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ORIGINAL ARTICLE

In Vitro and *In Vivo* Release Characteristics of Tacrolimus (FK506) from an Episcleral Drug-Delivery Implant

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Abstract

Purpose: To investigate the *in vitro* and *in vivo* release characteristics of Tacrolimus (FK506) from an episcleral drug-delivery implant.
Methods: For *in vitro* experiments, Tacrolimus-loaded implants (0.5 mL; at concentrations of 0.25, 0.5, and 1.0 mg/mL) were immersed in a balanced salt solution. Samples of the surrounding liquid were aspirated at different times over a 96-h period. For *in vivo* experiments, the experimental group received an implant loaded with Tacrolimus (0.5 mg/mL; 0.5 mL); the control group was given a subconjunctival injection of 0.5 mL Tacrolimus (0.5 mg/mL). On postoperative days 3, 7, 14, 28, and 56, 3 animals were sacrificed, and their eyes were enucleated. Tacrolimus concentrations were determined by liquid chromatographic–tandem mass spectrometry. Ocular toxicity was evaluated by slit-lamp photography, fundus photography, intraocular pressure (IOP), and histology.
Results: The implants released Tacrolimus in a biphasic pattern for 96 h in the *in vitro* study. The release kinetics were not dependent on the drug concentrations. The *in vivo* study showed statistically significant differences between the 2 treatment groups. Tacrolimus levels were particularly high in the conjunctiva, iris, ciliary body, cornea, sclera, choroid, and retina in the experimental group, while concentrations were low and only lasted for 1 week in the controls. Slit-lamp photography, fundus photography, IOP, and histology showed no evidence of toxic effects.
Conclusions: The episcleral drug-delivery implant mechanically released Tacrolimus through the apertures of capsules and, consequently, may be a promising drug vehicle for the treatment of immune-mediated ocular disorders.

Introduction

Immune-mediated ocular disorders, such as corneal graft rejection,[1] autoimmune uveoretinitis,[2] and graft-versus-host disease,[3] are important causes of visual impairment worldwide. In many refractory conditions, immunosuppressive therapies are administered systematically over a long period to enhance anti-inflammatory effects.[4] Tacrolimus (FK506) and cyclosporine A are 2 commonly used immunosuppressants.[4] Figure 1 shows the structure of Tacrolimus, which has a 10 to 100 times greater immunosuppressive potency than cyclosporine A.[5–7]

Tacrolimus was first isolated in 1984 from the fermentation broth of *Streptomyces tsukubaensis*[8,9] and effectively prevents acute rejection in organ transplants. The therapeutic mechanism of Tacrolimus involves the inhibition of T lymphocyte activation through binding to the immunophilin FKBP12,[10] reducing allergic symptoms. Tacrolimus has shown therapeutic effectiveness in ophthalmologic areas, such as allograft rejection in keratoplasty,[11] refractory inflammatory ocular surface diseases,[12] and noninfectious uveitis. The recommended oral dose is between 0.05 and 0.2 mg/(kg·day^{-1}).[13]

However, most immune-mediated ocular diseases require long-term administration to supply an effective concentration in the eyes.[14] Side effects caused by systemic immunosuppressants are common and include neurotoxicity, hepatitis, high blood pressure, and tremors.[14] The blood-ocular barriers[15] restrict delivery so that only low levels of drugs occur in ocular tissues following systemic administration, especially in the posterior segment. Consequently, ophthalmologic studies have primarily focused on the development of ocular administration strategies.

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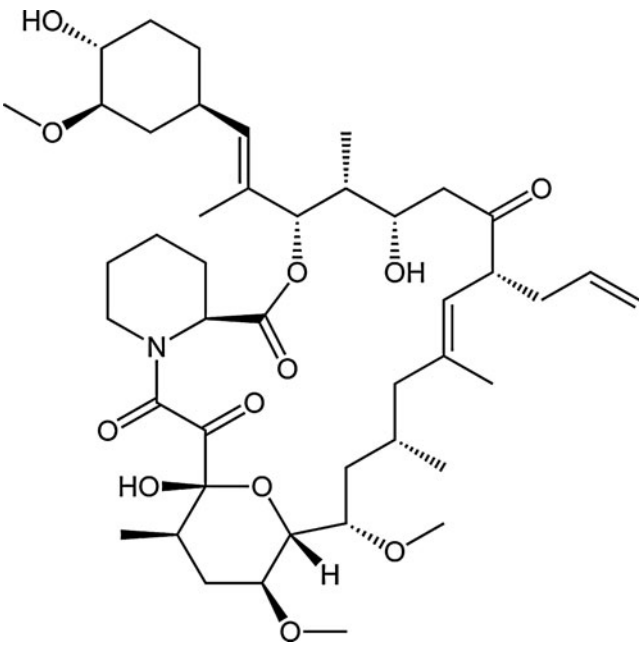

FIG. 1. Structure of Tacrolimus.

Ocular delivery types include intravitreal and periocular routes. Frequent intravitreal injections may lead to retinal detachment, hemorrhage, increased intraocular pressure (IOP), and endophthalmitis.[14] These complications can be minimized using novel drug-delivery systems via periocular routes.[16] Hence, recent studies have highlighted the administration of immunosuppressants via the periocular route.

Trans-scleral drug delivery is one option that uses the periocular route to the choroid and retina with negligible systemic absorption.[17] Therefore, trans-scleral delivery is a potential treatment approach for many chorioretinal disorders. The total surface of the sclera is 16–17 mm^2,[18] providing a large delivery area for drugs into the eyes. We designed a novel trans-scleral drug-delivery implant placed in the posterior of the eyeball. The injection of dexamethasone sodium phosphate into this implant resulted in sustained release from the device for 48 h and for 56 days under *in vitro* and *in vivo* conditions, respectively.[19] This approach is effective and noninvasive, as it is implanted directly into the episcleral space. In our study, we investigated the *in vitro* and *in vivo* release characteristics of Tacrolimus following encapsulation within this episcleral drug-delivery implant.

Materials and Methods

Preparation of tacrolimus

Working solutions containing 0.25, 0.5, and 1.0 g pure Tacrolimus powder (Fujisawa Pharmaceutical Co. Ltd.) were prepared by dilution in ethanol followed by the addition of polyethylene glycol, hydrogenated castor oils, polysorbate, and glycerin. Next, carboxymethylcellulose, previously dispersed in sterile water, Merthiolate, and sodium citrate were added. An acceptable pH level was 6.0–7.5. The suspensions were then filtered. Additional sterile water was replenished to bring the final drug concentrations to 0.25, 0.5, and 1.0 mg/mL.

Preparation of the episcleral drug-delivery implant

The raw material of liquid silicone rubber, Dow Corning Class VI elastomers, was supplied by Dow Corning Company. We manufactured the episcleral drug-delivery implants using tailor-made, modified liquid silicone rubber, which came as a 2-part kit (Part A and Part B). A series of 2-part platinum-catalyzed silicone elastomers were prepared by mixing Part A and Part B in equal proportions by weight. The resulting elastomer consisted of cross-linked dimethyl and methyl-vinyl siloxane copolymers and reinforcing silica. A specially designed mirror steel mold for casting the implant comprised an upper part, a lower part (both with heating holes), and a core. The core was linked to a drainage-tube pin, and the pin was connected with an injection channel. The elastomer was injected into the mold and heated to vulcanize to form a ''Y'' shape. The implant consisted of a capsule with a standard thickness of 0.01–5 mm, a 2–15-mm drainage tube, and a drainage valve. The valve had a larger end with an external diameter of 2–20 mm and a smaller end with an internal diameter of 1–20 mm. The Tacrolimus-loaded episcleral drug-delivery implant was a foldable capsular vitreous body drug-delivery system and had received a patent in China. Figure 2 shows the schematic cross-sectional (Fig. 2A) and top (Fig. 2B) views of a mold and a schematic view of an implant (Fig. 2C).

In vitro *release study*

Tacrolimus (0.25, 0.5, and 1.0 mg/mL; 0.5 mL) was injected into the capsules of the episcleral drug-delivery implants. Franz diffusion cells were applied to maintain the temperature at 98.6°F and the revolutions at 200 rpm. The capsules were subsequently immersed in 10 mL balanced salt solution (BSS; ALCON) in cups of modified Franz diffusion cells (Fig. 3). At 20, 40, 60, and 90 min, and 2, 4,

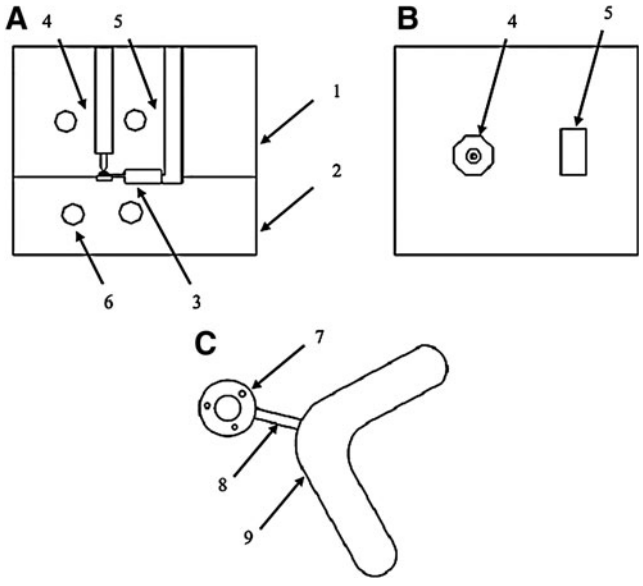

FIG. 2. Schematic cross-sectional **(A)** and top **(B)** views of a mold according to an embodiment of the episcleral drug delivery implant and schematic view of the implant **(C)**. 1. The upper mold; 2. The lower mold; 3. The core; 4. Plastic injection channel; 5. Positioning plate; 6. Heating holes; 7. Drain valve; 8. Drainage tube; 9. Capsular bag.

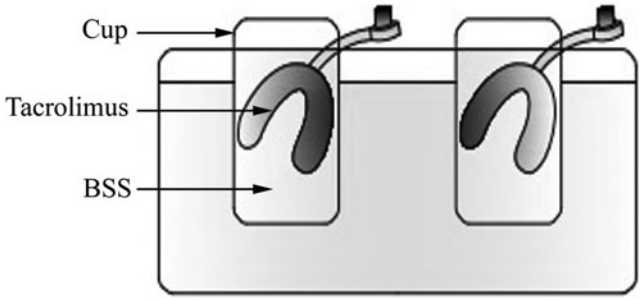

FIG. 3. Tacrolimus release study *in vitro*. Tacrolimus at concentrations of 0.25, 0.5, and 1.0 mg/mL in balanced salt solution (BSS) was injected into the capsules of the episcleral drug delivery implant, and then the capsules were immersed in cups of modified Franz diffusion cells; 100 μL of liquid in the cups was aspirated at time intervals of at 20, 40, 60, and 90 min, and 2, 4, 6, 12, 18, 24, 36, 48, 60, 72, 84, and 96 h.

6, 12, 18, 24, 36, 48, 60, 72, 84, and 96 h, 10-μL samples of the fluid surrounding the implants were removed by aspiration. At the same time, 10 μL of blank BSS was added to the cups to replenish the surrounding fluid. The amount of Tacrolimus released into the medium at each time point and the amounts of drug remaining in the capsules at 96 h were then determined by liquid chromatographic–tandem mass spectrometry (LC-MS/MS; Thermo-Finnigan).

Animals

New Zealand white rabbits weighing 1.5 to 2.0 kg each were used. Animal experiments were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Experimental Ethical Inspection to Ethics Committee of Zhongshan Ophthalmic Center at Sun Yat-sen University. The rabbits were divided into the experimental group (surgical implantation with Tacrolimus-loaded episcleral capsules) and the control group (subconjunctival injection of Tacrolimus).

Surgical procedures

Anesthesia was induced with an intramuscular injection of ketamine hydrochloride (30 mg/kg) and chlorpromazine hydrochloride (15 mg/kg) before the surgery. In the experimental group, the ocular surface of the right eye of each animal was anesthetized with a topical instillation of 0.5% proparacaine hydrochloride (S.A.ALCON-COUVREVR N.V Rijksmuseum 14,2870 Poors, Belgium). A conjunctival peritomy was created to expose a wide area of the superior bare sclera. Next, the capsule of the drug-delivery implant, sterilized by autoclaving for 1 h, was placed in the episcleral space to attach it to the posterior pole of the sclera. Approximately 0.5 mL of Tacrolimus (0.5 mg/mL) was then injected into the capsule through the tube-valve system. The valve was subsequently fixed under the conjunctiva, and the conjunctival wound was sutured with 8-0 silk. Figure 4 shows the surgical procedures and the relationship between

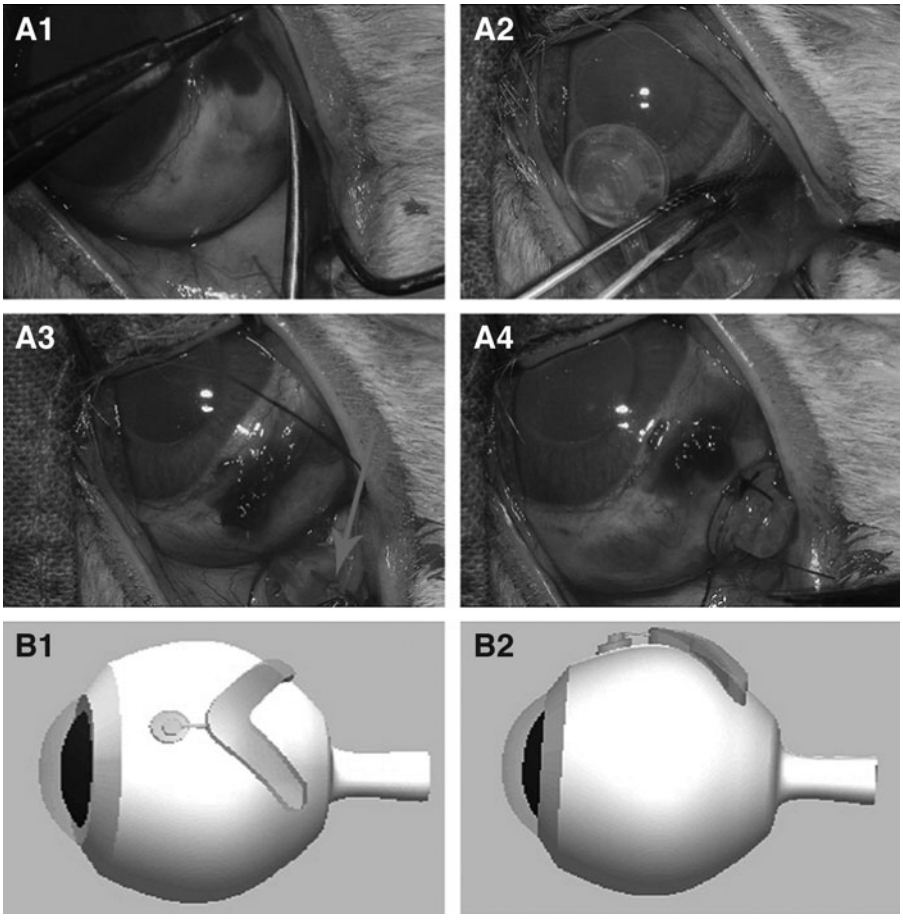

FIG. 4. The Tacrolimus-loaded drug delivery implant was placed in the episcleral space. **(A1–A4)** Surgical implantation of the episcleral drug delivery system. The arrow indicates that Tacrolimus was injected into the capsule through the valve by a syringe. Schematic top **(B1)** and cross-sectional **(B2)** views of the episcleral drug delivery implant that attaches to the posterior pole of the sclera.

the implant and eyeball. For controls, a subconjunctival injection of 0.5 mL Tacrolimus (0.5 mg/mL) was given under topical anesthesia in the right eye using a 29-gauge needle.

Clinical ophthalmic examination

The experimental group underwent a baseline slit-lamp examination (SL-D7; Topcon Co.) and fundus photography (TRC-50DX; Topcon Co.) before implantation surgery, and these procedures were repeated at 7, 14, 28, and 56 days postoperatively. IOP was recorded using A Tono-Pen (Tonopen Avia; Reichert Co.) at 3, 7, 14, 28, and 56 days after the implantation surgery.

Sample collection

On days 3, 7, 14, 28, and 56 after implantation, 3 rabbits in the experimental group were anesthetized, and 0.5 mL of whole-blood samples was collected from their marginal ear veins. The rabbits were then sacrificed, and their eyes were enucleated. The drug solutions in the capsules and samples of ocular tissues (aqueous humor, conjunctiva, cornea, iris, lens, vitreous body, ciliary body, sclera, retina, and choroid) were collected and immediately frozen at $-80°C$. Tacrolimus concentrations were determined with a sensitive HPLC-MS/MS method. In the control group, the whole-blood and ocular tissue samples were collected from 3 rabbits at 3, 7, and 14 days after the subconjunctival injections.

Assay of tacrolimus

The LC-MS/MS system consisted of an Agilent 1260 liquid chromatograph and a 6460 triple quadrupole mass spectrometer with electrospray ionization (ESI) source. Data were acquired with MassHunter B 05 software. Chromatographic separation was achieved at 40°C on a BDS Hypersil C18 column ($2.1 \times 50\,mm^2$ i.d., 2.4 μm; Thermo Scientific) with a Phenomenex C18 guard column ($4 \times 3\,mm^2$ i.d.). Mobile phase A was water with 0.1% formic acid; mobile phase B was methanol containing 0.1% formic acid. A mobile phase of 70% A:30% B (v/v) was initiated and

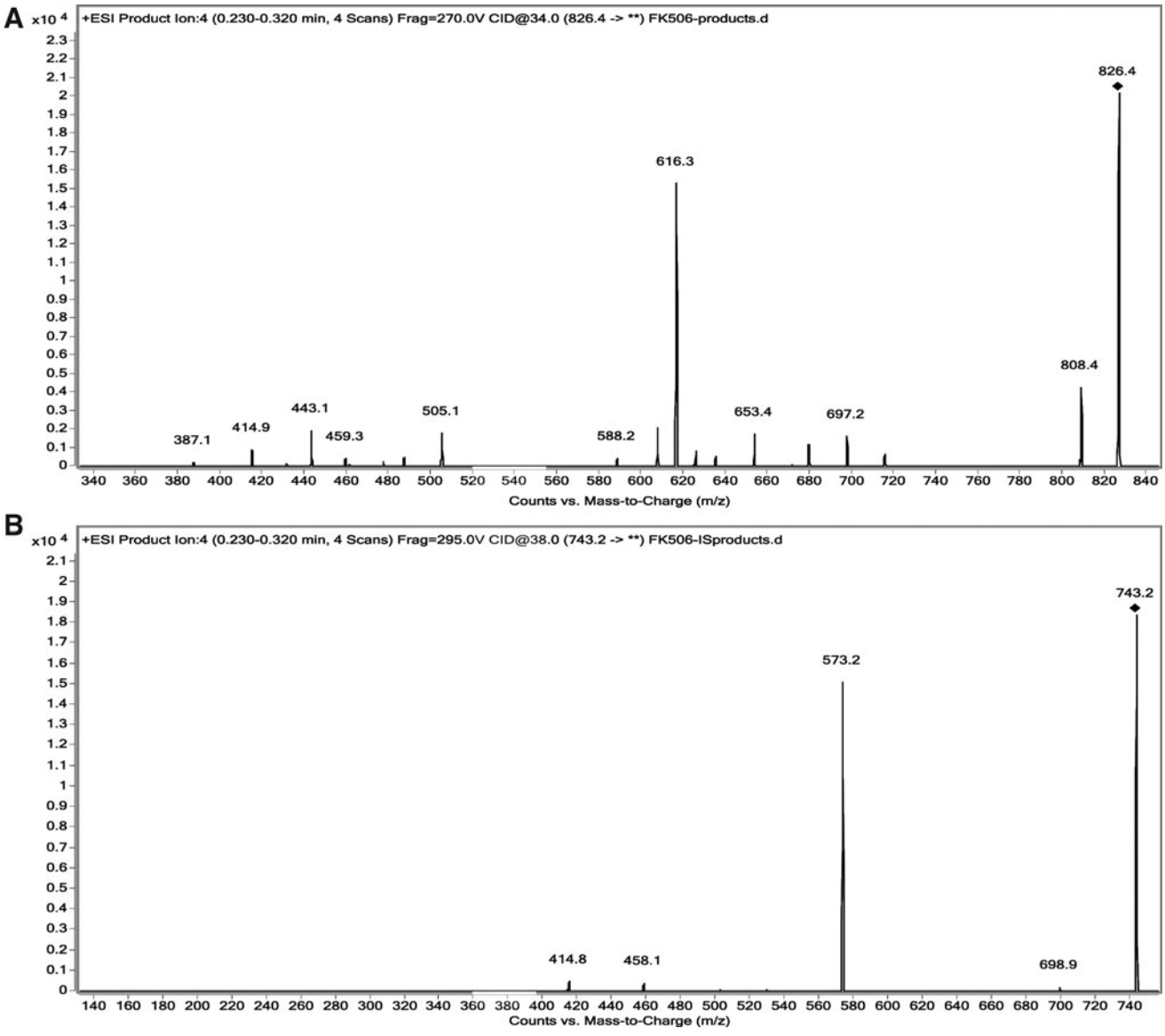

FIG. 5. Full-scan product ion spectra of [M + Na] + for **(A)** FK506 and **(B)** Ritonavir (IS, internal standard).

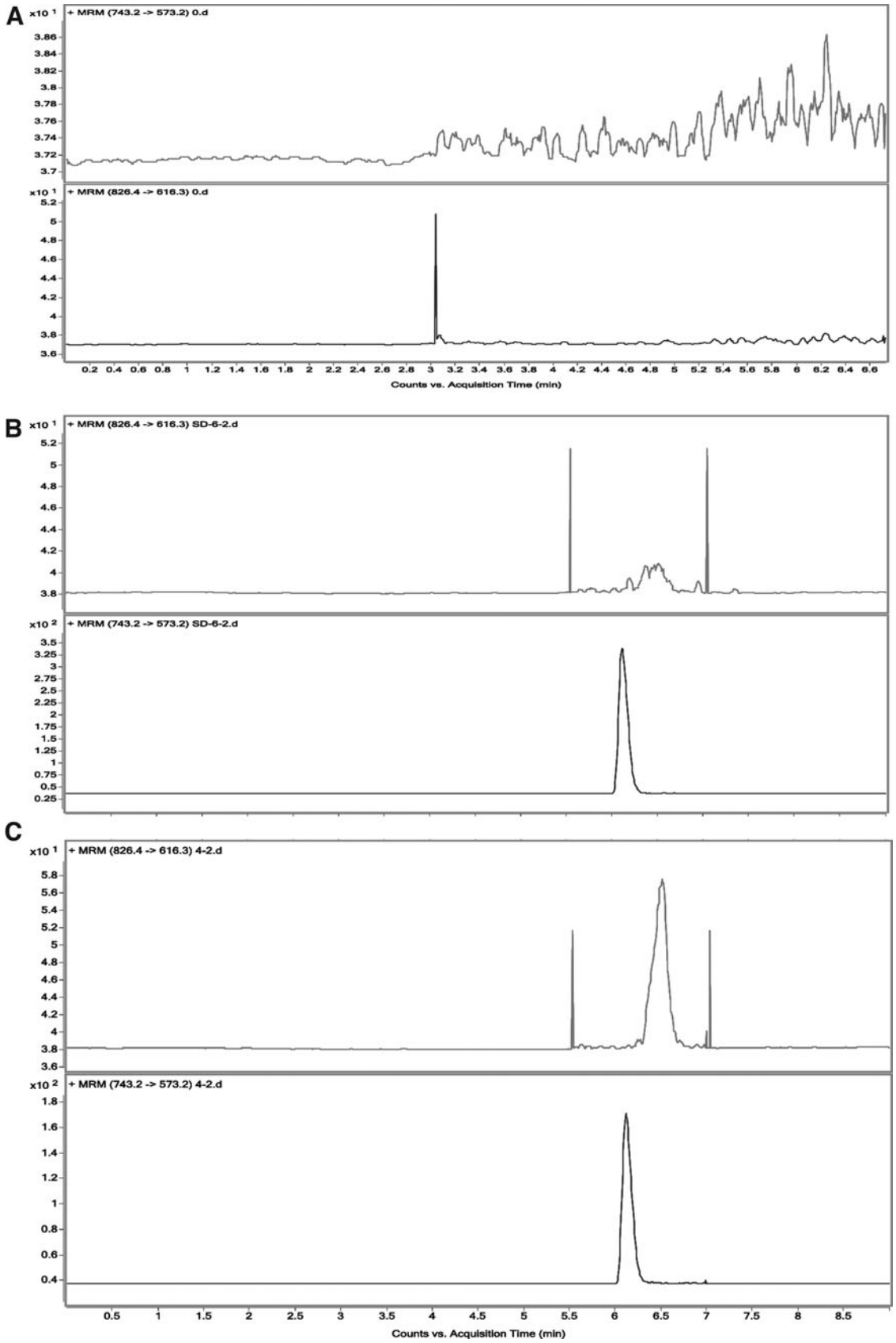

FIG. 6. Representative MRM chromatograms of **(A)** blank cornea, **(B)** blank cornea spiked with FK506 at LLOQ (0.5 ng/mL), and **(C)** a cornea sample containing FK506 on day 7 in the experimental group.

maintained for 1.1 min and then B was increased to 97% at 1.2 min. Between 1.2 and 5 min, the mobile phase was maintained at 97% B. From 5.1 to 9 min, the mobile phase was switched back to 30% B. The total flow rate of the mobile phase was 0.25 mL/min. The initial 5.5 min and the last 2 min were switched to the waste. The column temperature was 40°C, and the auto-sampler was conditioned at 4°C.

The mass spectrometer was operated in positive-ion mode for the analyte and internal standard (IS). Quantification was performed using the multiple reaction monitoring (MRM) mode, and the following transitions were recorded: FK506 m/z 826.4 $[M+Na]^+ \rightarrow$ m/z 616.3 and IS m/z $[M+Na]^+$ 743.2 $\rightarrow$ m/z 573.2, both at 35 eV. High-purity nitrogen served as both the nebulizing and drying gas. Other parameters of the mass spectrometer were set as follows: drying gas flow, 10 L/min; drying gas temperature, 350°C; nebulizer pressure, 40 psi; capillary voltage, 3500 V. The ESI source voltage was set at 3.5 kV, and the sheath gas flow rate and auxiliary gas flow rate were 35 and 5 psi, respectively; capillary temperature was 350°C.

Tacrolimus (FK506; purity >98.0%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ritonavir, the IS (MW 720.3), was obtained from Wyeth. High-performance liquid chromatography (HPLC)–grade formic acid was purchased from Sigma. HPLC-grade acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). Water was purified by a Milli-Q Water Purification System.

Stock solutions of known chemicals were prepared in methanol at concentrations of 400 μg/mL in 100-mL glass vials and were serially diluted with phosphate-buffered saline (*in vitro*) or blank tissue (*in vivo*) to prepare standard working solutions at the desired concentrations: 0.5, 1, 2, 5, 10, 20, 50, 100, 500, and 2000 ng/mL. Samples (50 μL) were transferred to 1.5-mL Eppendorf tubes, and 150 μL acetonitrile containing IS (Ritonavir 50 ng/mL) solution was added. The mixture was vortexed for 3 min and centrifuged at 16,000 rpm for 10 min. Approximately 120 μL of the supernatant was transferred to autosampler vials, and 5 μL was injected into the HPLC column.

Histology

Three rabbits in the experimental group were sacrificed at 56 days postoperatively. The operative eyes were enucleated and immersed in a mixture of 4% glutaraldehyde and 2.5% neutral buffered formalin for 48 h. The cornea, lens, and vitreous were then removed from the eyes. The retina-choroid and sclera were processed, embedded in paraffin, and sectioned at a thickness of 5 mm with a microtome. Sections were stained with hematoxylin-eosin and used for histologic examination by light microscopy.

Statistical analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 13.0. One-way analysis of variance (ANOVA) was used during the statistical analysis. A value of $P < 0.05$ was considered statistically significant.

Results

Method validation

LC-MS/MS parameters were optimized to produce the maximum response for FK506 in positive-ion mode. Figure 5 shows the production mass spectra of $[M+Na]^+$ of FK506. After ESI, positive-ion fragments of m/z 826.4 were detected in the SRM mode with a triple quadrupole tandem mass spectrometer, and the fragment ions of m/z 616.3 were chosen as the production for monitoring FK506. The MRM transitions selected for the detection of FK506 and IS were 626.4 $\rightarrow$ 616.3 and m/z 743.2 $\rightarrow$ 573.2, respectively. Representative LC-MS/MS chromatograms for FK506 and IS are shown in Fig. 6. Retention times of FK506 and IS were 6.6 and 6.2 min, respectively, without interference between them. Therefore, the method showed good selectivity and acceptability.

Table 1. Summary of Accuracy and Precision of Quality Control Samples in LC-MS/MS Detection

Added (ng/mL)	*Found (ng/mL)*	*RSD (%)*	*RE (%)*
1	0.98 ± 0.11	11.46	−2.67
10	9.53 ± 0.58	6.15	−4.67
100	96.40 ± 5.9	6.12	−3.60
1600	1621 ± 53.35	3.29	1.31

LC-MS/MS, liquid chromatographic–tandem mass spectrometry; RSD, relative standard deviation; RE, relative error.

The LC-MS/MS method was validated in accordance with the Guidance for Industry, Bioanalytical Method Validation of the Food and Drug Administration (FDA). The accuracy (RE%) and precision (RSD%) results for the quality control (QC) samples are summarized in Table 1. The results of RE ≤ 15% and RSD ≤ 15% confirmed an acceptable accuracy and precision for the proposed method.

In vitro *release study*

Figure 7 shows *in vitro* release profiles of Tacrolimus from the episcleral drug-delivery implant. The release rate was biphasic (faster during the first 12 h and slower thereafter). Initial bursts were observed from the 0.25, 0.5, and 1.0 mg/mL Tacrolimus-loaded implants, after which the concentrations fluctuated between 1 and 2 μg/mL. The concentrations of drugs remaining (0.11, 0.23, and 0.61 mg/mL, respectively) in the capsules at the 96-h time point in percentages relative to the initial amount were 44%, 46%, and 61%, respectively.

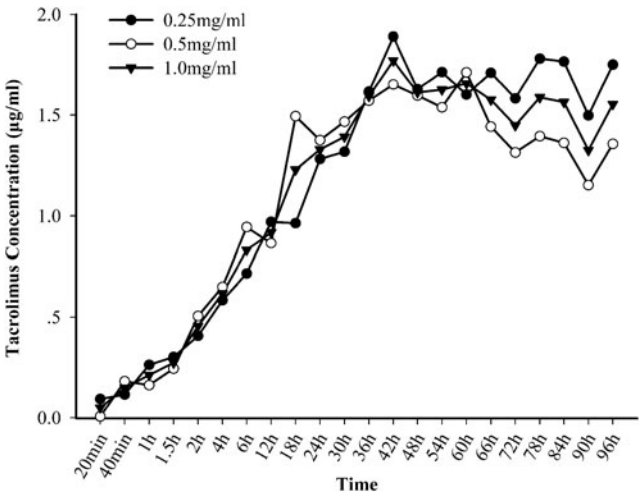

FIG. 7. *In vitro* release of Tacrolimus from the implant followed a biphasic pattern for 96 h.

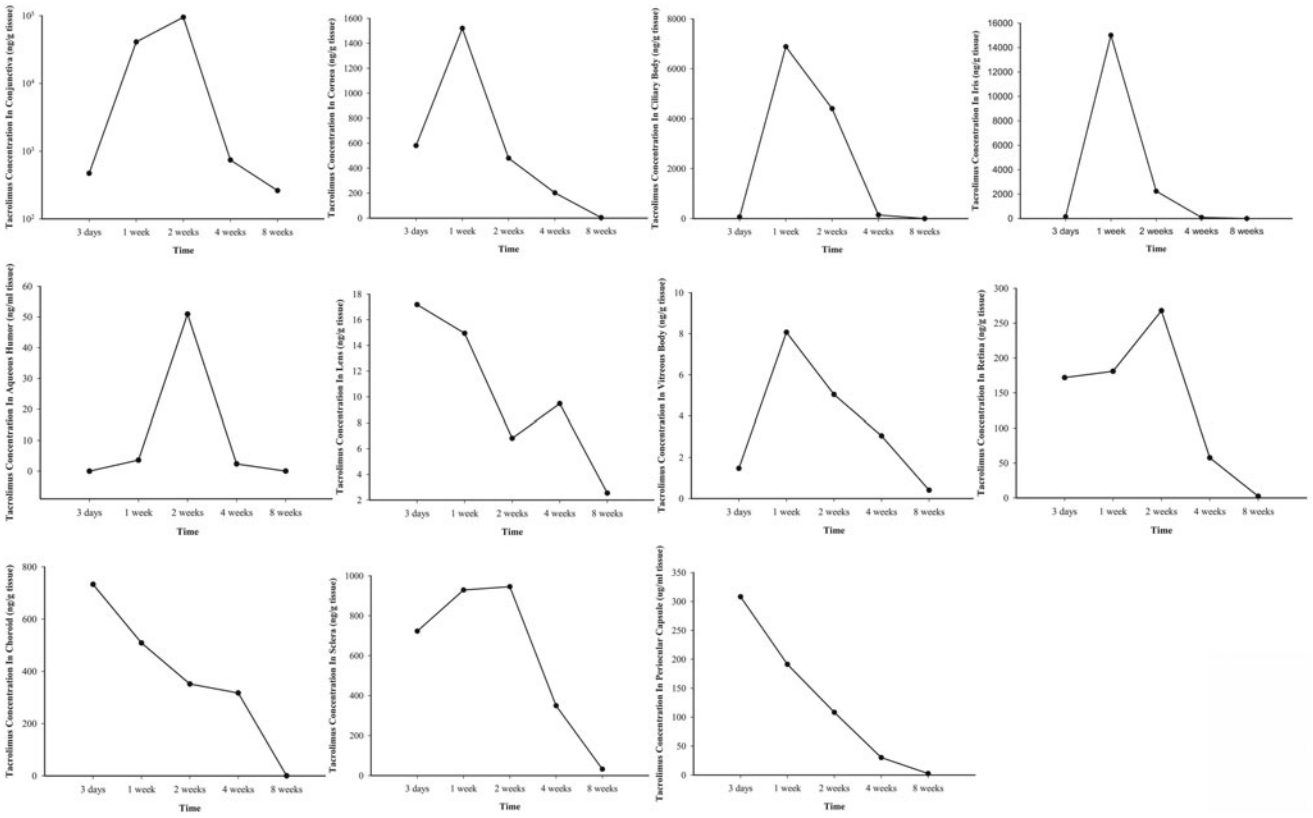

FIG. 8. The whole-blood and tissue concentrations of Tacrolimus (0.5 mg/mL) after implantation surgery in the experimental group. Each point represents the mean of data obtained from 3 rabbits.

In vivo *release study (experimental group)*

Figure 8 summarizes the *in vivo* release of Tacrolimus from the episcleral drug-delivery implant. The whole-blood concentration remained constant at a low level (0.31 to 1.83 ng/mL) throughout the study period. Tacrolimus was distributed predominantly in the conjunctiva, cornea, iris, and ciliary body, where the concentration increased sharply to reach peaks of 94.85 (on the 14th day), 1.52, 15.01, and 6.89 μg/g tissue (on the 7th day), respectively. In addition, relatively high concentrations of Tacrolimus were detected in the sclera, retina, and choroid, with initial bursts 3 days postoperatively (773.00, 171.84, and 732.97 ng/g tissue, respectively). The drug concentration in the choroid showed a steady decline over the experimental period, dropping to 0.44 ng/g tissue on day 56. In contrast, sclera and retina concentrations showed a substantial increase to the highest levels of 945.74 and 276.90 ng/g tissue, respectively, at 14 days, followed by a considerable decrease to the lowest levels on day 56 (32.16 and 2.89 ng/g tissue, respectively). The concentrations in the lens, vitreous body, and aqueous humor were low, with peaks of 17.18 ng/g tissue (on the third day), 8.07 ng/g tissue (on the seventh day), and 50.94 ng/g tissue (on the 14th day), respectively. The remaining concentration of Tacrolimus decreased significantly over the 56-day study period, dropping from 500 μg/mL initially to 2.52 μg/mL at 8 weeks after surgery. The pharmacokinetic parameters of Tacrolimus are shown in Table 2.

TABLE 2. OCULAR TISSUE AND WHOLE-BLOOD PHARMACOKINETIC PARAMETERS OF TACROLIMUS AFTER IMPLANTATION SURGERY IN THE EXPERIMENTAL GROUP

	Conjunctiva	*Cornea*	*Aqueous humor*	*Iris*	*Ciliary body*	*Lens*	*Vitreous body*	*Retina*	*Choroid*	*Sclera*	*Residual in capsule*	*Whole blood*
Cmax (ng/mL)	94849.60	1520.27	ND[a]	15009.57	6885.74	17.18	8.0720	267.90	732.97	945.74	308.19	ND[a]
Tmax (week)	1.00	1.00	ND[a]	1.00	1.00	0.43	1.00	2.00	0.43	2.00	0.44	ND[a]
T1/2 (week)	1.59	0.87	ND[a]	0.71	0.52	2.10	1.61	0.86	0.62	1.00	1.11	ND[a]
Ke (h-1)	0.44	0.79	ND[a]	0.98	1.34	0.33	0.43	0.81	1.12	0.70	0.63	ND[a]
AUC0t (ng·w/mL)	177240.09	2823.20	ND[a]	15410.91	12486.86	65.54	24.81	808.56	2246.46	3599.05	563.83	ND[a]

Values represent the mean of 3 rabbits.

[a]No data were available for the concentrations at some of the sampling points as they were under the limits of quantification.

Cmax, maximum concentration; Tmax, time to maximum concentration; T1/2, elimination half-life; Ke, elimination rate constant; AUC, area under the curve.

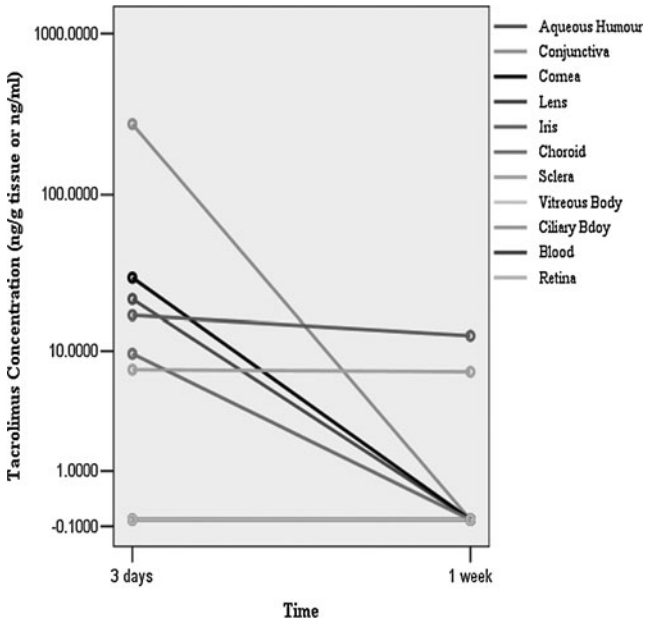

FIG. 9. The whole-blood and tissue concentrations of Tacrolimus (0.5 mg/mL) after subconjunctival injection in the control group. Each point represents the mean of data from 3 rabbits.

Drug distribution after subconjunctival injection of tacrolimus (control group)

As shown in Fig. 9, the Tacrolimus concentrations were generally low after subconjunctival injections, with relatively high levels at the 3-day time point (188.26 ng/g tissue in the conjunctiva, 28.96 ng/g tissue in the cornea, 18.57 ng/g tissue in the iris, 18.10 ng/g tissue in the aqueous humor, 8.47 ng/g tissue in the choroid, and 8.11 ng/g tissue in the sclera). Then, the concentrations in the conjunctiva, cornea, aqueous humor, and choroid declined dramatically until they were lower than the limits of quantification, while the iris and sclera concentrations nearly stabilized at low-concentration levels during a 7-day period (12.66 ng/g tissue in the iris and 7.20 ng/g tissue in the sclera on the seventh day). No Tacrolimus could be detected in the whole blood, ciliary body, lens, vitreous body, and retina after the subconjunctival injection.

Clinical observations and histology

No signs of complications were observed by slit-lamp biomicroscopy in the experimental group during the entire study. Figure 10 shows slit-lamp and fundus photographs. The IOP in the experimental group was maintained within normal limits during the 8-week follow-up period (Fig. 11), with no statistically significant difference ($P > 0.05$) between the experimental eyes and the contralateral eyes. Histological examination also demonstrated no obvious abnormalities at 8 weeks postoperatively.

Discussion

Immune-mediated diseases are prevalent causes of blindness for millions of patients around the world; therefore, identification of effective treatments is urgent. Tacrolimus, with its comparatively strong immunosuppressive potential, is a welcome new therapy. Nevertheless, systemic administration may lead to undesirable side effects. Therefore, ocular delivery should provide a safer approach for Tacrolimus therapy for immune-mediated disorders. Many studies have focused on the improvement of topical administration of Tacrolimus for use in ophthalmology. For example, Zhang et al.[20] reported that intravitreal injection of liposome-encapsulated Tacrolimus was effective in suppressing the process of experimental autoimmune uveitis, providing a vitreous concentration of 75 ± 16 ng/mL at 14 days after administration. Fang et al.[21] developed an implantable intravitreal device that could release Tacrolimus in a dosage-controlled manner; the device could maintain a drug concentration of $\sim$413.81 ng/g in the retinochoroid tissue. Sakurai et al.[22] studied the efficacy of a biodegradable polymeric scleral plug containing

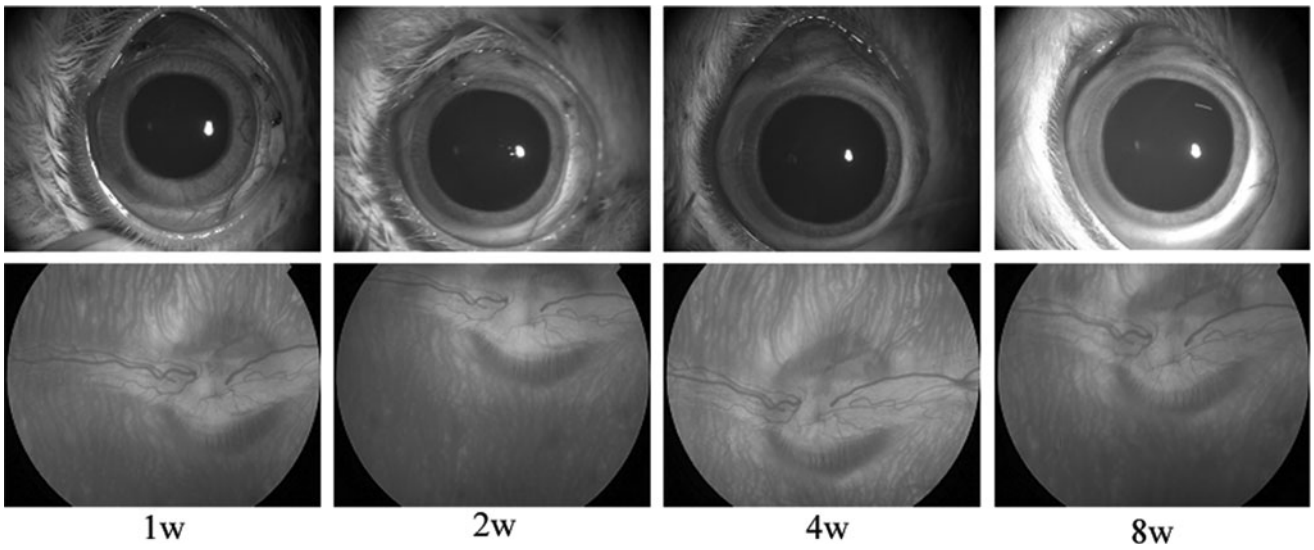

FIG. 10. Preoperative and 1-, 2-, 4-, and 8-week postoperative follow-up examinations using slit-lamp and fundus photography. No inflammation or other complications were observed in the anterior segments during the follow-up period. All eyes showed clarity in the vitreous cavities and the fundus.

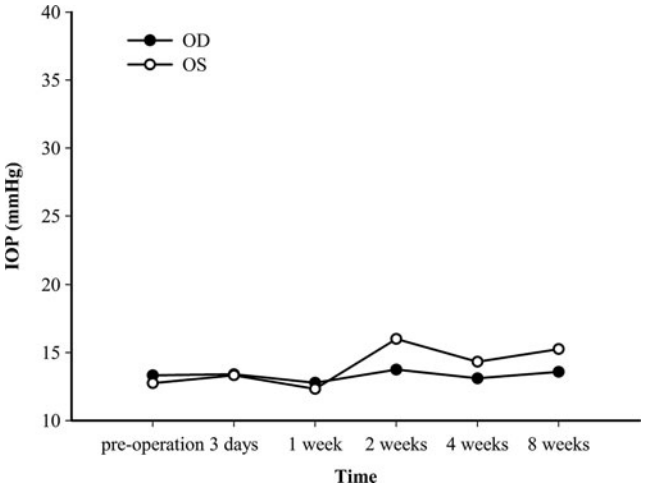

FIG. 11. Intraocular pressure changes during the 8-week follow-up in the experimental group. No significant differences ($P > 0.05$) were observed between the experimental and the contralateral eyes.

Tacrolimus in suppressing the inflammation of experimental uveitis in a rabbit model; the plug showed potential in the management of severe chronic uveitis in animals. These Tacrolimus delivery systems may elevate intraocular concentrations while reducing systematic effects; however, they are not perfect strategies due to complications, such as neurotoxicity, hepatitis, high blood pressure, and tremors, which decrease compliance. Thus, establishing a Tacrolimus delivery system that provides high efficacy and low invasiveness is an ongoing challenge in ophthalmology.

In the present study, we fabricated a Tacrolimus-loaded episcleral drug-delivery implant to investigate the *in vitro* and *in vivo* release characteristics of Tacrolimus. The Tacrolimus-loaded episcleral drug-delivery implant consists of a V-shaped capsule and a tube. The capability of this drug-delivery implant is attributed to its 300-nm apertures on the capsule. Immersion of the capsule injected with Tacrolimus suspension into a balanced salt solution forces movement of the drug molecules through the apertures to the circumference by drug concentration differences, as the *in vitro* experiment showed. Similarly, the released Tacrolimus is taken away by the blood or metabolized quickly in the *in vivo* condition, and the drug concentration difference still maintains the strain by constantly releasing Tacrolimus from the capsule to the episcleral space. The size of the apertures also limits the rapid loss of the drug and restricts the total flow rate; therefore, the drug is then released in a sustained fashion until it is completely consumed. Lee et al.[23] evaluated the clearance mechanisms with trans-scleral drug delivery, and they reported that the conjunctival blood and lymphatic vessel elimination are both important pathways of drug elimination. After implantation of the Tacrolimus delivery system, high drug levels were detected in the conjunctiva, iris, ciliary body, cornea, sclera, choroid, and retina (C max: 94849.60, 15009.57, 6885.74, 1520.27, 945.74, 732.97, and 267.90 ng/g tissue, respectively) in the experimental group. Then, according to Fujita et al.,[24] Tacrolimus in the eye would be absorbed into the nasolacrimal duct and distributed in the digestive tract via the nasal meatus and esophagus, after which it is excreted in the feces. To have a more profound understanding of the pathway of drug elimination from the eyes, a drug maker should be used to track the routes of Tacrolimus molecules. Further study would focus on the mechanism of the pathway of Tacrolimus elimination from the eyes.

The *in vitro* study showed that 0.25, 0.5, and 1.0 mg/mL Tacrolimus-loaded episcleral drug-delivery implants released the medication in a biphasic pattern for 96 h. The Tacrolimus release kinetics were divided in 2 phases: the quick elusion of Tacrolimus and diffusion into the buffer medium and the slow, sustained disposal when the drug concentration difference between within and outside the capsule became smaller. A higher dose of Tacrolimus did not lead to better effects; there was no proportional connection in release concentrations among 0.25, 0.5, and 1.0 mg/mL Tacrolimus-loaded implants. The release kinetics were not dependent on the drug concentrations.

In the first 24 h, Tacrolimus was released rapidly at all 3 test concentrations, with no significant difference in the release rate, suggesting that the loss of the drug and the total flow rate before achieving a balanced situation were mainly controlled by the size of the capsule's apertures and not by the concentration within the capsule. After 24 h, the concentrations of released drug were maintained at ~1.5 μg/mL, but the concentration was higher for the 0.5 mg/mL Tacrolimus-loaded implant than for the 1.0 mg/mL one. At 24 h, a small amount of cloudy white precipitate was observed at the bottom of the capsule injected with 1.0 mg/mL Tacrolimus. We inferred that the ingredients in the 1.0-mg/mL Tacrolimus-loaded implant were sufficiently unstable that they separated out after a short period. The precipitate then likely blocked some of the apertures in the capsule, resulting in a lower drug release.

The *in vivo* study showed significant differences in tissue concentrations between the experimental and control groups. Tacrolimus levels were particularly high in the conjunctiva, iris, ciliary body, cornea, sclera, choroid, and retina in the experimental group, while the drug concentrations in most ocular tissues in the control group were low and only detectable for 1 week. These data suggested that a conventional subconjunctival injection was an unsuitable therapy due to quick clearance by the nasolacrimal drainage system and poor penetration into the posterior eyeball.

Only a limited number of bench and clinical studies have shown the efficacy of Tacrolimus in immune-mediated diseases,[25] so its systemic and topical therapeutic concentration is still unknown. In organ-transplant recipients, the recommended minimum concentration is 2–10 ng/mL,[26] which may be the minimum effective level of Tacrolimus in treating immune-mediated ocular disorders. In our study, the episcleral drug-delivery implant maintained effective drug concentrations for nearly 8 weeks. Further, although the episcleral drug-delivery implant was placed directly in the posterior parts of the eyes, noticeably higher drug concentrations were detected in the anterior segment (conjunctiva, iris, ciliary body, and cornea) than in the posterior segment (sclera, choroid, and retina).

According to Ranta and Urtti,[27] 3 drug-penetration routes contribute to trans-scleral delivery: the anterior chamber, systemic circulation, and direct penetration routes. The direct penetration route is particularly important and is affected by the application side of the drug-delivery system on the sclera.[27] The barrier properties of the eye were

extremely crucial in the direct penetration route.[27,28] The retinal pigment epithelium (RPE) might be one of the rate-limiting permeation barriers to retinal delivery of macromolecules through the trans-scleral route.[27] Prausnitz and Noonan[29] concluded that the permeability in the RPE is determined by physicochemical factors (molecular weight, lipophilicity, charges, etc.) and the affinity for active transporters. As a part of the blood-ocular barrier, the RPE is equipped with a variety of transporters (eg, ABCB, ABCC, ABCG, SLC7, SLC16, SLC19, SLCO/SLC21A, SLC22A, and SLC29 transporters),[30] which regulate drug fluxes between the circulating blood and the retina. In our study, we detected that the concentrations of released Tacrolimus in the sclera, choroid, and retina were relatively low, which was surprising. Because the sclera, choroid, and retina are located at the shortest distance from the implant, and the sclera directly contacts the capsule; in theory, the drug concentrations should have been highest in these locations. However, that was not the case. Therefore, we proposed that the lower concentrations of released Tacrolimus seen in the sclera, choroid, and retina were related to the RPE barrier and active transporters in the RPE. The Tacrolimus-loaded drug-delivery implant was limited by efflux transporters in the RPE. Related studies in our group are being conducted by our group to confirm this hypothesis.

The normal results for slit-lamp photography, fundus photography, IOP, and histologic examinations indicated the safety of the Tacrolimus-loaded episcleral drug-delivery system and implantation surgery. The Tacrolimus-loaded episcleral drug-delivery implant provides favorable advantages over other ocular delivery formulations commercially used in clinics. First, traditional topical drug-delivery systems, such as eye drops, suspensions, ointments, and gels, are the most common drug-delivery strategies currently used. However, the need for multiple instillations may decrease patient compliance. In addition, topical drugs poorly permeated posterior lesions.[31] Tacrolimus eye drops are widely used in clinics, but studies indicate that 2.5 h after topical application of 0.05% (0.5 mg/mL) Tacrolimus, the drug concentrations could be detected only in the cornea (43.52 ng/g)[31]; while the episcleral drug-delivery implant, with the same initial concentration of Tacrolimus, released 580.73 ng/g of Tacrolimus into the cornea and continued to increase that release rate over time.

Intravitreal drug-delivery systems are another route for ocular delivery. Ozurdex[32] (Allergan) is one intravitreal strategy; it is a biodegradable dexamethasone implant currently undergoing clinical Phase III studies. The Phase II trial showed that this implant was effective in improving patients' vision, but it requires an invasive procedure and therefore may induce intraocular complications. In contrast, the episcleral drug-delivery implant does not damage the integral eyeball so that complications are less likely after the implantation surgery.

Our study has some limitations. First, only a small number of animals were evaluated at each time point, so any deviations between each rabbit during the observation period were negligible. The effect of Tacrolimus at different concentrations should also be determined by *in vivo* studies to further understand the pharmacokinetics of the drug-delivery system. Second, animal models of ocular diseases should be used to gauge the efficiency of the Tacrolimus-loaded episcleral drug-delivery implant in treating immune-mediated ocular disorders. Third, the 8-week observation period was not sufficiently long. The Tacrolimus concentrations in different ocular tissues were low or even lower than the limits of quantification at the end of the study. Fresh Tacrolimus solution could be administered via the valve at various time points to increase the ocular concentrations for longer observation periods. Fourth, the dosage released from the episcleral drug-delivery implant was not sufficiently stable. Future research will focus on improving the release rate and increasing the level of the released drug. For example, we can increase the numbers or sizes of the apertures on the capsule to raise the release rate of the drug.

The episcleral drug-delivery implant is a vehicle that could be used for other drugs (including antibiotics, anti-proliferation agents, and anti-VEGF, etc.) and even stem cells. Episcleral drug-delivery implants may therefore have potential for the treatment of many chronic ocular diseases.

Conclusion

Our study suggests that the episcleral drug-delivery implant can mechanically release Tacrolimus through the apertures of the capsule. The Tacrolimus-loaded episcleral drug-delivery system may be a promising drug vehicle for the treatment of immune-mediated ocular disorders.

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Author Disclosure Statement

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