

# Homemade lyophilized cross linking amniotic sustained-release drug membrane with anti-scarring role after filtering surgery in rabbit eyes

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## Abstract

• **AIM:** To investigate the antifibrotic effect of the freeze-dried bilayered fibrin-binding amniotic membrane as a drug delivery system on glaucoma surgery in rabbit model. The aim of this study was to prepare a novel local delivery system for the sustained and controllable release of 5-Fu.

• **METHODS:** Twenty-four Japanese white rabbits were randomized into three groups: the experimental group (ocular trabeculectomy in combination with 5-Fu loaded freeze-dried bilayered fibrin-binding amniotic membrane transplantation), the control group (ocular trabeculectomy in combination with 5-Fu) and the blank group (single trabeculectomy). HE staining, masson staining and immunohistochemistry for  $\alpha$ -SMA were performed on days 7, 14, 21 and 30 following surgery. The concentration of 5-Fu in rabbit aqueous humor was examined by high performance liquid chromatography (HPLC) 3 days after the surgery.

• **RESULTS:** Statistical differences were noted in intraocular pressure among groups on day 7, 14, 21 and 30 following surgery. Histology further demonstrated that trabeculectomy in combination with freeze-dried bilayered fibrin-binding amniotic membrane yielded well wound healing and no scar formation and was beneficial for long term effect.

• **CONCLUSION:** HPLC showed a good slow-release effect with freeze-dried bilayered fibrin-binding amniotic membrane.

• **KEYWORDS:** freeze-dried bilayered fibrin-binding amniotic membrane; drug delivery system; filtering surgery; glaucoma; inhibition of scarring formation

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## INTRODUCTION

Filtration channel fibrous tissue hyperplasia after glaucoma filtering surgery leading to scar adhesion is the main reason for surgical failure<sup>[1]</sup>.

There are two methods for clinical scar reduction, one is to use the drugs which could inhibit scarring<sup>[2]</sup>, the second is to place the implants under conjunctival flap after trabeculectomy, mechanical isolating the conjunctiva and sclera of the operating area, avoiding or reducing scar formation, and maintaining filtration channel smooth<sup>[3]</sup>.

Improvement of the implant material is another main direction of glaucoma filtration surgery research. It is urgent to choose biopolymer functional materials with the growth inhibition role of fibroblast and with excellent biocompatibility and tardo biological degradation; or to add scar formation inhibition drugs on the implants, thus effectively reducing bleb scarring and improving the success rate of surgery<sup>[4-6]</sup>.

The amniotic membrane is an excellent tissue engineering materials. Firstly, the normal amniotic membrane is thin and transparent, without antigenicity, blood vessels, nerve growth and rejection. Secondly, the amniotic membrane is rich in a variety of cytokines, which could resistant to infection, reduce inflammation, inhibit angiogenesis formation and stimulate the differentiation of epithelial and goblet cells. Thirdly, the amniotic membrane can enhance the adhesion of basal cell, prevent apoptosis of epithelial cells and maintain normal epithelial surface. Fourthly, the

amniotic membrane as an effective drug release system, so that releasing drugs slowly [7,8]. In addition, the amniotic membrane with drawn wide, simple preparation and can be long-term preserved.

Our experiment is to put 5-Fu into the fibrin glue, using the characteristics of fibrin glue [9], to make freeze-dried crosslinking amniotic sustained-release drug membrane.

Animal experiments were performed to compare the effects of trabeculectomy surgery and trabeculectomy surgery with lyophilized crosslinking amniotic release membrane under conjunctival flap and trabeculectomy surgery with 5-Fu cotton under conjunctival flap 5-Fu cotton under conjunctival flap for 5 minutes. Our data provided theoretical basis for clinical treatment.

### MATERIALS AND METHODS

**Materials** Twenty-four healthy adult Japanese white rabbits weighing 2.5kg-3.5kg, were provided by the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology. All experiments were performed in accordance with the applicable national regulations and with the Association for Research in Vision and Ophthalmology (ARVO) guidelines regarding the care and use of animals for ophthalmic and vision research.

These rabbits were randomly divided into three groups, including experimental group (8 rabbits with 16 eyes): trabeculectomy surgery with lyophilized crosslinking amniotic release membrane under conjunctival flap; control group (8 rabbits with 16 eyes): trabeculectomy surgery with 5-Fu cotton under conjunctival flap; blank group (8 rabbits with 16 eyes): trabeculectomy only. The two eyes of each rabbit received the surgery at the same time.

### Methods

**Source and preparation of amniotic membrane** The fresh placenta of non-infectious diseases (viral hepatitis, AIDS, syphilis, etc) and systemic disease pregnant women were chose by caesarean section. The amniotic surface was washed by saline water to remove blood under sterile conditions, soaked for 20 minutes with sterile saline containing antibiotics (concentration of the antibiotic mixture containing 50µg/mL penicillin, 50µg/mL streptomycin, 100µg/mL neomycin and the 2.5µg/mL amphotericin B.) The chorion was separated from the amnion by blunt dissection. The amnion was put on a nitrocellulose filter paper with epithelial side up.

The amnion together with the paper was placed in a container equipped with sterilized glycerol for 24 hours, and then transferred to another closed glycerol-containing bottle in a refrigerator at 4°C.

Preparation of lyophilized crosslinking amniotic drug membrane: All the operations were under sterile conditions. Two amniotic membrane maintained in glycerol were chose and put into 0.9% saline water to full rehydration for 20

minutes, and cut into 3.0cm ×3.0cm small pieces. One amniotic membrane was turned with basal surface down and 5-Fu containing fibrin glue was spread evenly on the epithelial surface, and then another amniotic membrane with basal surface down was put on it with neat involution. It was put into the -80°C refrigerator for 12 hours, quickly placed in the lyophilizer for 12 hours, packaged under vacuum at room temperature packaging and sterilized by gamma 60 ray.

**Surgery** Experimental animals were general anesthetized with ketamine (50mg/kg) and Toluene thiazide (15mg/kg). 1% lidocaine was used for subconjunctival anesthesia. Conjunctival flap was used for 3mm × mm scleral flap. In group A, freeze-dried glue was spread on the scleral flap and below the conjunctival flap, repaired with 4 sutures using 10-0 suture needle. In group B, 5-fu cotton was spread on the scleral flap and below the conjunctival flap, five minutes later, washed by 0.9% saline water and repaired with 4 sutures using 10-0 suture needle. In group C, only trabeculectomy was performed.

**Intraocular pressure measurement** Preoperative intraocular pressure (IOP) and postoperative IOP at 7, 14, 21, 30 days were determined by Tono-pen tonometer (by Tono-Pen XL, Mentor, USA), and Double-Factor Variance Analysis was performed.

**Histopathological analysis** Each of eight animals of three groups from 7, 14, 21 and 30 days after surgery were injected a lethal dose of pentobarbital sodium, evisceration was performed. Eyes were cut into 10mm×10mm, filtered to remove blebs, fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned and HE stained for general histological analysis. Massion staining was used to observe the distribution of collagen deposition. Immunohistochemistry assay of α-SMA was used to evaluate muscle fibroblasts distribution.

**Pharmacokinetics study of 5-FU in rabbit aqueous humor** The concentration of 5-FU in rabbit aqueous humor was examined by high performance liquid chromatography (HPLC) 3 days after the surgery. The UV wavelength was 268nm. HPLC separation was performed on a C18 analytical column (250×4.0mm, 5µm). The mobile phase consisted of ammonium phosphate buffer (50mmol/L, pH 6.0) with a flow rate of 1.0mL/min. The rabbits were generally anaesthetized (intramuscular injected with 50mg/kg ketamine and 15mg/kg xylazine for general anaesthesia, and 1% lidocaine for surface anaesthesia) 3 days after the surgery. 1mL syringes were used to puncture from rabbit cornea into anterior chamber and 100µL aqueous humor was extracted at each time point and was preserved immediately in -80°C refrigerator. Method of detection: added 500µL acetonitrile into 50µL aqueous humor sample to precipitate the protein, vortexed 3 minutes, centrifuged at 3 000r/min for 10 minutes,

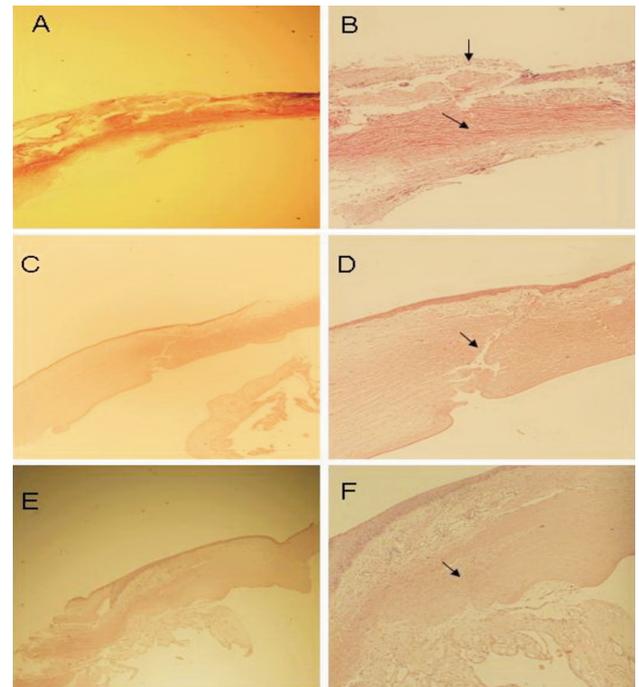
extracted 480 $\mu$ L supernatant, dried in an evaporimeter (UT-80, Eyela, Tokyo, Japan), added 200 $\mu$ L ammonium phosphate buffer into the residues to form the mobile phase and used 20 $\mu$ L mobile phase with automatic pipette for HPLC detection. T-test was performed.

## RESULTS

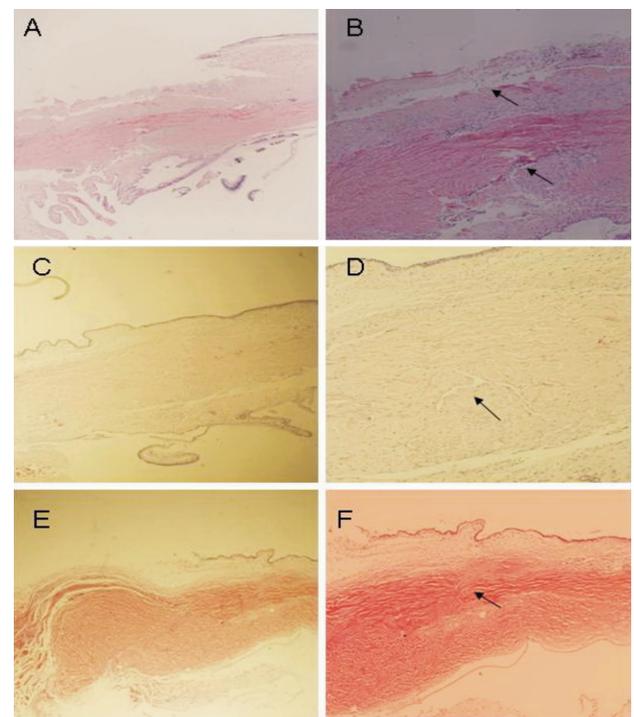
**HE Staining** With light microscope, we found that the filtration tracks from the experimental groups at 14 days and 30 days after the surgery were both clear. No obvious proliferation of fibroblasts and tissue fibrosis were observed in conjunctival flaps and superficial sclera. Some irregular window-like sclera gaps were observed in filtration fistula. Obvious conjunctival filtration blebs with some fibers and cells surrounded were seen in most of pathological sections, suggesting the good function of filtration tracks and the integrity of amniotic membranes (Figure 1A, 1B) (Figure 2A, 2B). Consist with the experimental groups at 14 days after surgery, no obvious proliferation of fibroblasts and tissue fibrosis were observed in conjunctival flaps and superficial sclera from the control groups at 14 days. The function of filtration was fine and filtration blebs existed. No macrophages, foreign body giant cells or tissue exudates were observed in operation fields (Figure 1C, 1D). However, in the control groups at 30 days after surgery, we observed obvious proliferation of fibroblasts and tissue fibrosis in both conjunctival flaps and superficial sclera. Proliferated fibroblasts and scar tissues could also be observed in filtration tracks (Figure 2C, 2D). In the blank groups, no matter at 14 days or 30 days after the surgery, filtration tracks and the interspaces between conjunctiva and sclera were filled with lots of granulation tissues. A large number of fibroblasts could be seen and filtration tracks were blocked (Figure 1E, 1F and Figure 2E, 2F).

**Massion Staining** With light microscope, little collagen was observed in filtration tracks and conjunctiva from both experimental groups and control groups at 14 days after the surgery. The amniotic membranes of experimental groups were integrated (Figure 3A-D). Obvious filtration fistulas and a small amount of collagen could be seen under conjunctiva in experimental groups at 30 days after surgery (Figure 4A, 4B), while some amount of collagen were deposited in filtration tracks and conjunctiva in control groups (Figure 4C, 4D). In blank groups at both 14 days and 30 days after the surgery, a great amount of intensive and parallel collagen could be observed in filtration tracks and conjunctiva (Figure 3E, 3F, and Figure 4E, 4F).

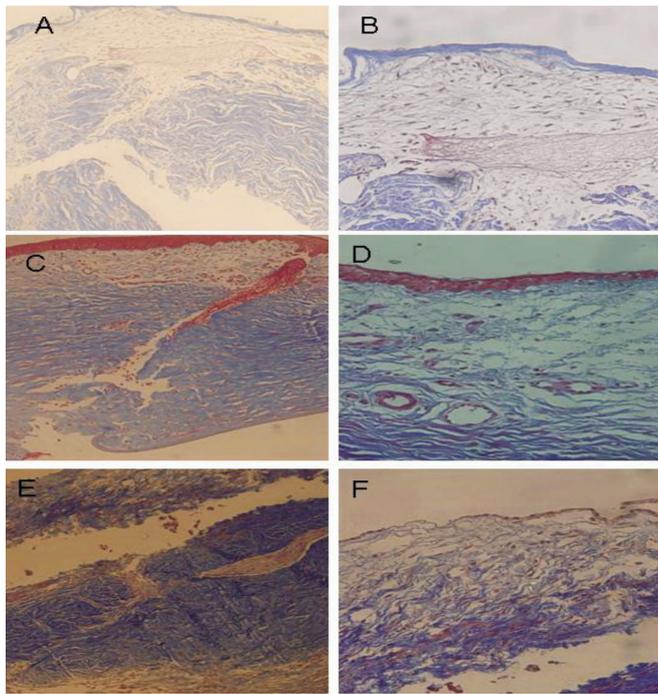
**$\alpha$ -SMA Immunohistochemistry Staining** With light microscope, we found that at 14 days after the surgery, no apparent expression of  $\alpha$ -SMA was observed in experimental groups, little expression in control groups and great expression in blank groups (Figure 5). At 30 days after the surgery,  $\alpha$ -SMA was little expressed in experimental groups, obviously expressed in control groups and highly expressed in blank groups.



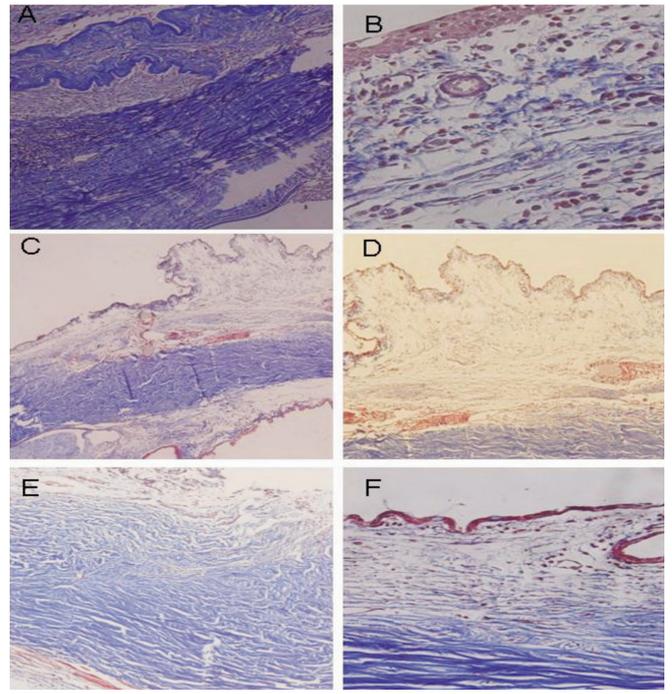
**Figure 1 Staining of rabbit corneoscleral region 14 days** A, B: after trabeculectomy and implantation of lyophilized crosslinking amniotic sustained-release drug membrane under the conjunctival flap; C, D: after trabeculectomy and implantation of 5-Fu cotton under the conjunctival flap; E, F: after trabeculectomy. (The existence of amniotic membranes and the good function of filtration tracks were shown in A, B; the good function of filtration tracks was shown in C, D; the existence of fibroblasts in filtration tracks was shown in E, F).



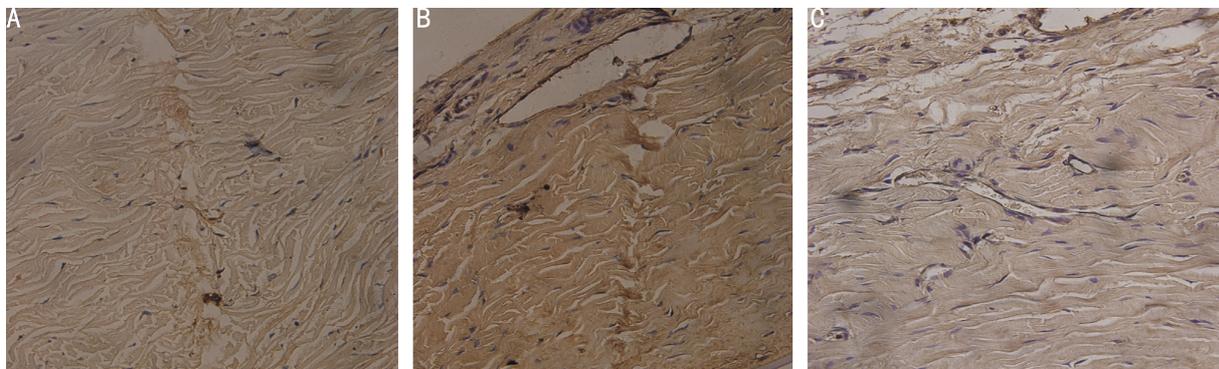
**Figure 2 HE staining of rabbit corneoscleral region 30 days** A, B: after trabeculectomy and implantation of lyophilized crosslinking amniotic sustained-release drug membrane under the conjunctival flap; C, D: after trabeculectomy and implantation of 5-Fu cotton under the conjunctival flap; E, F: after trabeculectomy. (The existence of amniotic membranes and the good function of filtration tracks were shown in A, B; most of the filtration tracks were blocked in C, D; the filtration tracks were blocked completely, and large amount of fibroblasts in E, F).



**Figure 3 Massion staining of rabbit corneoscleral region 14 days** A, B: after trabeculectomy and implantation of lyophilized crosslinking amniotic sustained-release drug membrane under the conjunctival flap; C, D: after trabeculectomy and implantation of 5-Fu cotton under the conjunctival flap; E, F: after trabeculectomy. (The existence of filtration tracks and a small amount of collagen in A-D; the existence of amniotic membranes in A, B; a great amount of intensive and parallel collagen in filtration tracks and conjunctiva in E, F).



**Figure 4 Massion staining of rabbit corneoscleral region 30 days** A, B: after trabeculectomy and implantation of lyophilized crosslinking amniotic sustained-release drug membrane under the conjunctival flap; C, D: after trabeculectomy and implantation of 5-Fu cotton under the conjunctival flap; E, F: after trabeculectomy. (The existence of amniotic membranes, filtration bleb and fistulas in A, B; a great amount of collagen in filtration fistulas and a small amount of in C, D; a great amount of collagen in conjunctiva and filtration fistulas in E, F).

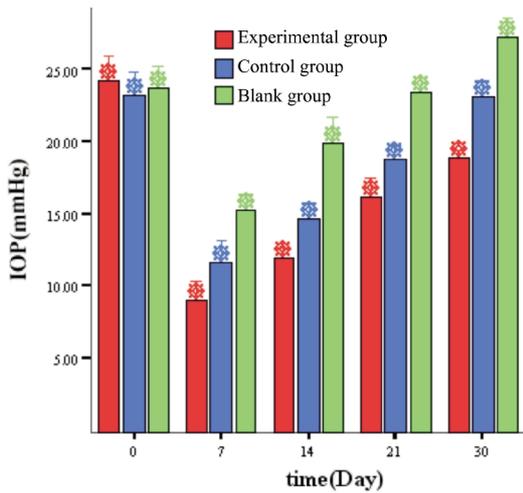


**Figure 5 Immunohistochemical staining of  $\alpha$ -actin protein: expression of  $\alpha$ -SMA 14 days after operation** A: without obvious expression. Experimental group, trabeculectomy surgery with lyophilized crosslinking amniotic release membrane under conjunctival flap; B: small amount of expression. Control group, trabeculectomy surgery with 5-Fu cotton under conjunctival flap; C: highly expressed  $\alpha$ -SMA. Blank group, trabeculectomy.

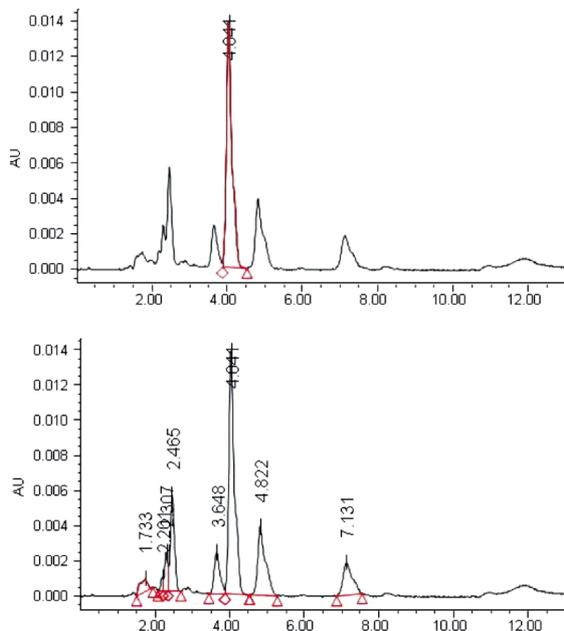
**Intraocular pressure measurement** We measured intraocular pressure with contact tonometer (Tono-Pen XL, Mentor, USA) at the same time point at 7, 14, 21, 30 days before and after the surgery, respectively and recorded the average of 3 measurements (Figure 6). Analyzed by two-way ANOVA, the differences among the experimental groups, control groups and blank groups are statistically significant ( $P < 0.001$ ). Further analyzed by a Fisher's least significant difference (LSD) post hoc test, we found there was a significant difference between the experimental

groups and the control groups ( $P < 0.001$ ), the experimental groups and the blank groups ( $P < 0.001$ ) and the control groups and the blank groups ( $P < 0.001$ , Figure 6).

**Pharmacokinetics of 5-Fu in Rabbit Aqueous Humor** HPLC standard curve was showed (Figure 7). 5-Fu was released more in experimental groups than in control groups at 3 days after the surgery. The absorbance was higher in experimental groups than in control groups at 3 days after the surgery.



**Figure 6** Experimental group, control group and blank group was statistically significant using 2-way ANOVA. The experimental group and control group was statistically significant using the LSD method. The control group and blank group were statistically significant.



**Figure 7** Standard chromatograms by the detection of high performance liquid chromatography (HPLC).

## DISCUSSION

In our experiments, we use the amniotic membrane as the substrate and the fibrin glue as the sustained release carrier of the 5-Fu to make the freeze-dried fibrin glue-amniotic membrane with the aim of investigating the anti-scarring role of this new biomaterial in glaucoma filtering operation in rabbits. Thanks to the network structure of fibrin sealant, the drug can be wrapped into the glue easily and released slowly to avoid the sudden release. Once reported that various cytokines or drugs were loaded in the fibrin glue which can play its sustained releasing role [10-14]. Olsen did experimental study about the epichoroidal space cannulation system and proved the safety and feasibility of this system.

He presumed this method would be the effective, targeted intra-infusion approach<sup>[15]</sup>.

Freeze-drying technology in the state of very low pressure low temperature can lead to dehydration and drying, maintain the protein anhydrous without affecting the quality of quantity of the major chemical bonds. It can restore the characteristics of the raw materials after adding water without affecting their biological activity. Freeze-drying technology has been widely used in our daily lives, such as: food preservation. In medicine, the freeze-drying technology also plays an increasingly prominent role. Through this technology, tissue extracts, bacteria, vaccines and plasma keep in a dry state, which keeps from the happening of enzymatic, bacteria and chemical changes<sup>[16-18]</sup>.

It is known that the direct application of 5-Fu at the wound leads to toxicity and various complications such as corneal punctate epithelial shedding, corneal erosion, wound leakage, bleb leakage and infection; shallow anterior chamber, anterior chamber inflammatory response<sup>[19,20]</sup>.

At present, studies focus on the treatment of glaucoma in the two following aspects. One is the angiogenesis inhibition and cytotoxicity reduction through drugs [21]. Another is the reduction of intraocular pressure through the application of biomedical materials as well as reduction of the wound adhesion after glaucoma surgery<sup>[22,23]</sup>.

We dissolve the fibrin glue and 5-Fu as a whole and crosslink it with the amniotic membrane. Through the use of freeze-drying technology, we have made sustained-release membrane. This new membrane can avoid the direct contact of 5-Fu with wounds at operating area and keep slow release of 5-Fu. Moreover, it effectively prevents the formation of scleral scar for a long time; maintains filtration channel and the aqueous outflow channel under conjunctiva in an unobstructed status; maintain intraocular pressure at a relatively low range and extend the function of filtration after surgery.

The lyophilized cross linking amniotic release membrane is not significant different with natural state of amniotic membrane in morphology. It maintains a good biocompatibility of the original amniotic membrane with non-antigenic and anti-infective functions. In addition, the toughness of the membrane is increased, so that the amniotic membrane can be long-term preserved at room temperature, and the characters of the double-layer amniotic membrane is kept and degradation time is prolonged. We found that the glue in the lyophilized crosslinking amniotic membrane persists for two weeks in the body<sup>[24]</sup>.

To identify whether sustained-release membrane could maintain the status and function of the bleb after trabeculectomy, intraocular pressure was measured. Histopathology analysis was carried out and aqueous 5-Fu pharmacokinetics was examined.

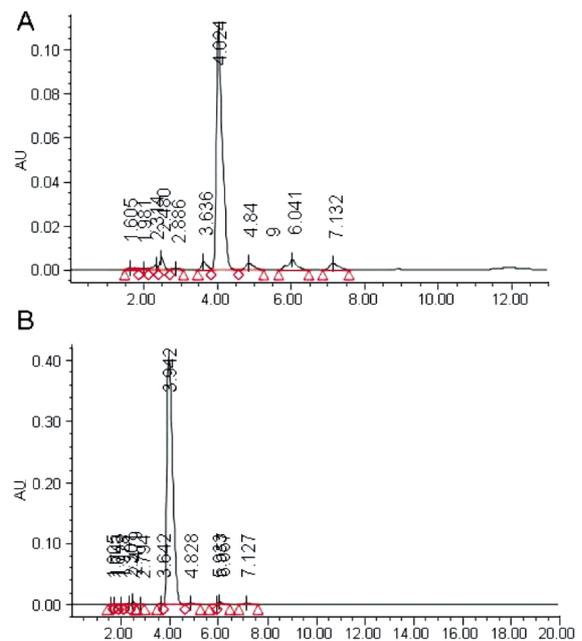
At the same time point on day 7, 14, 21 and 30 before and after surgery, intraocular pressure was measured by using contact tonometer (Tono-Pen XL, Mentor, USA), and the average values were recorded (Figure 6). The experimental group, control group and blank group were statistically significant ( $P < 0.001$ ) by the analysis of two-way ANOVA. These differences was more significant ( $P < 0.001$ ) when using LSD method.

HE staining results showed that on day 14 and day 30 after surgery, filtration channel was unobstructed, and the blebs and amniotic membrane were integrated in the experimental group (Figure 1A, 1B and Figure 2A, 2B), suggesting that filtration channel had a good function. Similar to control group, experimental group had unobstructed filtration channel on day 14. The conjunctival flap and the episclera displayed no significant fibroblast proliferation and tissue fibrosis. Macrophages, foreign-body giant cells and tissue exudates were not seen in the surgical area (Figure 1C, 1D). However, fibroblast proliferation and scar tissues were observed in the conjunctival flap and the episclera in the control group after 30 days. Proliferation of fibroblast cells and scar tissue could be seen in filtration channel (Figure 2C, 2D).

In blank group, 14 days or 30 days after surgery, filtration channel and the gap between conjunctiva and sclera were filled with a lot of granulation tissues. A large number of fiber fibroblast cells were seen and filtration channel was obstructed (Figure 1E, 1F and Figure 2E, 2F). Immunohistochemistry and Masson staining were further used to analyze the morphology changes of filtration fistula and bleb after filtration surgery. Myofibroblasts play an important role in wound repair process, and like smooth muscles, they can make the cells connected to each other through mechanical contraction, eventually leading to the formation of wound contraction and formation of extracellular matrix [25,26]. The main characteristic of myofibroblasts is the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)<sup>[27]</sup>.

Massion staining: a very small amount of collagens were observed in filtration channel and conjunctiva through light microscopy on day 14 in the both experimental group and the control group. In the both experimental group, amniotic membrane was integrated (Figure 3A-D). In experimental group on day 30, filtration fistula and a small amount of collagen deposited under the conjunctival were still observed (Figure 4A, 4B).

In control group on day 30, certain amounts of collagens deposition in the filtration path and within conjunctiva (Figure 4C, 4D) were seen. A large number of dense-parallel collagen deposition in filtration path and conjunctiva were seen in blank group on day 14 and day 30 (Figure 3E, 3F and Figure 4E, 4F). Immunohistochemical staining of  $\alpha$ -actin protein: (Figure 5).  $\alpha$ -SMA was seldom expressed in



**Figure 8 HPLC chromatograms of rabbit aqueous humor 5-Fu 3 days post operation** A: in the experimental group; B: in the control group. Absorbance: A>B. 5-Fu release was higher in group A than group B after three days post operation.

the experimental group, markedly expressed in control group and highly expressed on day 30 after operation. 5-Fu pharmacokinetics in the rabbit aqueous humor: 5-FU release in experimental group was greater than that in the control group 3 days after operation. The absorbance of experimental group was greater than control group 3 days after operation (Figure 8).

Our results demonstrate that sustained-release membrane maybe act as a novel drug delivery system to be used in glaucoma filtration surgery. We have two main issues which need further study: 1) How to extend the time of the FG degradation in order to increase its *in vivo* release properties and effectively avoid the burst release. 2) A larger sample size and the longer time of the animal experiments are needed to further confirm its effectiveness.

In our experiments, the sustained-release amniotic membrane prolongs the survival time of blebs, inhibits the degree of tissue scarring under the conjunctival, and maintains the post-operative curative effect for a long time. This new material will be possibly applied in the glaucoma filtration surgery.

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