The PLGA Implant as an Antimitotic Delivery System After Experimental Trabeculectomy

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Purpose. To investigate the effect of poly (lactic-co-glycolic acid) (PLGA) implants loaded with mitomycin C (MMC) and with different adjuvant treatments after glaucoma filtration surgery (GFS), in comparison to standard treatments.

Methods. Forty-two New Zealand White rabbits underwent bilateral GFS and received different treatments: topical MMC (group 1); topical 5-fluorouracil (5-FU; group 2); PLGA implant (group 3); MMC-loaded and -coated PLGA implant (group 4); MMC-loaded and 5-FU–coated PLGA implant (group 5); subconjunctival bevacizumab (group 6); MMC-loaded PLGA implant and subconjunctival bevacizumab (group 7); and no treatment (right eye of all animals; control group). Intraocular pressure (IOP) and filtering bleb were evaluated on different days after GFS. Histology was performed to examine the conjunctiva, sclerotomy, filtering bleb, and persistence of the implant.

Results. The best hypotensive results were achieved in the MMC-loaded and -coated PLGA implant group, which presented the lowest IOP values on days 1, 5, 7, 14, and 28 after GFS. Excluding the implant groups, in which the bleb could not be properly measured, bleb survival was superior to controls in groups 1, 2 and lower in group 6. Group 7 presented greater extension, height, and vascularization of the bleb. Epithelial thinning and lymphoplasmacytic infiltrate were observed in groups 1, 2, 4, 5, and 7. The rates of closure of the sclerotomy and bleb were 100% and 76%, respectively, and implant persistence was 95%.

Conclusions. MMC-loaded and -coated implants have optimal surgical results, followed by topical MMC application. In this experimental model, bevacizumab could interact with MMC.

Keywords: glaucoma, antimitotics, trabeculectomy, PLGA, implant, ocular drug delivery systems

Glaucoma filtration surgery (GFS) is currently one of the most effective methods for treating glaucoma. Unfortunately, this surgery tends to fail over time due to excessive scarring at the filtering bleb.[1] Numerous strategies have been employed to modulate wound healing and increase surgical success (beta radiation,[2] 5-fluorouracil [5-FU],[3] mitomycin C [MMC],[4] growth factor inhibitors,[5,6] etc.). However, these drugs may be relatively ineffective or give rise to an increase in complications, such as persistent postoperative hypotony, corneal problems, filtering bleb leakage, endophthalmitis, etc. The development of drug delivery systems to release antimitotics and facilitate the administration of these agents at appropriate doses and for the required time could lead to improved surgical outcomes.

An ideal drug delivery system is biocompatible and biodegradable, and should facilitate the local release of drug without local or systemic toxicity. To date, diverse materials have been studied as drug delivery systems for antimitotics in experimental filtration surgery. Polyanhydrides are biodegradable polymers, which have been studied as a vehicle for the release of 5-FU[7] and MMC.[8] One of their inconveniences is their association with dehiscence processes of the wound and implant extrusion.[9] Vinyls have also been used as 5-FU vehicles in primates.[10] Despite the fact that they are not biodegradable, they do not need to be removed, since they are inert materials. Collagen has also been used as a drug delivery system for antimitotics in animals[11] and humans.[12] However, despite its biodegradable nature, it has been associated with local inflammatory phenomena.

Poly(ortho esters) are biodegradable polymers that have prolonged GFS survival in rabbits, by means of 5-FU release.[13] Their principal limitation consists in the relatively long time required to produce them and their unreliable release kinetics.

Poly(hydroxy esters) (polylactic acid [PLA]; poly [lactic-co-glycolic acid] [PLGA] and polycaprolactone [PLC]) are biodegradable polymers that have been used in implant, microparticle, and nanoparticle treatments.[14] PLA has been used in experimental models of GFS in rabbits as a drug delivery system for 5-FU. Despite its good results, it has the

inconvenience of persistence and long-term encapsulation.[15] PLGA can be easily produced and has predictable decomposition kinetics. It is degraded by the hydrolysis of its corresponding monomers (lactic and glycolic acids), which are eliminated with minimal systemic toxicity. It has been used previously as a drug delivery system for 5-FU in experimental GFS in rabbits, with satisfactory short-term results.[16,17] PLGA has a shorter degradation half-life than PLA and it has been found to have minimal toxicity in rabbits as a drug delivery system for 5-FU.[16,18,19]

Thus, the purpose of this study was to investigate the clinical and histological outcomes of an MMC-loaded PLGA implant analyzed with different adjuvant treatments after experimental trabeculectomy in the rabbit eye.

Methods

Animals and Anesthesia

All animal experiments were conducted according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and approved by the Ethics Committee of the Basque Country University. Forty-two New Zealand White female rabbits (weight, 2.5–2.7 kg) were used in the study. Rabbits were anesthetized using a combination of ketamine (40 mg/kg; Ketolar 50 mg/mL; Pfizer, Madrid, Spain) and xylazine (8 mg/kg; Rompun 20 mg/mL; Bayer, Barcelona, Spain), administered by intramuscular injection before surgery.

Glaucoma Filtration Surgery

GFS was performed by the same surgeon in both eyes of each animal, considering the right eye as the control. Briefly, after placing a partial-thickness corneal traction suture, a fornix-based conjunctival flap was performed on the superior lateral quadrant of the eye. A half-thickness, rectangular, 4- × 3-mm scleral flap was then dissected, and a clear corneal paracentesis was carried out. Then, a 2- × 1.5-mm sclerectomy followed by peripheral iridectomy was performed. The scleral flap was closed with two 10-0 nylon sutures (Alcon Surgical, Fort Worth, TX). Finally, one drop of both atropine 1% eye drops (Colircusi Atropina; Alcon-Cusí, El Masnou, Barcelona, Spain) and the combination of tobramycin and dexamethasone (Tobradex; Alcon-Cusí) were instilled.

Implants were obtained by aggregation of PLGA 50:50 microspheres, prepared using the double emulsion/solvent extraction method (w/o/w).[20] Four sets of implants were obtained: drug-free implants, MMC-loaded implants, MMC-loaded and -coated implants, and MMC-loaded and 5-FU–coated implants.

Rabbits were randomly allocated to seven groups ($n = 6$ eyes per group), in relation to the adjuvant treatment administered to the left eye, described as follows:

1. Group 1: topical intraoperative MMC (surgical sponge soaked in 0.4 mg/mL solution, applied for 5 minutes);
2. Group 2: topical intraoperative 5-FU (surgical sponge soaked in 50 mg/mL solution, applied for 5 minutes);
3. Group 3: PLGA drug-free implant without adjuvant treatment;
4. Group 4: MMC-coated PLGA implant containing MMC microparticles;
5. Group 5: 5-FU-coated PLGA implant containing MMC microparticles;
6. Group 6: postoperative subconjunctival bevacizumab (0.05 mL; Avastin, Roche Farma, Madrid, Spain); and
7. Group 7: noncoated PLGA implant containing MMC microparticles and subconjunctival bevacizumab.

For the control group, the right eye of each animal was treated with trabeculectomy without adjunctive treatment (i.e., all animals in groups 1–7; $n = 42$ eyes).

Topical MMC and 5-FU were applied over the filtration site prior to the dissection of the scleral flap and irrigated thoroughly with 20 mL of balanced salt solution. Implants were placed adjacent to the surgical site, subconjunctivally, just before conjunctival closure. The concentration of MMC employed in the manufacture of the implants, as well as on their coating, was 1.25 mg/mL. The concentration of 5-FU employed as coating was 66.25 mg/mL. Bevacizumab was administered in a single subconjunctival injection at the bleb site, once surgery had concluded. The PLGA implant was prepared by accumulation of microspheres, obtained through the solvent evaporation/extraction method.

Clinical Examination

Evaluations were performed preoperatively (5 minutes before the surgery) and postoperatively at 0, 1, 5, 7, 14, 21, and 28 days after trabeculectomy. These examinations included measurement of intraocular pressure (IOP) and analysis of the bleb.

IOP was determined by rebound tonometry (Icare VET; Icare Finland, Helsinki, Finland) after instillation of a mixture of oxybuprocaine and tetracaine (Colircusi anestésico doble; Alcon-Cusí). Two sets of six measurements of IOP were performed, yielding an average value for both series. All determinations were performed by the same observer and at the same time of the day (10:00–12:00 AM).

The characteristics of the filtering bleb were analyzed in a blinded way according to the Moorfields bleb grading system (Clarke JC, et al. *IOVS* 2003;44:ARVO E-Abstract 1201). This classification scores the bleb with respect to a set of reference photographs, taking into account bleb height, extension, and vascularity.

Histological Analysis

On day 30, animals were killed by means of anesthetic overdose. After the enucleation of both eyes, specimens were fixed in 4% formaldehyde, cryopreserved in 30% sucrose, and included in OCT. Then, they were cut into 14-μm sections on a cryostat and stained with hematoxylin-eosin and Verhoeff (Verhoeff's hematoxylin). Sections were cut taking as a reference the suture points of the trabeculectomy, in such a way that the sections included the operated area and the control area (opposite limbal region). These preparations were examined and photographed using a microscope (Axioskop 2; Carl Zeiss Microscopy, LLC, Thornwood, NY), with particular attention to the characteristics of the conjunctival epithelium, stromal cell infiltration and persistence of the sclerotomy, filtering bleb, and implant.

Statistical Analysis

Statistics were performed using statistical software (SPSS; SPSS Sciences 19.0, Chicago, IL). Normality of variables was analyzed with the Kolmogorov-Smirnov test. IOP values were analyzed using parametric tests (Student's *t*-test, ANOVA of repeated measurements and Tukey's contrast test). The bleb was analyzed using nonparametric tests (Mann-Whitney *U* test, Wilcoxon W test, and the Kruskal-Wallis H test). The correlation between IOP and the filtering bleb variables was

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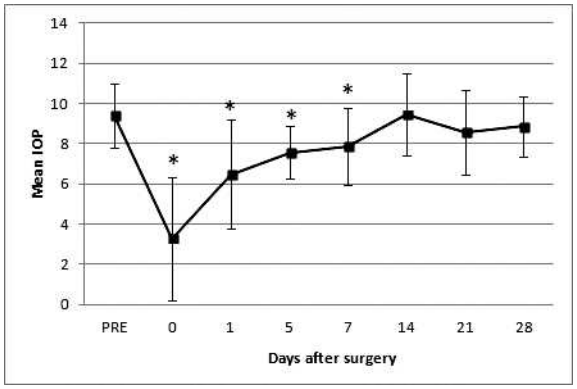

Figure 1. Evolution of mean IOP (mm Hg) over time, in the control group. *Statistically significant differences with respect to basal IOP.

evaluated using the Spearman correlation test. We also analyzed GFS survival using the Kaplan-Meier method, defining surgical failure as the return to the basal IOP ± 1 mm Hg or the disappearance of the filtering bleb (grade 1 extension bleb) and the log rank test to make comparisons between groups. The level of statistical significance was considered to be $P < 0.05$.

Results

IOP

Control Group. The control group (no adjunctive treatment; $n = 42$ eyes) exhibited IOP reduction with respect to preoperative values, at all stages of the study, with the exception of day 14. This reduction was statistically significant at 0, 1, 5, and 7 days after GFS ($P < 0.01$; Fig. 1, Table 1). Mean surgical survival in this group, as a function of IOP, was 11.9 days ± 1.14 (mean ± SE; range, 1–30 days; Fig. 2, Table 2).

Study Groups. Group 1 (topical MMC; $n = 6$ eyes) showed improved results with respect to the control group throughout the study period (Fig. 3, Table 3) and particularly at 14 ($P = 0.006$) and 21 days ($P = 0.005$) after surgery. Paradoxically, IOP was higher than control immediately after surgery ($P = 0.016$). Mean surgical survival for this group was 21.6 days ± 4.51 (mean ± SE; range, 5–30 days; Fig. 2, Table 2).

In group 2 (topical 5-FU; $n = 6$ eyes), IOP was found to decrease in a similar manner (Fig. 3, Table 3), although this decrease was significantly different with respect to the control group at 7 ($P = 0.03$) and 14 days ($P = 0.049$) postop. The only difference with group 1 was found at day 1, in which IOP with 5-FU was also higher than control. Mean surgical survival for this group was 16.6 days ± 4.08 (mean ± SE; range, 5–30 days), which was longer than that for the control group, but shorter than that of the topical MMC group (Fig. 2, Table 2).

In group 3 (empty PLGA implant; $n = 6$ eyes), IOP was reduced at 1, 5, 7, and 14 days postsurgery, with respect to the control group (Fig. 3, Table 3), with this reduction being statistically significant at 5 ($P = 0.012$) and 14 ($P = 0.029$) days. Surgical survival in this group was similar to that of the control group (13.1 days ± 3.32; mean ± SE; range, 7–30 days; Fig. 2, Table 2). These results are demonstrative of an isolated hypotensive effect due to the spacing effect of the implant.

Group 4 (MMC-loaded and -coated PLGA implant; $n = 6$ eyes) presented IOP reduction at all stages of the study (Fig. 3, Table 3), and significantly at 5 ($P = 0.002$); 7 ($P = 0.004$); 14 ($P = 0.001$); and 28 days ($P = 0.001$). The longest survival results are associated with this group (22 days ± 3.89; mean ± SE; range, 7–30 days; Fig. 2, Table 2).

Table 1. IOP Values in the Control Group

Time, d	Mean	SD
Pre	9.39	1.61
0	3.29	4.07
1	6.49	2.73
5	7.56	1.32
7	7.88	1.93
14	9.44	2.05
21	8.59	2.11
28	8.86	1.49

Group 5 (MMC-loaded, 5-FU–coated, PLGA implant; $n = 6$ eyes) exhibited values inferior to those of the control group throughout the study (Fig. 3, Table 3). These differences were statistically significant at 28 days postsurgery ($P = 0.027$). Mean surgical survival for this group was 16.5 days ± 4.45 (mean ± SE; range, 1–30 days), which is longer than that of the control group (Fig. 2, Table 2).

Group 6 animals (subconjunctival bevacizumab; $n = 6$ eyes) experienced IOP reduction after 1, 5, 14, and 28 days of GFS (Fig. 3, Table 3), with this reduction being statistically significant at 1 ($P = 0.03$); 14 ($P = 0.049$); and 28 days ($P = 0.004$). Despite this, mean GFS survival for this group was 11.3 ± 3.07 days (mean ± SE; range, 5–21 days); shorter than that of the control group (11.9 days; Fig. 2, Table 2).

In group 7 (MMC-loaded PLGA implant and subconjunctival bevacizumab; $n = 6$ eyes), we observed IOP reduction throughout the study (Fig. 3, Table 3), which was statistically significant only after 28 days ($P = 0.032$), as was observed in group 5. Mean survival for this group was 16.6 days ± 4.08 (mean ± SE; range, 5–30 days; Fig. 2, Table 2).

Upon comparing the different coadjuvant treatments, we found statistically significant differences between groups. The groups that contributed to these differences were group 4 (MMC-loaded and -coated PLGA implant) and group 8 (control). The most significant results were obtained in group 4, which presented the lowest values of IOP at days 1, 5, 7, 14, and 28. These results were exceeded only by group 2 at day 21 and by group 5 immediately postsurgery.

Bleb Grading

Control Group. In this group, we observed a progressive disappearance of the filtering bleb (Fig. 4), with an associated mean survival of 12.9 days ± 1 (mean ± SE; range, 5–30 days). In statistical terms, the bleb was patent in its extension on days 1, 5, and 7 ($P < 0.001$) and in its height on days 1 ($P < 0.001$) and 5 ($P = 0.005$). More intense vascularization of the bleb was seen on days 1, 5, 7, 14 ($P < 0.001$), and 21 ($P = 0.02$).

Study Groups. Similarly, we observed a decreasing bleb grade pattern in all study groups (Fig. 4). Mean surgical survival, as function of bleb extension, was longer than that of the control group in topical MMC group (22 days ± 3.26; mean ± SE; range, 14–30 days) and topical 5-FU group (14.1 days ± 3.03; mean ± SE; range, 7–30 days), and shorter in subconjunctival bevacizumab group (11.1 days ± 3.48; mean ± SE; range, 5–30 days). In the other groups, survival could not be determined due to the bias induced by the spacing effect of the implant.

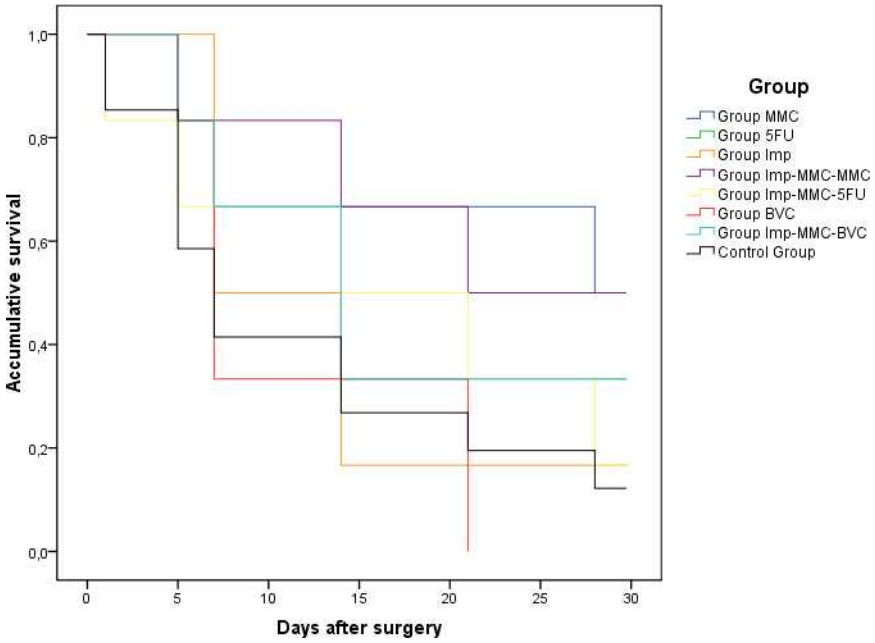

Figure 2. Analysis of surgical survival as a function of IOP by means of the Kaplan-Meier method.

Filtering bleb vascularization presented a decreasing pattern in all groups, which was not statistically different to that of the controls, with the exception of the implant groups, with higher hyperemia ($P < 0.05$ in all cases, from day 1) and of subconjunctival bevacizumab group, which was less hyperemic on day 1 ($P = 0.003$; Fig. 4).

Group comparison revealed a larger bleb size in the groups with implant. Of these, the MMC-loaded implant and subconjunctival bevacizumab groups presented larger values of height, extension, and vascularization of the bleb conjunctiva.

Analysis of correlation revealed a negative correlation between IOP and the area and height of the filtering bleb. In the case of area, this correlation was significant at day 28 ($r_s = -0.259$; $P = 0.03$). In the case of height, significance was found on days 5 ($r_s = -0.262$; $P = 0.017$); 14 ($r_s = -0.28$; $P = 0.032$); and 28 ($r_s = -0.329$; $P = 0.005$).

Similar GFS survival periods were found independent of the criterion of surgical survival that was employed (IOP or bleb grade). Thus, once we had excluded the implant groups, due to the bias introduced in bleb grading, we found better results in group 1, followed by groups 2, 8, and 6, respectively.

Anatomopathology

Histological analysis of the eye globes revealed closure of the surgical fistula in all cases. Upon comparing the sclera of the operated area with that of the control area in both experimental and control eyes, an increase in the density of scleral collagen could be seen in the operated area (Fig. 5).

We observed epithelial thinning in groups 1, 2, 4, 5, and 7, accompanied by lymphoplasmacytic stromal infiltration. In contrast, no signs of this thinning were apparent in group 3, despite presenting the same type of stromal infiltration. Group 6 showed clear epithelial alterations, lymphoplasmacytic infiltration, and stromal fibrosis (Fig. 6). In implant-containing animals, the infiltrate consisted principally of lymphoplasmacytic cells, occasionally macrophages, polymorphonuclear cells, and multinucleated gigantic cells (Fig. 7). In the majority of animals (76% of cases), we did not find evidence of filtering bleb at the time of necropsy.

We verified implant persistence in 95% of eyes. However, despite the different degree of persistence of the implant (Fig. 8), there was no evidence of statistical differences among groups in this regard. In group 3, we found a negative correlation between IOP and implant persistence ($r_s = -0.956$; $P = 0.003$). In contrast, in groups 4, 5, and 7 (pharmacologically loaded implants), we found a positive IOP-implant persistence correlation ($r_s = 0.67$, 0.53, and 0.73, respectively; $P > 0.05$; Fig. 9). Finally, it should be noted that some of the implants were found to be encapsulated, principally those of group 7 (85% of cases; Fig. 8).

Table 2. Mean Survival (Days) of the GFS as a Function of IOP in Each Group

Group	Mean Survival
1	21.6
2	16.6
3	13.1
4	22
5	16.5
6	11.3
7	16.6
8	11.9

Discussion

In the present study, we have used the albino rabbit, since it is the most typically used animal in experimental studies of GFS. In addition, this animal is easy to manage and house and is inexpensive. Given its efficient wound healing characteristics, it allows a shortening of follow-up times and would be equivalent to a model of high-risk surgical failure, with accompanying antimitotic treatment.[21]

The standard in wound healing studies after GFS consists in the intervention of a healthy eye, without prior induction of glaucoma. In this way, the iatrogenic effects produced by the induction of glaucoma are avoided. However, by intervening normotensive eyes, IOP measurement as a

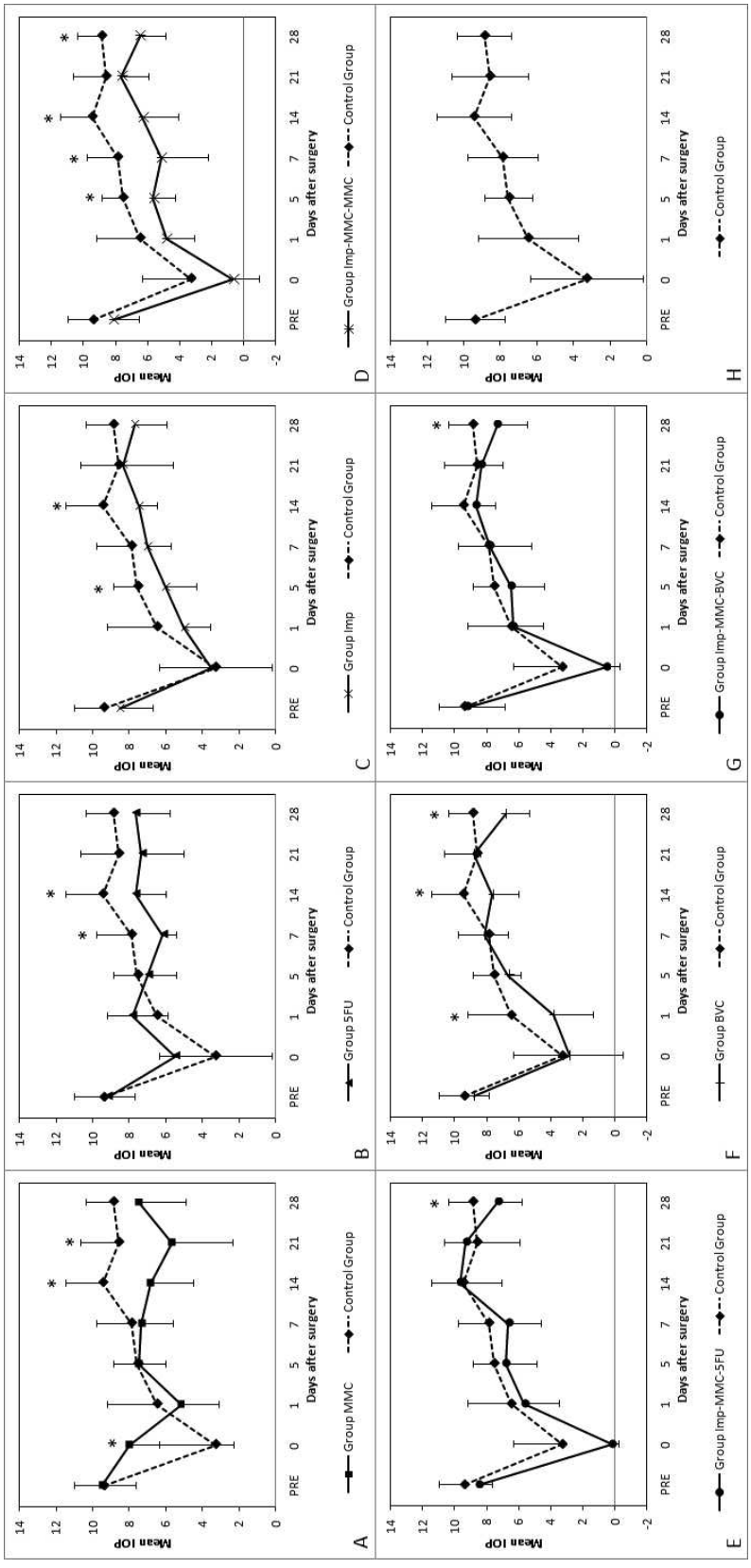

Figure 3. Comparison over time of the mean IOP values (mm Hg) between control and each study group. (**A**) Topical MMC (MMC). (**B**) Topical 5-FU (5FU). (**C**) Implant (Imp). (**D**) MMC-loaded and -coated implant (Imp-MMC-MMC). (**E**) MMC-loaded and 5-FU-coated implant (Imp-MMC-5FU). (**F**) Subconjunctival bevacizumab (BVC). (**G**) MMC-loaded implant and subconjunctival bevacizumab (Imp-MMC-BVC). (**H**) Graphic showing IOP values in the control group. *Bars* indicate the standard deviation. *Indicates statistically significant differences in comparison with the control group.

Table 3. Evolution of IOP (Mean ± SD) Over Time in All Study Groups

	Days Postsurgery							
	Pre	**0**	**1**	**5**	**7**	**14**	**21**	**28**
Control	9.39 ± 1.61	3.29 ± 3.07	6.49 ± 2.73	7.56 ± 1.32	7.88 ± 1.93	9.44 ± 2.05	8.59 ± 2.11	8.86 ± 1.49
Group 1 (MMC)	9.50 ± 1.87	8.00 ± 5.72	5.17 ± 2.04	7.50 ± 1.51	7.33 ± 1.75	6.83 ± 2.31*	5.67 ± 3.32*	8.86 ± 1.49
Group 2 (5FU)	9.17 ± 1.47	5.50 ± 5.28	7.83 ± 1.94	7.00 ± 1.54	6.17 ± 0.75*	7.67 ± 1.63*	7.33 ± 2.33	7.67 ± 1.86
Group 3 (Imp)	8.50 ± 1.76	3.50 ± 3.32	5.00 ± 1.41	6.00 ± 1.67*	7.00 ± 1.26	7.50 ± 1.04*	8.33 ± 2.73	8.50 ± 1.76
Group 4 (Imp-MMC-MMC)	8.17 ± 1.60	0.67 ± 1.63	4.83 ± 1.72	5.67 ± 1.36*	5.17 ± 2.92*	6.33 ± 2.25*	7.67 ± 1.75	6.50 ± 1.64*
Group 5 (Imp-MMC-5FU)	8.50 ± 0.83	0.17 ± 0.40	5.67 ± 2.16	6.83 ± 1.94	6.67 ± 2.06	9.67 ± 2.58	8.33 ± 3.38	7.33 ± 1.50*
Group 6 (BVC)	8.83 ± 0.98	2.83 ± 3.35	3.83 ± 2.48*	6.67 ± 0.82	8.17 ± 1.47	7.67 ± 1.63*	8.83 ± 0.41	6.83 ± 1.47*
Group 7 (Imp-MMC-BVC)	9.17 ± 2.31	0.50 ± 0.83	6.33 ± 1.86	6.50 ± 2.07	7.83 ± 2.63	8.67 ± 1.21	8.33 ± 1.36	7.33 ± 1.86*

* $P < 0.05$ with respect to the control group.

parameter during follow-up can be invalidated.[22] In fact, in models similar to ours, some authors reject IOP as a criterion of surgical functionality, considering only filtering bleb,[13,23–26] while others claim that the IOP would be higher than the bleb measurement.[18,26] In the present study, we found that both parameters are efficacious, presenting—as expected—an inverse correlation. It should be recognized, however, that in measuring bleb size, we excluded rabbits with implants, since the anatomical effect of the implant was indistinguishable from that of the filtering bleb.

The surgical technique employed herein has been used previously and it is very similar to what we routinely use in daily clinical practice.[8,13] Some other authors tend to use other techniques such as posterior-lip sclerotomy[7,27] or perform sclerotomy with the placing of a cannula draining into the subconjunctival space.[23,28,29] Upon comparing the different techniques, Esson concluded that sclerotomy with cannula induces lower synthesis of wound-healing mediators than posterior-lip sclerotomy.[30] This study demonstrates that the GFS experimental model described herein can efficaciously reduce IOP, and facilitate the formation of the filtering bleb, with similar surgical survival rates, independent of the failure criterion employed. We believe that the influence of anesthesia on IOP values has been minimal in the study, since the examinations were performed under topical anesthesia. However, the immediate postoperative determination could be influenced by the anesthesia, since it has been reported that the effect of ketamine-xylazine mixture remains 30 minutes after the anesthetic induction.[31]

The doses and administration routes of 5-FU, MMC, and bevacizumab have been adopted from other studies.[6,23,32–34] In the case of bevacizumab, we have chosen to study its administration in a single dose,[6] in order to be able to compare its effects with those of other treatments. Other authors have recommended its repeated administration, due to its short half-life.[25]

All of the evaluated treatments exhibit a hypotensive effect that was superior to that found in the control group. Excluding the implant-containing groups, bleb formation was also higher in the experimental groups than in the control group. The bevacizumab group is the only excep-

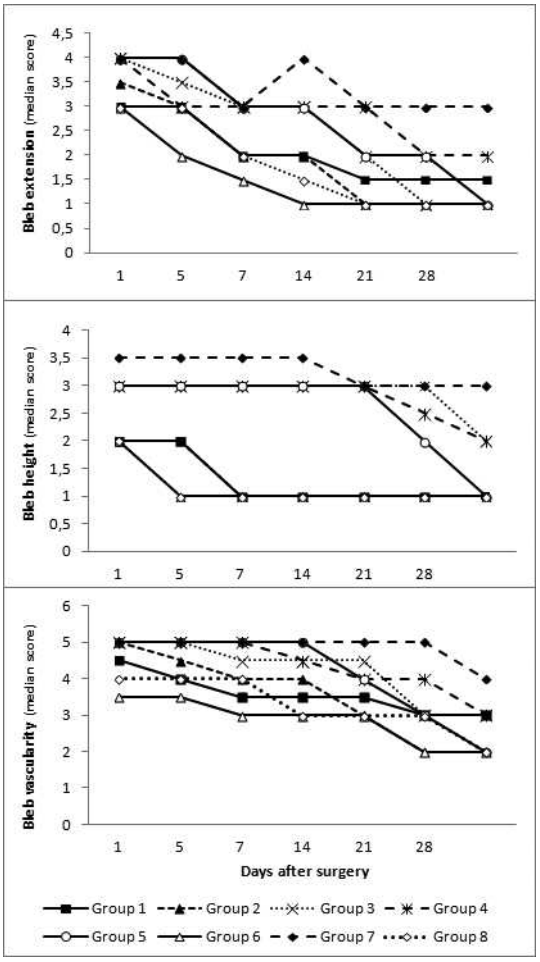

Figure 4. Evolution of median bleb grading as a function of extension (**A**), height (**B**), and vascularity (**C**). Note the bias induced by the spacing effect of the implant on bleb extension and height in groups 3 (implant); 4 (MMC-loaded and -coated implant); 5 (MMC-loaded and 5-FU-coated implant); and 7 (MMC-loaded implant and subconjunctival bevacizumab).

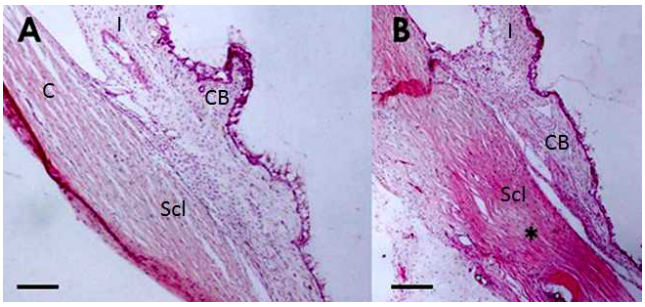

Figure 5. Anatomical view of the iridocorneal angle in opposite limbus (**A**) and operated (**B**) zones in all the animals. An increase in scleral density can be observed (*). Hematoxylin-eosin staining. C, cornea; CB, ciliary body; I, iris; Scl, sclera. *Scale bars*: 200 μm.

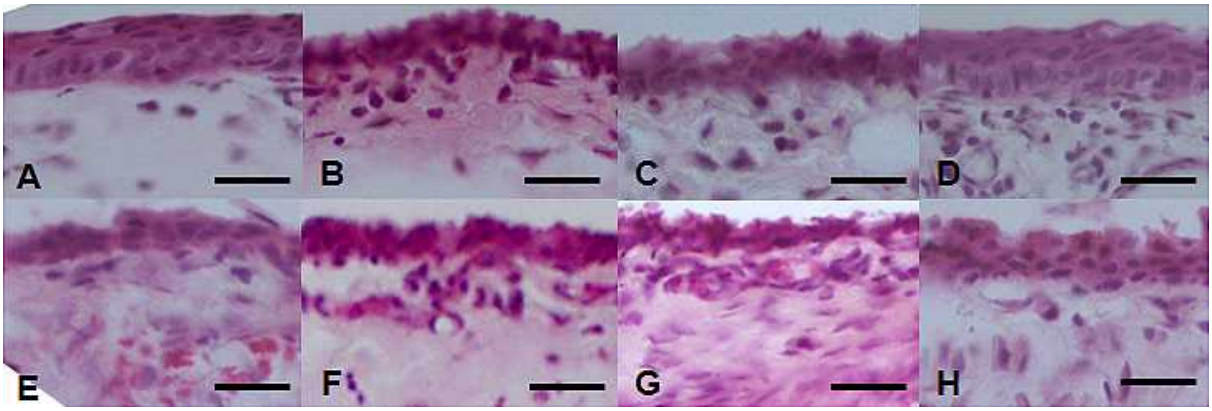

Figure 6. View of the conjunctival epithelium and stroma in the filtering bleb. (**A**) Epithelium in the control group. (**B**, **C**) Thinned epithelium in groups 1 and 2, which involve topical antimitotics. (**D**) Normal thickness epithelium in the empty implant (group 3). (**E**, **F**, **H**) Thinned epithelium with underlying infiltration in groups 4, 5, and 7. (**G**) Thinned epithelium, with infiltration and subepithelial fibrosis in the bevacizumab group 6. Hematoxylin-eosin staining. *Scale bars*: 50 μm.

tion, since surgical survival in this group was shorter than that of the control group, independently of the failure criterion employed.

Topical MMC was surpassed in reducing IOP only by implants that were loaded and covered with MMC. The initial elevation of IOP could be due to postoperative inflammation, whose peak occurs in the first week.[35] The subsequent IOP reduction could be caused by the maximum effect of the drug in this experimental model. We found no evidence of corneal complications (opacity and neovascularization), hypotony, or endophthalmitis, in contrast to that reported by other authors.[18,33] The histological alterations observed in the conjunctiva are very similar to those reported by Sherwood, despite the difference in the time of analysis (2 weeks vs. 1 month in our study).[28]

Topical 5-FU achieved a hypotensive effect that was lower than that of MMC administered topically or via implant, but higher than that associated with the isolated implant and bevacizumab. These results corroborate those of other authors in terms of hypotensive effects,[13,15] but differ in terms of bleb formation. Thus, Einmahl et al.[13] reported an 83% persistence of bleb 1 month after injection of poly(ortho esters) loaded with 5-FU. As in the case of MMC, we did not find any complications such as corneal edema[13,15] or avascular filtering bleb.[33]

Subconjunctival bevacizumab treatment achieved reduced IOP in comparison with controls. However, this reduction was somewhat irregular and consequently, surgical survival was not improved. In addition, we found no improvement with bevacizumab in terms of filtering bleb formation. These findings contrast with those of other authors who have reported a longer life of the bleb, in the absence of IOP differences, when administered either alone[36] or repeatedly.[25] As reported previously,[25,37] the blebs of this group were found to be less hyperemic at the early postoperative stage. Histologically, we found more substantial conjunctival alterations than those reported by Memarzadeh et al.,[25] despite the fact that these authors performed multiple postoperative injections.

The use of drug-free PLGA implants was also associated with reduced IOP, corroborating the spacing effect of the implant, as has been previously reported.[17] Our implants led to hyperemia throughout the study period, without associated complications arising, in line with the findings of Cui et al. for PLA.[15] We did not observe implant encapsulation in any cases of this group, as might occur with other experimental groups. Other authors have already reported the presence of multinucleated gigantic cells,[10,13,16,27] which likely represent a foreign body response to the implants.

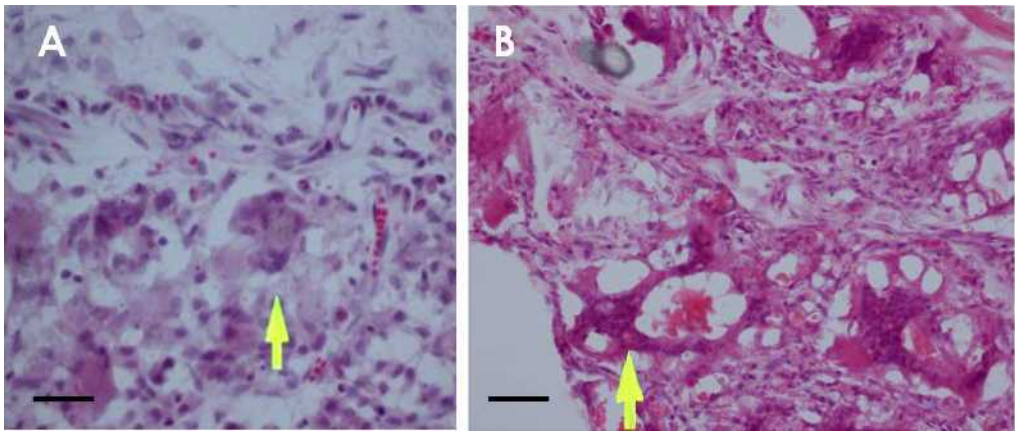

Figure 7. Presence of multinucleated gigantic cells (*arrows*) during the degradation phase of the implant, 1 month postsurgery. Hematoxylin-eosin staining. *Scale bars*: 50 μm (**A**, **B**).

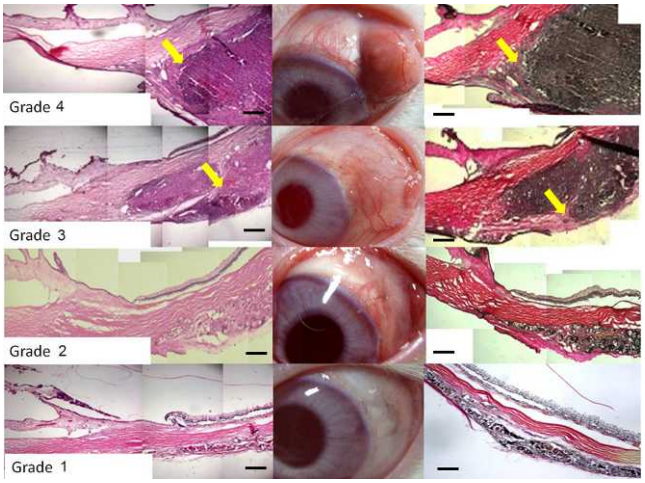

Figure 8. Degree of persistence of the implant, 30 days postsurgery. A fibrous capsule can be observed (*arrows*). The examples shown belong to groups Imp-MMC-MMC (grade 2); Imp-MMC-5FU (grade 1); and Imp-MMC-BVC (grades 3 and 4). Hematoxylin-eosin (*left column*) and Verhoeff (*right column*) staining. *Scale bars*: 300 μm.

The mixed MMC application (encapsulated and covering the implant) was found to be the treatment with highest hypotensive efficacy. Given our previous in vitro results, the release of MMC from the microspheres starts from the first week (data not shown). Thus, it is likely that the MMC that covers the microspheres acts during the earlier stages, and is substituted by that liberated by the microspheres at a later stage. As detected in the other groups involving drug-loaded implants, we observed a correlation between IOP and the degree of persistence of the implant. This may be due to the encapsulation of the implant, which in turn would lead to inhibition of MMC diffusion to tissue, resulting in increased IOP.

MMC implants coated with 5-FU exhibit improved IOP levels with respect to the control group. However, these improvements are not as large as those associated with the MMC-loaded and -coated implants group and the topical MMC group. Lu obtained similar results upon comparing the effect of PLGA microparticles loaded with 5-FU and covered with MMC versus topical MMC, despite the fact that the MMC dose in these studies was different.[18] The similarity between this group and the topical 5-FU group would indicate the absence of an additive effect between MMC and 5-FU in our study. Curiously, in this group, we observed a higher rate of implant degradation at the end of the study in comparison with the other implant groups. This finding has been previously reported by others regarding PLGA[16] and polyorthoesters.[13]

Finally, the group with MMC implants and bevacizumab exhibited reduced IOP, but to a lesser extent than that observed with the other implant groups, indicating that there may be a certain interaction between these drugs. In fact, Hilgert reported a higher degree of fibrosis upon combining both drugs, in comparison to using MMC alone (Hilgert CR, et al. *IOVS* 2011;52:ARVO E-Abstract 634). This interaction can be seen histologically as a higher degree of implant encapsulation in this group. In contrast, other authors have reported a synergistic effect of combining topical MMC and subconjunctival bevacizumab in humans.[38] However, in our study, we cannot exclude a possible role of PLGA in the interaction between the two drugs.

In conclusion, in the present animal model, MMC-loaded and -coated PLGA implants provide optimal hypotensive results after GFS, indicating that this methodology may provide improved surgical outcomes in humans.

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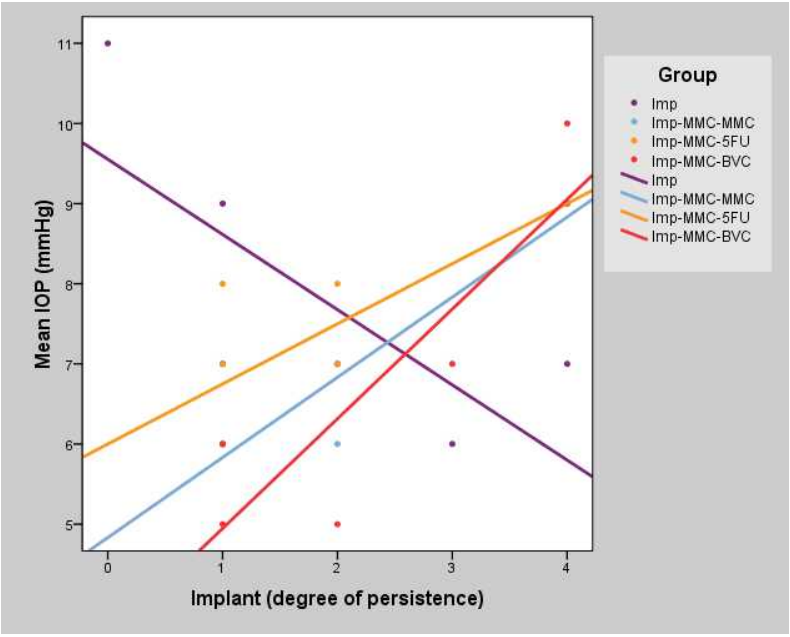

Figure 9. Scatter plot showing the relation between IOP and the degree of persistence of the implant in each group.

References

1. Hitchings RA, Grierson I. Clinicopathological correlation in eyes with failed fistulizing surgery. *Trans Ophthalmol Soc U K*. 1983;103:84–88.
2. Kirwan JF, Rennie C, Evans JR. Beta radiation for glaucoma surgery. *Cochrane Database Syst Rev*. 2009;2:CD003433.
3. Heuer DK, Parrish RK II, Gressel MG, Hodapp E, Palmberg PF, Anderson DR. 5-fluorouracil and glaucoma filtering surgery. II. A pilot study. *Ophthalmology*. 1984;91:384–394.
4. Chen CW, Huang HT, Bair JS, Lee CC. Trabeculectomy with simultaneous topical application of mitomycin-C in refractory glaucoma. *J Ocul Pharmacol*. 1990;6:175–182.
5. CAT-152 0102 Trabeculectomy Study Group; Khaw P, Grehn F, Hollo G, Overton B, et al. A phase III study of subconjunctival human anti-transforming growth factor beta (2) monoclonal antibody (CAT-152) to prevent scarring after first-time trabeculectomy. *Ophthalmology*. 2007;114:1822–1830.
6. Grewal DS, Jain R, Kumar H, Grewal SP. Evaluation of subconjunctival bevacizumab as an adjunct to trabeculectomy a pilot study. *Ophthalmology*. 2008;115:2141–2145.
7. Lee DA, Leong KW, Panek WC, Eng CT, Glasgow BJ. The use of bioerodible polymers and 5-fluorouracil in glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 1988;29:1692–1697.
8. Charles JB, Ganthier R Jr, Wilson MR, et al. Use of bioerodible polymers impregnated with mitomycin in glaucoma filtration surgery in rabbits. *Ophthalmology*. 1991;98:503–508.
9. Jampel HD, Leong KW, Dunkelburger GL, Quigley HA. Glaucoma filtration surgery in monkeys using 5-fluorouridine in polyanhydride disks. *Arch Ophthalmol*. 1990;108:430–435.
10. Blandford DL, Smith TJ, Brown JD, Pearson PA, Ashton P. Subconjunctival sustained release 5-fluorouracil. *Invest Ophthalmol Vis Sci*. 1992;33:3430–3435.
11. Hasty B, Heuer DK, Minckler DS. Primate trabeculectomies with 5-fluorouracil collagen implants. *Am J Ophthalmol*. 1990;109:721–725.
12. Herschler J. Long-term results of trabeculectomy with collagen sponge implant containing low-dose antimetabolite. *Ophthalmology*. 1992;99:666–670.
13. Einmahl S, Behar-Cohen F, D'Hermies F, et al. A new poly (ortho ester)-based drug delivery system as an adjunct treatment in filtering surgery. *Invest Ophthalmol Vis Sci*. 2001;42:695–700.
14. Athanasiou KA, Niederauer GG, Agrawal CM. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic and copolymers. *Biomaterials*. 1996;17:93–102.
15. Cui LJ, Sun NX, Li XH, Huang J, Yang JG. Subconjunctival sustained release 5-fluorouracil for glaucoma filtration surgery. *Acta Pharmacol Sin*. 2008;29:1021–1028.
16. Gould L, Trope G, Cheng YL, et al. Fifty: fifty (DL glycolic acid-lactic acid) copolymer as a drug delivery system for 5-fluorouracil: a histopathological evaluation. *Can J Ophthalmol*. 1994;29:168–171.
17. Trope GE, Cheng YL, Sheardown H, et al. Depot drug delivery system for 5-fluorouracil after filtration surgery in the rabbit. *Can J Ophthalmol*. 1994;29:263–267.
18. Lu DW, Chang CJ, Chiang CH, Yeh MK, Chou PI. Wound modulation after trabeculectomy by different formulations of antimetabolites in rabbits. *J Ocul Pharmacol Ther*. 2000;16:529–538.
19. Chiang CH, Tung SM, Lu DW, Yeh MK. In vitro and in vivo evaluation of an ocular delivery system of 5-fluorouracil microspheres. *J Ocul Pharmacol Ther*. 2001;17:545–553.
20. Igartua M, Hernández RM, Rosas JE, Patarroyo ME, Pedraz JE. Gamma-irradiation effects on biopharmaceutical properties of PLGA microspheres loaded with SPf66 synthetic vaccine. *Eur J Pharm Biopharm*. 2008;69:519–526.
21. Miller MH, Grierson I, Unger WI, Hitchings RA. Wound healing in an animal model of glaucoma fistulizing surgery in the rabbit. *Ophthalmic Surg*. 1989;20:350–357.
22. McGuigan LJ, Mason RP, Sanchez R, Quigley HA. D-penicillamine and beta-aminopropionitrile effects on experimental filtering surgery. *Invest Ophthalmol Vis Sci*. 1987;28:1625–1629.
23. Cordeiro MF, Constable PH, Alexander RA, Bhattacharya SS, Khaw PT. Effect of varying the mitomycin-C treatment area in glaucoma filtration surgery in the rabbit. *Invest Ophthalmol Vis Sci*. 1997;38:1639–1646.
24. Mead AL, Wong TT, Cordeiro MF, Anderson IK, Khaw PT. Evaluation of anti-TGF-beta2 antibody as a new postoperative anti-scarring agent in glaucoma surgery. *Invest Ophthalmol Vis Sci*. 2003;44:3394–3401.
25. Memarzadeh F, Varma R, Lin LT, et al. Postoperative use of bevacizumab as an antifibrotic agent in glaucoma filtration surgery in the rabbit. *Invest Ophthalmol Vis Sci*. 2009;50:3233–3237.
26. Grisanti S, Szurman P, Warga M, et al. Decorin modulates wound healing in experimental glaucoma filtration surgery: a pilot study. *Invest Ophthalmol Vis Sci*. 2005;46:191–196.
27. Kimura H, Ogura Y, Moritera T, et al. Injectable microspheres with controlled drug release for glaucoma filtering surgery. *Invest Ophthalmol Vis Sci*. 1992;33:3436–3441.
28. Sherwood MB. A sequential, multiple-treatment, targeted approach to reduce wound healing and failure of glaucoma filtration surgery in a rabbit model (an American Ophthalmological Society thesis). *Trans Am Ophthalmol Soc*. 2006;104:478–492.
29. Wong TT, Mead AL, Khaw PT. Matrix metalloproteinase inhibition modulates postoperative scarring after experimental glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 2003;44:1097–1103.
30. Esson DW, Neelakantan A, Iyer SA, et al. Expression of connective tissue growth factor after glaucoma filtration surgery in a rabbit model. *Invest Ophthalmol Vis Sci*. 2004;45:485–491.
31. Ding CH, Wang P, Tian N. Effect of general anesthetics on IOP in elevated IOP mouse model. *Exp Eye Res*. 2011;92:512–520.
32. Khaw PT, Doyle JW, Sherwood MB, Grierson I, Schultz G, McGorray S. Prolonged localized tissue effects from 5-minute exposures to fluorouracil and mitomycin C. *Arch Ophthalmol*. 1993;111:263–267.
33. Khaw PT, Doyle JW, Sherwood MB, Smith MF, McGorray S. Effects of intraoperative 5-fluorouracil or mitomycin C on glaucoma filtration surgery in the rabbit. *Ophthalmology*. 1993;100:367–372.
34. Oh SY, Youn, DH, Kim DM, Hong Ch. The effects of intraoperative mitomycin-C or 5-fluorouracil on glaucoma filtering surgery. *Korean J Ophthalmol*. 1994;8:6–13.
35. Esson DW, Popp MP, Liu L, Schultz GS, Sherwood MB. Microarray analysis of the failure of filtering blebs in a rat model of glaucoma filtering surgery. *Invest Ophthalmol Vis Sci*. 2004;45:4450–4462.
36. Li Z, Van Bergen T, Van de Veire S, et al. Inhibition of vascular endothelial growth factor reduces scar formation after glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 2009;50:5217–5225.
37. How A, Chua J, Charlton A, et al. Combined treatment with bevacizumab and 5-fluorouracil attenuates the postoperative scarring response after experimental glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 2010;51:928–932.
38. Choi JY, Choi J, Kim YD. Subconjunctival bevacizumab as an adjunct to trabeculectomy in eyes with refractory glaucoma: a case series. *Korean J Ophthalmol*. 2010;24:47–52.

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