation, which is why only a small number of rabbits received AS-OCT imaging. Histological analysis was always planned to be limited because enucleation and histology precluded using postmortem tissue for RT-PCR experiments. In future studies, AS-OCT should be acquired more frequently for all rabbits across multiple time points. Additionally, increasing sample size and following IOP and bleb characteristics further in time will be important. Last, a future study will evaluate the impact of mitomycin C, as well given its wider clinical use in humans.

In conclusion, this study shows that larger blebs and improved IOP trends were observed in rabbit GFSs after subconjunctival injection of VEGF-C plus 5-FU. Future studies are needed to determine the best timing and strategy for applying these GFS adjunctive treatments. Additional lymphoproliferative agents[40] and molecular pathways known to impact conjunctival lymphatics[41] should be tested as well. Ultimately, with optimal scar mitigation added to lymphoproliferation, the investigation in human clinical trials of lymphoproliferation to improve trabeculectomy surgery is needed.

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