• Basic Research •

Single subconjunctival injection formulation with a 5-fluorouracil-poly(lactic) acid controlled-release system for glaucoma filtration surgery

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Received: 2025-04-27 Accepted: 2025-07-09

Abstract

● **AIM:** To develop a 5-fluorouracil (5-FU) mesoporous poly(lactic) acid (PLA) delivery system for glaucoma filtration surgery suitable for a single subconjunctival implantation.

● **METHODS:** The 5-FU was infiltration-loaded into mesoporous PLA. *In vitro* and *in vivo* release experiments and ocular toxicology evaluation of the formulation were performed. The antiproliferative effect of this 5-FU-PLA tablet after glaucoma filtration surgery in rabbits was evaluated. Pathology, immunohistochemistry, and Western blot were used to further validate the inhibitory effect of this sustained release system.

● **RESULTS:** Various drug formulations were tested, and two 5-FU-PLA tablets, namely 1.5P15 (5-FU 1.5 mg+PLA 15 000 Da) and 2.5P15 (5-FU 2.5 mg+PLA 15 000 Da), had the most suitable release profiles *in vitro*. Further *in vivo* studies confirmed the safety and sustained-release profiles of both drugs. Both 5-FU-PLA tablets, relative to the free drugs, significantly inhibited tissue proliferation after glaucoma filtration and improved surgical success. Western blot showed that transforming growth factor-β (TGF-β) and connective tissue growth factor (CTGF) were inhibited by 5-FU after filtration surgery, with the effects of the 5-FU-PLA tablets being more lasting.

● **CONCLUSION:** The tested 5-FU-PLA tablets provide a sustained release of 5-FU, which may be used for a single subconjunctival implantation to inhibit proliferation after filtration surgery.

● **KEYWORDS:** 5-fluorouracil mesoporous poly(lactic) acid; sustained release system; filtration surgery; glaucoma
DOI:10.18240/ijo.2025.10.02

Citation: Zhang JJ, Gao F, Deng KX, Guan WX, Sun YY. Single subconjunctival injection formulation with a 5-fluorouracil-poly(lactic) acid controlled-release system for glaucoma filtration surgery. *Int J Ophthalmol* 2025;18(10):1823-1833

INTRODUCTION

Glaucoma, characterized by elevated intraocular pressure (IOP) and progressive visual field defects, is the most common cause of permanent blindness globally[1-2]. According to estimates, glaucoma affects 3.5% of persons between the ages of 40 and 80y globally. In 2040, it is anticipated that 111.8 million individuals will have glaucoma globally due to the aging of societies[3-4].

Reducing IOP remains the core of glaucoma treatment to date. Drug therapy is the first line of defense against glaucoma, followed by laser therapy. If IOP is uncontrollable with standard treatment, surgical interventions, such as filtering surgery and drainage device implantation, become necessary[5-6]. Currently, filtration surgery is the most effective of glaucoma surgeries because of its better control of IOP[7]; it reduces IOP by creating a new filtration channel for aqueous humor outflow. The patency of the filtration channel and the formation of functional filter blebs are keys to the success of the surgery. Patients with glaucoma may experience filtration failure due to subconjunctival scarring and scleral bleb fibrotic adhesion over time, which can increase IOP. According to the literature, the incidence of filter channel obstruction after surgery can be up to 30%.

Table 1 Drug loading and encapsulation efficiencies of different 5-FU-PLA tablets

Sample	Composition, 5-FU/PLA (mg/Da)	5-FU content (mg)	Mass of each tablet (mg)	Mass of dosing (mg)	Loading efficiency (%)	Encapsulation efficiency (%)
1.5P3	1.5/3000	1.503±0.039	5.983±0.117	1.803±0.024	25.12	83.36
1.5P6	1.5/6000	1.497±0.015	6.083±0.075	1.790±0.018	24.61	83.63
1.5P10	1.5/10000	1.507±0.022	6.050±0.105	1.781±0.025	24.91	84.61
1.5P15	1.5/15000	1.502±0.010	6.167±0.083	1.782±0.021	24.36	84.29
1.5P20	1.5/20000	1.513±0.007	6.117±0.075	1.778± 0.018	24.73	85.01
2.5P3	2.5/3000	2.500±0.013	5.933±0.121	2.933±0.030	42.19	85.24
2.5P6	2.5/6000	2.505±0.018	6.000±0.110	2.931±0.027	41.75	85.47
2.5P10	2.5/10000	2.512±0.013	6.083±0.117	2.922±0.029	41.39	86.17
2.5P15	2.5/15000	2.502±0.015	6.183±0.075	2.920±0.018	40.47	85.68
2.5P20	2.5/20000	2.510±0.011	6.200±0.089	2.915±0.023	40.65	86.11
3.0P3	3.0/3000	3.016±0.123	6.033±0.197	3.688±0.049	51.15	81.78
3.0P6	3.0/6000	3.036±0.128	6.000±0.089	3.685±0.022	50.60	82.39
3.0P10	3.0/10000	3.029±0.120	6.017±0.183	3.680±0.046	50.34	82.31
3.0P15	3.0/15000	3.036±0. 080	6.183±0.075	3.678±0.018	48.62	82.54
3.0P20	3.0/20000	3.066±0.077	6.267±0.082	3.672±0.021	48.44	83.50

5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

The first anti-proliferative drug used during or after filtration surgery is 5-fluorouracil (5-FU); it has anti-metabolic activity and inhibits the wound-healing response, which, in turn, improves the surgery success rate[8-9]. However, the short half-life of 5-FU necessitates frequent postoperative subconjunctival injections, which increases the incidence of side effects such as bleb leakage, persistent ocular hypotony, endophthalmitis, and patient discomfort[10-12]. Therefore, finding a sustained release system for 5-FU is important for the success of glaucoma surgery.

Poly(lactic) acid (PLA) is a synthetic polymer that has been employed as a carrier material for injectable microcapsules, microspheres, and implants. However, its usage in ophthalmology is limited. It has been shown that a 5-FU-PLA sustained release system with high 5-FU concentrations can sustain 5-FU release for more than three months[13]. However, a prolonged sustained release has negative effects on both wound healing, and 5-FU can be toxic to the cornea and conjunctiva. As a result, this study investigated various long-acting 5-FU polylactic acid extended-release system parameters and assessed the effects of this extended-release system related to reducing local toxic reactions after glaucoma filtration surgery and inhibiting the proliferation of filtration channel fibroblasts *in vivo* and *in vitro*.

MATERIALS AND METHODS

Ethical Approval All animal experiments were performed in strict accordance with the Association for Research in Vision and Ophthalmic and Vision Resolution for the Use of Animals in Ophthalmic and Vision Research and with the guidelines provided by the Animal Care Use Committee of Peking University (Beijing, China, IACUC number: 2016PHC022).

Preparation of 5-FU-PLA Tablets Different masses of 5-FU and different molecular weights of PLA (Table 1) were weighed and dissolved in 3 mL of CH_2CL_2 containing 0.02 g span-80, and 15 mL of petroleum ether was added dropwise with high-speed stirring. Fifteen milliliters of petroleum ether was added after the microspheres were aggregated and precipitated for 10min for solidification. After centrifugation, the precipitate was removed and placed in a glass mold coated with glycerol. The surface was dried and cut in a 3-mm diameter tubular mold. The prepared 5-FU-PLA tablets were oven-dried at 50°C and stored at 4°C. The surface characteristics was observed under light microscopy and scanning electron microscopy (SEM).

Determination of Loading and Encapsulation Efficiency One dried 5-FU-PLA tablet was weighed and dissolved in 2 mL of CH_2Cl_2, followed by extraction with 0.1 mol/L and redissolution in 5 mL HCl solution three times. The corresponding 5-FU concentration was determined with a ultraviolet radiation (UV) spectrophotometer, and the loading and encapsulation efficiencies of the 5-FU-PLA tablets were calculated according to the following formulas:

$$Drug\ loading\ efficiency = \frac{mass_{5FU}}{mass_{5FUPLA}} \times 100\%$$

where $mass_{5FU}$ refers to the 5-FU concentration in the 5-FU-PLA tablet, and $mass_{5FUPLA}$ refers to the mass of the 5-FU-PLA tablet.

$$Encapsulation\ rate = \frac{mass_{5FU}}{total\ mass_{5FU}} \times 100\%$$

where $massc_{5FU}$ refers to the 5-FU concentration in the 5-FU-PLA tablet, and $total\ mass_{5FU}$ refers to the total mass of 5-FU in the loading system. Six tablets were randomly selected from

the same batch, and their average 5-FU concentration was calculated after repeated measurements.

***In vitro* Release** Six tablets were randomly selected from the same batch, weighed, and placed at the bottom of a 3-mm diameter tubular release device and incubated with 1 mL of simulated body fluid at 37°C. The entire solution was changed after 2, 4, 8, 16, and 24h on the first day; daily in the first week; every other day in the second week; every 4d in the third to fifth weeks; and weekly after the sixth week. The concentration of 5-FU was measured using a UV spectrophotometer. The concentrations of six samples were averaged to calculate the cumulative release and plot the *in vitro* release curve for the 5-FU-PLA release tablet. According to previous reports, the minimum effective 5-FU concentration was 10 μg/mL. Thus, the sampling was discontinued if the daily average release concentration was <5 μg/mL and lasted for 2 observation time points.

***In vivo* Release** Adult New Zealand rabbits were used (mean body weight of 4.2 kg). Based on the results of *in vitro* release experiments, two 5-FU-PLA tablets with effective concentrations for 30–45d and release rates of <50% within the first week, including 1.5P15 and 2.5P15, were selected for further *in vivo* pharmacokinetic studies. Twelve rabbits were investigated, and the left eye of each rabbit was selected for examinations. The tablets were surgically implanted under the conjunctiva. Paracentesis was performed on the rabbit eyes under general anesthesia on days 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42 after implantation, and 0.1 mL of aqueous humor was collected each time. The concentration of 5-FU in the aqueous humor was measured the same way as for the in vitro release assay.

***In vivo* Ocular Safety and Drug Pharmacodynamics** Forty-eight adult New Zealand rabbits were randomly divided into 4 groups (12 rabbits in each group), and the left eye was selected for examinations. All animals received a standardized filtration surgery. Basically, the surgical procedure involved meticulous dissection to access the subconjunctival space, followed by creation of a patent fistula connecting the anterior chamber to the subconjunctival space to facilitate aqueous outflow. The groups were treated as follows. Forty-eight adult New Zealand rabbits were randomly divided into 4 groups (12 rabbits in each group), and the left eye was selected for examinations. The groups were treated as follows: Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group and Group D: phosphate buffer saline (PBS) group. A 5-FU-PLA tablet was implanted subconjunctivally at the end of the filtration procedure for Groups A and B. Subconjunctival injection of 0.2 mL of 5-FU (25 mg/mL) and PBS was administered at the end of the filtration surgery and on the third and sixth days after the surgery for Groups C and D, respectively.

The postoperative IOP was measured on days 1, 3, 5, 7, 10, 14, 21, and 42 after surgery using a Perkin's tonometer. Slit lamp biomicroscopy was used to observe the postoperative changes in the rabbit eyes.

Histology Two rabbits from each group were randomly killed humanely on days 7, 14, and 42 after filtration surgery, and the conjunctival and corneoscleral tissues within 0.5 cm of the surgical area were quickly dissected after eye enucleation and placed in 10% formalin for hematoxylin-eosin staining. The proliferation of fibroblasts in the filtration area at different time points were compared. The proliferating cell nuclear antigen (PCNA) immunohistochemical staining was performed to observe the proliferating fibroblasts and non-proliferating cells. The number of PCNA-positive cells was counted and presented as a percentage of the total number of cells in the field.

Western Blot Conjunctival and corneoscleral tissues were collected from each group on days 7, 14, and 42 after filtration surgery. A Bio-Rad test kit was used to extract the total protein from the tissues and determine the protein concentration (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (30 μg) were dissolved in polyacrylamide gels containing 12% Tris-HCl before being transferred to a polyvinylidene fluoride blotting membrane (Millipore, Billerica, MA, USA). After blocking, specific antibodies against transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF), and beta-actin (1:2000, Abcam, Cambridge, MA, USA) were treated with the membranes. The protein bands were detected by chemiluminescence after the blots had been carefully washed and treated with peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:1,000, ZSGB-Bio, Beijing, China). The experiment was conducted three times[14].

Statistical Analysis All the data were analyzed using SPSS version 17.0, and *P*-values of <0.05 denoted statistical significance. The groups were compared using one-way analysis of variance; the counting data were compared using the Chi-squared test.

RESULTS

Physical Characteristics of the 5-FU-PLA Tablets The 5-FU-PLA tablet was prepared as a white circular tablet with a homogeneous texture and smooth surface. In the upper petroleum ether smear, a few microspheres (5-FU-PLA-MS) were visible, having diameters of approximately 5–7 μm. The microspheres were regularly spherical and had smooth surfaces and uniform sizes. The smaller the molecular weight of 5-FU-PLA tablets, the rougher the surface was, and the larger the molecular weight, the denser and smoother the surface was. For those with low drug content, the surface showed stripes, which were the traces of polylactic acid precipitation; while for

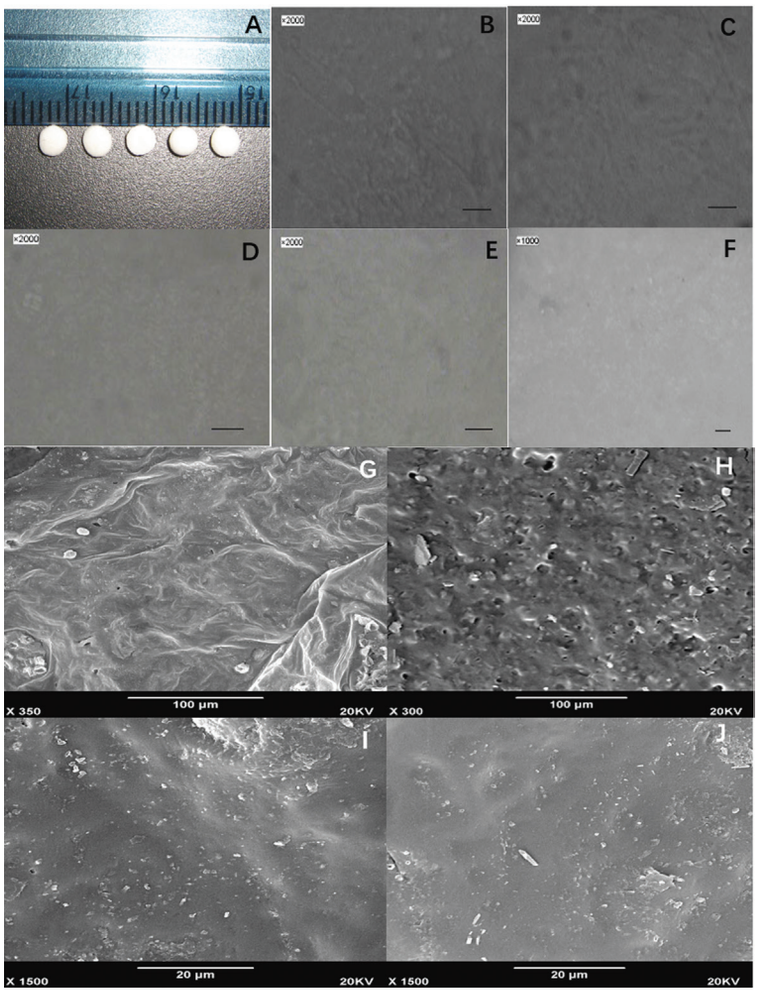

Figure 1 Surface morphology of the 5-FU-PLA tablets under an optical microscope and scanning electron microscope A: Direct observation of 5-FU-PLA; B–F: Surface morphology of 5-FU-PLA tablets with different molecular weights under a light microscope. From B to F, the molecular weights were 3000 D, 6000 D, 10 000 D, 15 000 D, and 20 000 D, respectively. G–J: Surface morphology of 5-FU-PLA-DS with different molecular weights under SEM. From G to J, the molecular weights were 3000 D, 3000 D, 20 000 D, and 20 000 D, respectively. 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid; SEM: Scanning electron microscopy.

those with high drug content, granular microsphere traces were visible on the surface (Figure 1).

Loading and Encapsulation Efficiencies The loading and encapsulation efficiencies of 5-FU-PLA tablets with different molecular weights and drug dosing amount are provided in Table 1. Overall, the loading weight percentage increased with increasing drug dosing amount, and the encapsulation rate fluctuated between 81.78% and 86.17%.

***In vitro* and *in vivo* Release Results** Overall, all 5-FU-PLA tablets had a high release rate within 1wk, reaching approximately 50% of the entire drug release profile and gradually reaching the release plateau according to time. With the increase in the drug loading weight percentage, the release was prolonged accordingly. A burst release profile of 5-FU (exceeding 50% cumulative release within the initial postoperative week) was associated with increased risks of delayed wound healing. Consequently, our formulation development prioritized sustained drug delivery systems maintaining therapeutic release for over 4wk while eliminating initial burst release characteristics. Based on the results of the *in vitro* release experiments, drugs with an effective concentration for 30–45d and release rates of <50% within the first week, including 1.5P15 and 2.5P15, were selected for further *in vivo* pharmacokinetic studies (Figure 2). For the *in vivo* release, the concentration-time curve demonstrated a very short burst release on day 1, reaching a plateau within 2wk. This was followed by a sustained release with drug concentrations of 67–25 μg/mL for the 1.5P15 group and 60–18 μg/mL for the 2.5P15 group for up to 42d. Figure 3 showed the tablets at different time points after subconjunctival implantation (1d and 4wk), which were stably placed in the subconjunctival position at the time of implantation and their volume decreased with time. Figure 3E showed the *in vivo* release curves for both drugs.

***In vivo* Ocular Safety and Drug Pharmacodynamics**

Safety On postoperative day 1, all operated eyes in all

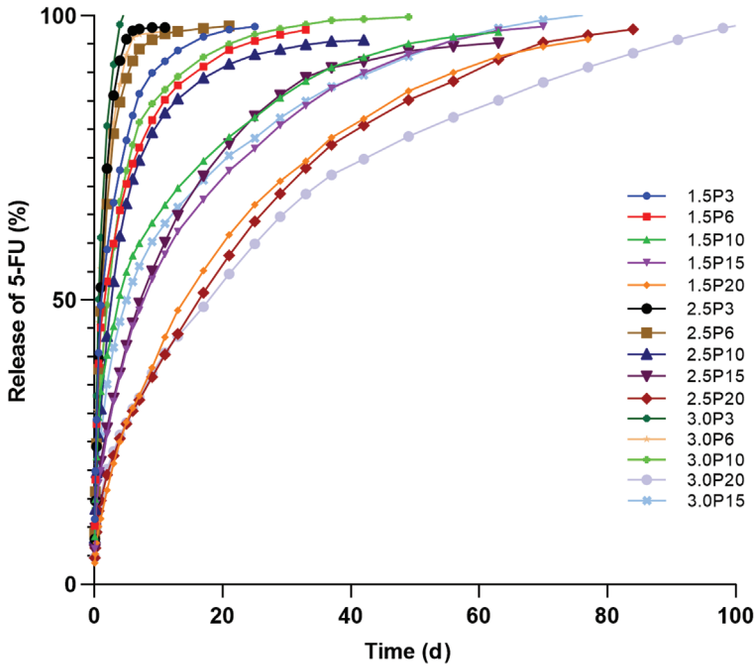

Figure 2 *In vitro* release of different 5-FU-PLA tablets For each 5-FU-PLA tablet, there was an initial burst release followed by a sustained release of 5-FU. The release duration increased with the increase in drug loading weight percentage. 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

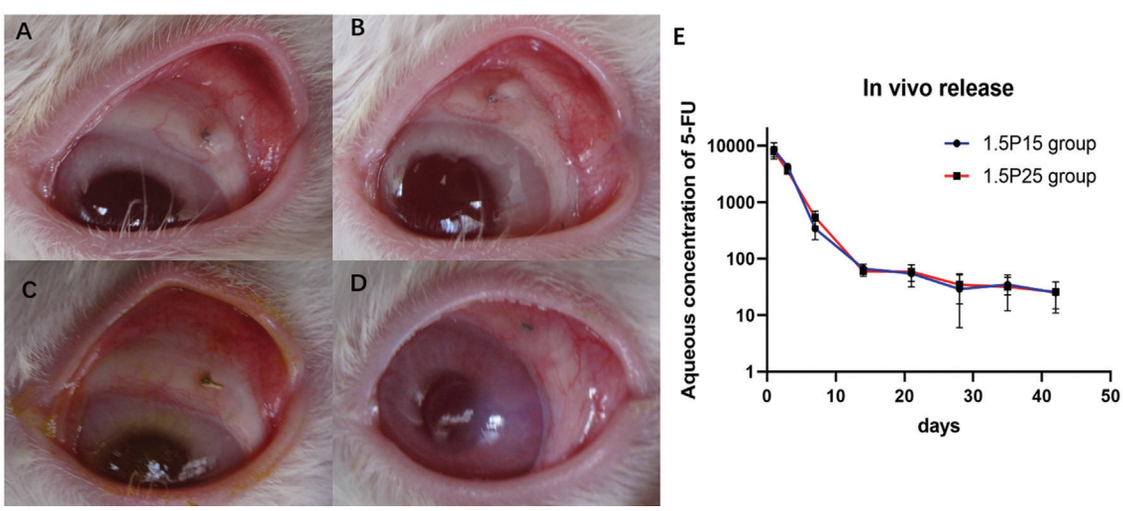

Figure 3 *In vivo* release results in rabbits A, B: The images show the tablets 1d after subconjunctival implantation; white plates were observed under the conjunctiva (A: 1.5P15; B: 2.5P15); C, D: The volumes of the tablets decreased with no conjunctival congestion 4wk after the implantation (C: 1.5P15; D: 2.5P15); E: *In vivo* release curves of both drugs. 1.5P15: 5-FU 1.5 mg+PLA 15 000 Da; 2.5P15: 5-FU 2.5 mg+PLA 15 000 Da; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

experimental groups showed significant conjunctival congestion. By postoperative day 7, the proportion of conjunctiva that showed congestion was 75%, 83.33%, 91.67%, and 58.33% for groups A, B, C, and D, respectively. By postoperative day 42, the proportion of conjunctiva that showed congestion was 25%, 25%, 12.5%, and 0 for Group A, B, C and D, respectively. All groups showed the same degree of postoperative corneal epithelial defects with corneal edema; the corneal edema and defect gradually improved with time. The proportion of eyes with corneal epithelial defects on day 42 was 12.5%, 12.5%, 0, and 0 for groups A, B, C, and D, respectively. Postoperative conjunctival degeneration, endophthalmitis, filtering bleb fistula, and cataract were not observed in any of the operated eyes, and there was no prolapse of the 5-FU-PLA tablets in any of the operated eyes in groups A and B (Table 2).

Filtering bleb situation Filtering bleb formation is the most important indicator of the success of the surgery and the effect of 5-FU. A thin-walled microcystic bulging bleb or a diffuse flattened bleb was considered functional, while the presence of subconjunctival scarring adhesions or a thick-walled sclerotic bleb with a restricted height and cystic hyperplasia was considered nonfunctional. On postoperative day 1, bulging blebs were formed in all experimental groups. The bleb

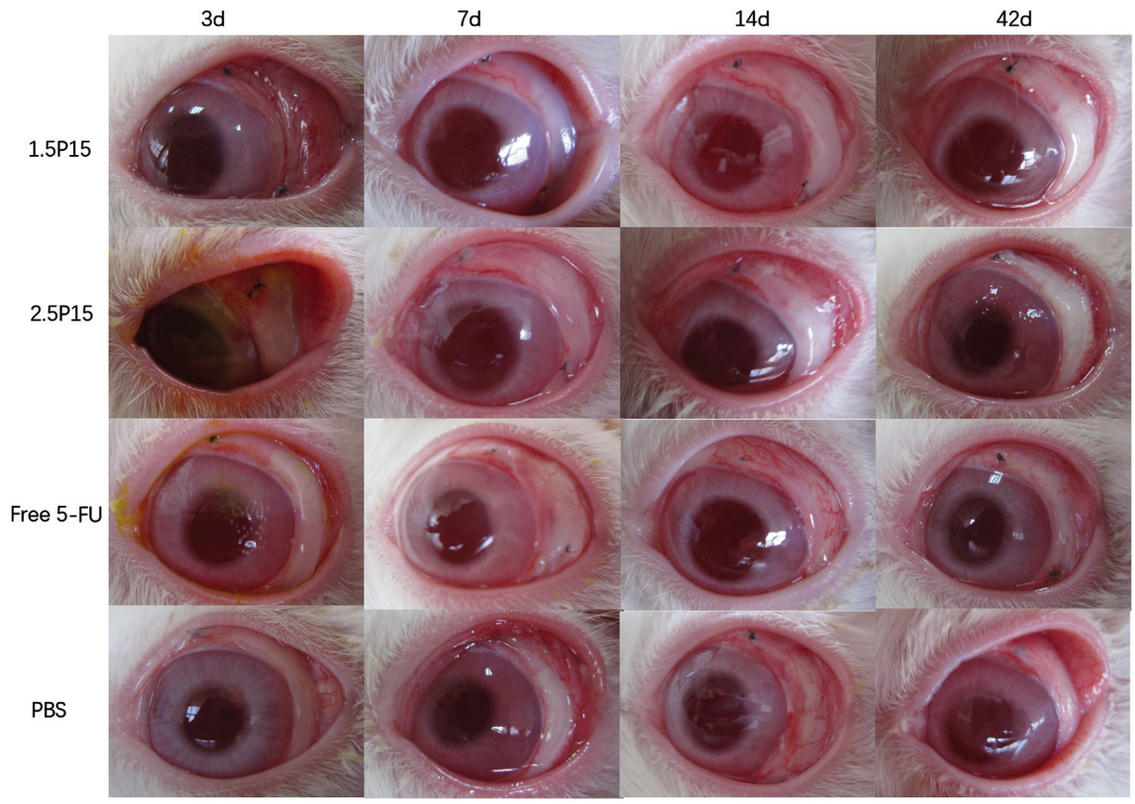

Figure 4 Filtering bleb conditions of different groups at different time points The volumes of the subconjunctival implants in groups A and B decreased with time. There were no significant differences between conjunctival congestion and corneal edema in the groups. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group. 1.5P15: 5-FU 1.5 mg+PLA 15 000 Da; 2.5P15: 5-FU 2.5 mg+PLA 15 000 Da; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

morphology was most visible on postoperative days 5–7 for both groups A and B (Figure 4); most eyes formed bulging, clear, large, and avascular blebs. The blebs changed slowly thereafter, with some blebs becoming flattened by day 14 and sustained for up to day 42. In group C (Figure 4), the blebs were most visible on postoperative day 3 and disappeared in some eyes by day 7. Functional blebs were no longer visible in most eyes on postoperative day 14 and lasted until day 42. In group D (Figure 4), the blebs became progressively smaller and flatter, with thickened walls and subconjunctival scar adhesions becoming visible from day 3 postoperatively. On day 14 postoperatively, all the blebs became non-functional blebs, and this lasted until day 42. The changes in the functional blebs of the various groups with time were shown in Figure 5.

IOP changes There were no statistical differences between the IOPs of the groups before surgery (P>0.05). On postoperative day 1, the IOPs of all groups decreased significantly (P<0.01), but there were no statistical differences between them (P>0.05). On postoperative day 3, the IOPs of groups A, B, and C decreased to 5.51±1.23, 5.82±1.69, and 6.24±2.88 mm Hg, respectively, while the IOPs of group D began to increase (7.64±2.17 mm Hg); the differences between the IOPs of all groups were significant (P<0.01). IOPs of groups A and B remained significantly lower on postoperative day 42 than preoperatively at 9.62±1.86 and 10.23±1.54 mm Hg, respectively (P<0.05). IOPs of groups C (16.22±2.08 mm Hg) and D (16.34±1.56 mm Hg) increased on postoperative day 42, but the differences were not significant (P>0.05); this suggested that the surgery failed. The IOP fluctuations in each group were shown in Figure 6.

Table 2 Eyes with conjunctival congestion in each group after surgery

n (%)

Days	Eyes	Group A	Group B	Group C	Group D
1	12	16 (100)	12 (100)	12 (100)	12 (100)
7	12	9 (75)	10 (83.3)	11 (91.7)	7 (58.3)
14	10	6 (60)	7 (70)	5 (50)	3 (30)
42	8	2 (25)	2 (25)	1 (12.5)	0

Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

Histology On postoperative day 7, spacious sub-scleral filtration channels were observed in groups A and B, with little fibrous tissue around the incision. In group C, there were fibroblasts and vascular tissue proliferation in the sub-scleral filtration channels, but the drainage channels were still open. In group D, there was marked fibrous connective tissue proliferation under the conjunctival and scleral flaps

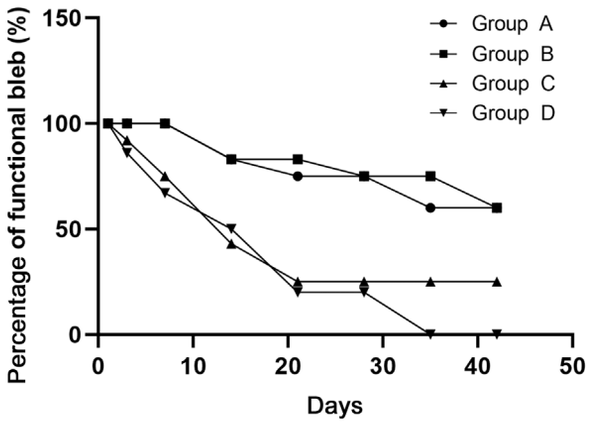

Figure 5 The changes in the functional blebs of different groups at different time points The number of functional blebs decreased with time in all groups, but stabilization was achieved after 14d in groups A and B and continued until 42d. The percentages of functional blebs in groups C and D were less than 50% after 21d. Only 12.5% of the rabbit eyes in group C had functional blebs by 42d; all functional blebs in group D had disappeared. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group.

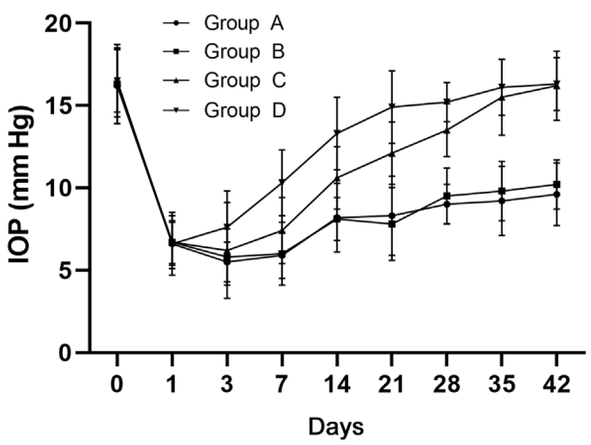

Figure 6 IOP changes in the groups at different time points IOP decreased in all groups on the first postoperative day and improved after 3d. The IOPs of groups A and B remained significantly lower on postoperative day 42 than preoperatively, and the IOPs in groups C and D returned to preoperative levels, suggesting surgical failure. IOP: Intraocular pressure. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group.

accompanied by neovascularization. On postoperative day 14, a few fibroblasts and inflammatory cells were found in the channels of groups A and B, but they were sparse. In group C, there was marked fibrous connective tissue proliferation under the conjunctival and scleral flaps, which blocked the filtration channels; there were also several disorganized fibroblasts. In group D, the tissue proliferation resulted in blockage of the filtration channels, and strips of collagen fibers were observed. The number of fibroblasts in groups A and B

Table 3 Percentage of PCNA-positive cells in the groups at different time points mean±SD

Days	Group A	Group B	Group C	Group D
7	17.60±3.45	15.90±4.06	32.60±5.09	60.50±5.30
14	21.78±3.16	19.08±4.74	52.78±6.55	43.20±5.26
42	5.54±1.47	3.98±2.47	10.04±5.20	14.32±5.59

PCNA: Proliferating cell nuclear antigen. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group.

stabilized on postoperative days 42 with a wide gap left under the scleral flap. In group C, the scleral incision disappeared on postoperative day 42; however, only a very narrow gap remained under the scleral flap. In group D, the scleral incision was filled with layers of fibrous tissue, and the filtration channel disappeared on postoperative day 42 (Figure 7).

In groups A and B, the filtration channels were clear without any large, dark brown nuclei, and the PCNA-positive cells were mostly elongated and lightly stained. The proportion of positive cells peaked at 14d after surgery and gradually decreased until day 42. In group C, the number of PCNA-positive cells on both sides of the filtration channel increased on postoperative day 7, and the nuclei became larger and rod-shaped with darker staining. The number of positive granules further increased on postoperative day 14, and the cells significantly proliferated; the proportion of positive cells gradually decreased on day 42. In group D, the fibroblasts on both sides of the filtration channel proliferated significantly on postoperative day 7, and a large number of dark brown PCNA-positive granules appeared; the nuclei were large and dark-stained. The brown granules began to decrease on postoperative day 14, and the filtration tract was mostly filled with blue-stained fibroblasts. On postoperative day 42, the filtration channel had been blocked by fibrous tissue, and the proportion of PCNA-positive cells further decreased (Figure 8). The percentages of PCNA-positive cells in the groups were shown in Table 3.

Western Blot Factors related to cell proliferation, including TGF-β and CTGF, were analyzed. The expressions of those two factors were upregulated after surgery but could be inhibited by 5-FU. On postoperative day 7, the expressions of TGF-β and CTGF decreased significantly in groups A, B, and C relative to those in group D. By postoperative day 14, the expressions of both proteins were still significantly lower in groups A and B, but there was no significant difference between them in groups C and D. On postoperative day 42, the expressions of TGF-β and CTGF were stabilized in all groups, suggesting that initial treatment with 5-FU can inhibit proliferation and fibrosis in the filtration channels, with the effect of 5-FU-PLA tablets being more sustainable (Figure 9).

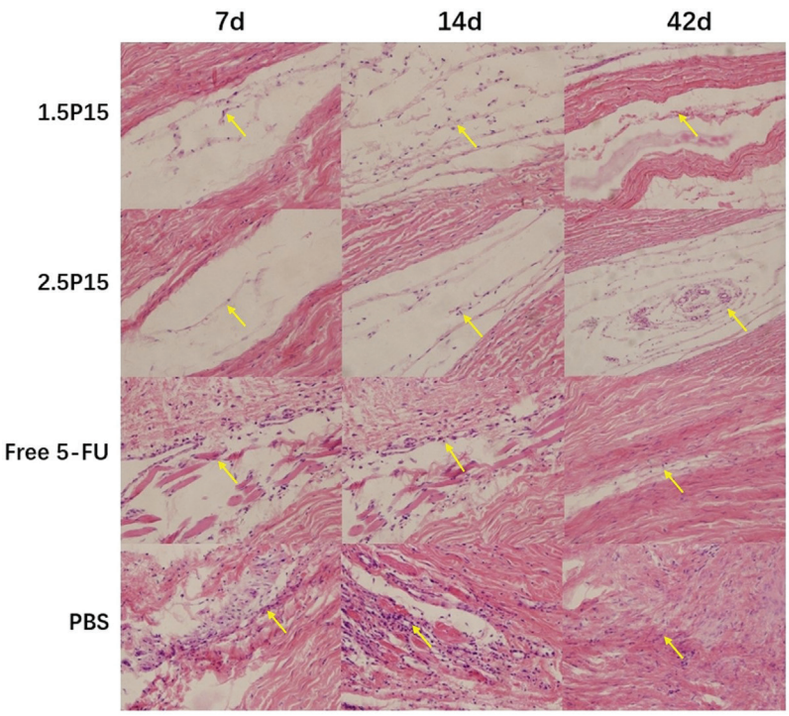

Figure 7 Histology results for the groups at different time points For groups A and B, fiber proliferation was not significant with time. In contrast, the fibroproliferation in groups C and D increased significantly over 42d; the filtration channels in groups C and D were already blocked by fibrovascular tissue. Fibroblasts and inflammatory cells are indicated by yellow arrows. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group. 1.5P15: 5-FU 1.5 mg+PLA 15 000 Da; 2.5P15: 5-FU 2.5 mg+PLA 15 000 Da; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid.

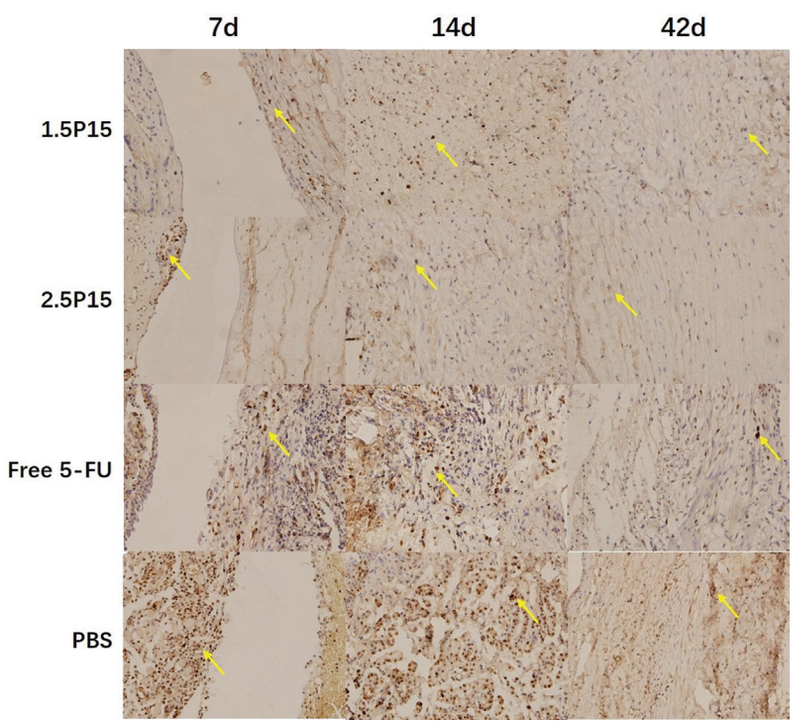

Figure 8 Histology results of the groups at different time points In groups A, B, and C, the proportions of PCNA-positive cells peaked 14d after surgery and gradually decreased till postoperative day 42. In group C, more PCNA-positive cells were observed all the time. In group D, the fibroblasts on both sides of the filtration channel proliferated significantly on postoperative day 7, and the nuclei were large and stained dark; the brown granules began to decrease on postoperative day 14, and the filtration tract was predominantly filled with blue-stained fibroblasts. On postoperative day 42, the filtration channel had been blocked by fibrous tissue. PCNA-positive cells are indicated by yellow arrows. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group. 1.5P15: 5-FU 1.5 mg+PLA 15 000 Da; 2.5P15: 5-FU 2.5 mg+PLA 15 000 Da; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid; PCNA: Proliferating cell nuclear antigen.

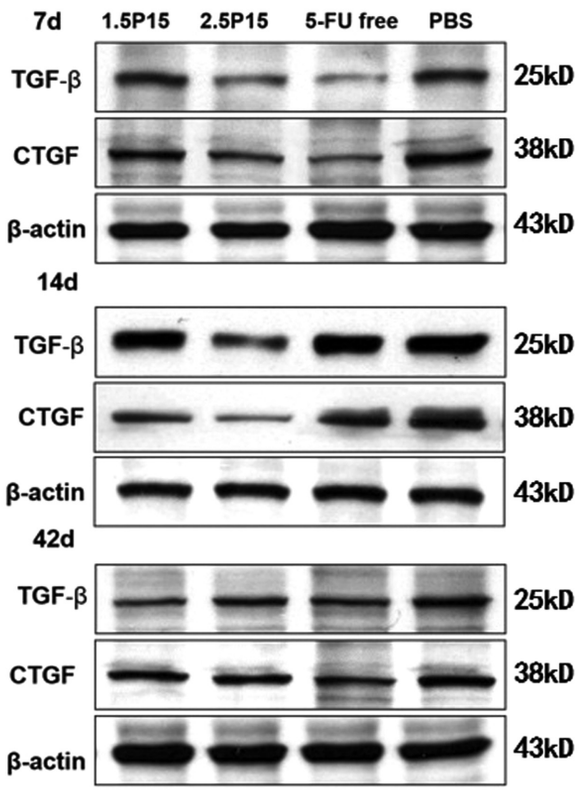

Figure 9 Western blot results for CTGF and TGF-β On postoperative day 7, the expressions of CTGF and TGF-β decreased in all three groups. On day 14, the expressions of CTGF and TGF-β were still decreased in both groups A and B, but they returned to the preoperative levels in group C. On day 42, the expressions of CTGF and TGF-β returned to the preoperative levels in all groups. The CTGF and TGF-β expressions did not decrease in group D. Group A: 1.5P15 5-FU-PLA tablets group; Group B: 2.5P15 5-FU-PLA tablets group; Group C: 5-FU free drug group; Group D: Phosphate buffer saline (PBS) group. 1.5P15: 5-FU 1.5 mg+PLA 15 000 Da; 2.5P15: 5-FU 2.5 mg+PLA 15 000 Da; 5-FU: 5-Fluorouracil; PLA: Poly(lactic) acid; CTGF: Connective tissue growth factor; TGF-β: Transforming growth factor-β.

DISCUSSION

In this study, we developed a 5-FU-PLA sustained delivery system, investigated the *in vitro* release of various drug delivery tablets, and chose appropriate candidates for further *in vivo* research. The 5-FU-PLA sustained delivery system considerably increased the success rate of glaucoma filtration surgery with a satisfactory intraocular safety profile based on the postoperative IOP, filtration bubbles, and related complications in rabbit eyes undergoing glaucoma filtration surgery. This study is the first to conduct systematic *in vitro* and *in vivo* analyses and test various 5-FU-PLA tablet characteristics.

There are three main causes of glaucoma surgery failure: surgery-related trauma results in the release of several cytokines that encourage the growth of fibroblasts and scar tissue; blood-atrial aqueous barrier; and neovascularization[15]. Strategies to control postoperative fibrosis, such as the topical application of steroids and anti-fibrotic agents such as mitomycin C (MMC) and 5-FU, can improve the success rate of filtration surgery[16]. However, subconjunctival injections of free drugs usually result in burst release, instead of sustained release, and frequent injections are necessary[17]. Besides, the high concentration of a burst release inevitably disturbs the physiological structure of the surrounding normal tissues, which delays wound healing and causes ocular toxicity reactions, among other consequences[13,18]. Therefore, the development of an efficient and low-toxicity slow-release drug delivery system loaded with anti-fibrotic agents is important to improve the success rate of glaucoma filtration surgery[10].

Some 5-FU release systems have been reported with release times between 10d and 3mo[10,19-20]. However, the control of the release time to facilitate post-filtration surgery proliferation control by 5-FU while reducing long-term toxicity needs to be explored[10]. Additionally, the rapid release results in high local drug concentrations, which can increase the risk of complications. PLA is an ideal carrier for slow- and controlled-release formulations. Attempts have been made to prolong the sustained release of 5-FU; however, an excessively sustained release may be nonessential and increase ocular toxicity. Almost all current 5-FU sustained-release agents using PLA as a carrier are microsphere formulations that are easily diffused under the conjunctiva; this makes it challenging to achieve low-dose, uniform, long-term release under the conjunctiva[21]. In our study, 5-FU and PLA were molded into tablets in this experiment to facilitate sustained low-dose, uniform, long-term release in the confined space under the conjunctivas of rabbits.

It was established earlier in this study that 5-FU-PLA extended-release tablets were beneficial for sustained release since the peak period of scarring after filtration surgery is 14–21d. However, an optimum drug-loaded formulation that can inhibit scarring while minimizing toxicity is a problem to be solved in this study. Various drug-loading doses and molecular weights of PLAs were compared to discover the best formulation for the development of 5-FU-PLA. The drug-loading weight percentage increased with the increase in 5-FU delivery; however, it was discovered that the overall encapsulation rate was steady. Analysis of the drug release profile showed that higher PLA molecular weights were associated with smoother drug release, which was consistent with the observation under SEM. The effective concentration increased with the drug-loading dose for a given PLA molecular weight. Given that too or short release durations after glaucoma surgery can compromise surgical results, 1.5P15 and 2.5P15 were acceptable candidates. Further, we chose 5-FU-PLA tablets that had a 50% release in the first week for subsequent *in vivo* trials.

The sustained releases of both 5-FU-PLA tablets were less prolonged *in vivo* than *in vitro*, and an explanation may be that the clearance *in vivo* was faster than that *in vitro*. For the long-term result of the treatment, we discovered that the inhibition of fibrosis was quite satisfactory. To ensure that the corneal endothelium is not harmed, it is crucial to measure the concentrations of 5-FU in the aqueous humor. In our study, we found that the concentration of 5-FU in the aqueous humor at each time point was considerably lower than the threshold recommended by Mannis *et al*[22] for 5-FU-related toxicity of the corneal endothelium (1-10 mg/mL). As a result, the cases of conjunctival hyperemia and corneal edema were milder than what would have occurred with a free 5-FU administration.

Based on the postoperative IOP and bleb morphology, the 5-FU-PLA tablets effectively inhibited post-filtration trauma healing; inflammatory cells and fibroblasts in the filtration channel were significantly inhibited. Immunohistochemical PCNA staining also demonstrated the inhibitory effect of 5-FU-PLA tablets on fibroblasts. Western blot results showed that the expressions of inflammatory factors, including TGF-β and CTGF, were reduced, confirming that this release system facilitates the long-term inhibition of scar formation through the inhibition of relevant proliferative pathways. The sustained-release 5-FU formulation mitigates scar formation by suppressing fibroblast proliferation and myofibroblast transdifferentiating, downregulating pro-fibrotic mediators (TGF-β, CTGF), and inhibiting extracellular matrix overproduction, pathological angiogenesis, and aberrant wound contraction through sustained modulation of key signaling pathways.

There are some limitations of this study. Current safety assessment was confined to *in vivo* evaluations, warranting future *in vitro* toxicity profiling of 5-FU-PLA's dose-response effects on subconjunctival cell populations using real-time monitoring systems (*e.g.*, XCelligence). Furthermore, the relatively short observation period necessitates extended monitoring to elucidate the formulation's long-term biocompatibility and polymer degradation kinetics.

In this study, we screened drug formulations for *in vitro* studies and further validated them with *in vivo* studies rather than performing *in vivo* experiments for all formulations. More *in vivo* studies may be required in the future to investigate the impact of various pharmacological characteristics on *in vivo* release in light of the differences between the *in vivo* and *in vitro* conditions. Longer follow-ups are also required. Additionally, PLA has a steady release; however, it has a burst release during the first week. Future chemical surface modifications of the sustained release system may result in a more gradual release.

In conclusion, the tested 5-FU-PLA tablets provided a sustained release of 5-FU, which may be used for a single subconjunctival implantation to inhibit proliferation after filtration surgery. It will be ideal to determine how to reduce the initial release rate in future research with longer follow-ups.

ACKNOWLEDGEMENTS

We thank the Beijing Key Laboratory of Ocular Disease and Optometry Science, Peking University People's Hospital for providing the facilities and equipment necessary for the conduct of this study.

Authors' Contributions: Zhang JJ, Gao F, and Deng KX performed the preparation and *in vitro* study of 5-FU-PLA tablets. Guan WX and Sun YY performed the *in vivo* study. Zhang JJ and Sun YY performed the histology study. All authors contributed to the study conception and design, manuscript writing, and final approval of manuscript.

Foundations: Supported by the National Natural Science Foundation of China (No.82301211); Beijing Natural Science Foundation (No.J230028).

Conflicts of Interest: Zhang JJ, None; **Gao F,** None; **Deng KX,** None; **Guan WX,** None; **Sun YY,** None.

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