

Impact of application of bio-amniotic membrane immersed in 5-fluorouracil solution in trabeculectomy on rabbit retina

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Background: To observe the impact of application of bio-amniotic membrane immersed in 5-fluorouracil solution in trabeculectomy on the retina in a rabbit model. **Materials and Methods:** Healthy white New Zealand rabbits were randomly assigned into three groups with 20 in each group. Bio-amniotic membranes of 4 × 5 mm immersed in either physiological saline/water for 10 min, or 25 mg/mL 5-fluorouracil solution for 5 and 10 min, respectively, were applied on rabbit eyes during trabeculectomy. At 7, 14, 21, and 28 days of postoperation, five rabbits from each group were examined with electroretinogram (ERG). After being examined for eye pressure and bleb morphology, rabbits were sacrificed by air embolism and their retinas were collected and examined by transmission electron microscopy (TEM). In addition, 5-fluorouracil amount in bio-amniotic membranes was measured using high-performance liquid chromatography. **Results:** Each bio-amniotic membrane could absorb 59.004 μg and 75.828 μg 5-fluorouracil after being immersed in 5-fluorouracil solution for 5 and 10 min, respectively. Application of these bio-amniotic membranes in trabeculectomy could promote the formation of well-functioning bleb and maintain intraocular pressure, although it had no effect on retina structures as examined with ERG and TEM. **Conclusion:** Application of 5-FU soaked bio-amniotic membrane in rabbit eye trabeculectomy is effective and safe.

Key words: Biological amniotic membrane, 5-fluorouracil, retina, trabeculectomy

The aim of penetrating filtering surgery for glaucoma is to reduce intraocular pressure (IOP) and preserve residual visual function by forming filtering bleb through establishment of a new passage of aqueous humor from the anterior chamber to the subconjunctival space. Thus, postoperative maintenance of bleb function is of importance to ensure the success of operation. Bleb failure is most often resulted from fibroblast proliferation-induced scarring of filtration passage.^[1] Surgery-caused damage and its following repair are the key issues in surgical scar region. Intraoperative placement of amniotic membrane beneath conjunctiva or scleral flap can effectively prevent scar formation,^[2] thus keeping the filtration pathway smooth and effectively maintaining long-term bleb functioning.

Currently, intraoperative and postoperative application of mitomycin is still considered as the most effective and commonly used method to improve success rate.^[3] However, application of antimetabolites could lead to complications such as corneal epithelial defects, bleb leakage, persistent ocular hypotony, and endophthalmitis^[4] and is effective only for a short period. 5-Fluorouracil can inhibit fibroblast proliferation and help form and maintain functional filtering bleb.^[5] In previous clinical courses, antiproliferative drugs were not applied to the filtration passage after trabeculectomy to avoid drug-induced ophthalmic tissue damage. Application of bio-amniotic membrane immersed or not immersed with

antiproliferative drugs to filtration passage and its effects on eyes, especially on retina, have not been reported. In this study, we applied 5-fluorouracil-immersed bio-amniotic membranes during trabeculectomy and explored its impacts on retinal structure and function.

Materials and Methods

Materials

5-Fluorouracil standard and 5-fluorouracil injection solution (25 mg/mL) were procured from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. High pressure liquid chromatography (HPLC) grade methanol and ultra-pure water were procured from J.T. Baker Company (USA). Lyophilized and Co⁶⁰ sterilized bio-amniotic membranes with size of 4 × 5 mm and free of hepatitis B, hepatitis C, syphilis, Human immunodeficiency virus (HIV), and other pathogens were procured from Jiangxi Rui-Ji Medical Devices Co. Ltd. They were prepared as follows: Clean amniotic membranes were soaked in 30% glycerol. After prefreezing them for 10 h at 0°C to -56°C, they were lyophilized at -10°C to -56°C till their water content reached 1–2%. The membranes were then repacked and sterilized by Co⁶⁰. Prior to use, the membranes were rehydrated for 5 min. Healthy adult New Zealand white rabbits (2–3 kg, 30 males and 30 females) were procured from Jinan Experimental Animal Center and kept under normal conditions. The study was approved by the Ethics Committee of Jinan Second People's Hospital.

High pressure liquid chromatography

5-fluorouracil was analyzed on Diamonsil C₁₈ column (5 μm, 4.6 × 250 mm) using methanol–water (2:98) as mobile phase at flow rate of 1.0 mL/min and column temperature of 30°C, and monitored by absorption at 266 nm. 5-fluorouracil

Access this article online

Website:

www.ijo.in

DOI:

10.4103/0301-4738.121151

Quick Response Code:

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Manuscript received: 28.10.12; **Revision accepted:** 17.06.13

standard was first dissolved in methanol and then prepared as 100 µg/mL methanol–water (2:98) solution. Twenty bio-amniotic membranes were immersed in 2 mL of 25 mg/mL 5-fluorouracil solution for 5 or 10 min, respectively, transferred into 1 mL mobile phase, and vortexed for 2 min. Supernatant was collected by centrifugation and subjected to HPLC analysis. 5-fluorouracil was identified by comparing its retention time to that of the standard, and its amount per bio-amniotic membrane was calculated by comparing peak areas of supernatant and standard.

Animal experiments

Male and female rabbits were randomly assigned into three groups with 20 in each group. Rabbits in group A, B, and C were implemented trabeculectomy and intraoperatively transplanted rehydrated bio-amniotic membranes that had been immersed in 5-fluorouracil solution for 5 or 10 min, or in saline for 5 min, respectively. Two hours prior to the operation, the operative eye of each rabbit was examined by electroretinogram (ERG). In detail, the body hair in the central part was removed using depilatory and the pupil was fully zoomed by alternately dropping 1% atropine and 10% phenylephrine eye drops for three times. Rabbits were adapted to dark environment for 40 min and injected 3% pentobarbital sodium solution (1 mL/kg) from the ear vein. Under general anesthesia, the experimental eyelid was fully opened and placed right in the front of full-field stimulating ball. The control eye was carefully covered to avoid all light stimulation. The operation eye was further anesthetized using 0.5% tetracaine eye drops. Then the corneal contact electrode, reference electrode, and grounding electrode were placed on the corneal surface, surface skin of center forehead, and surface skin of middle ear edge, respectively. The parameters of electrophysiological diagnostic equipment were set as 13.7 cd/m² for flash intensity of stimulating light, 2 s for flash interval, 0.1-75 Hz for passband to detect b-wave, and five times for magnifying recording waveform. The results were automatically processed, analyzed, and printed by a computer and the amplitudes (aA, bA) of b-waves were recorded. After examination, conjunctiva was treated with chloramphenicol eye drops.

The operation was performed under general anesthesia by intramuscularly injecting 50 mg/kg ketamine hydrochloride and 10 mg/kg chlorpromazine. After washing with sterile saline, the operative eye was routinely disinfected with iodine disinfectant and covered with sterile towels. Conjunctival flap based on the fornix was created at the superior temporal quadrant 5-7 mm behind the corneoscleral edge of eyeball. Conjunctiva and Tenon's capsule were bluntly separated to 1 mm in cornea transparent zone and a sclera flap with size of about 3 × 4 mm and thickness of 1/2 of the sclera was created. Deep scleral tissues equivalent to 1 × 2 mm trabecular along with iris root were excised and a preimmersed bio-amniotic membrane was placed underneath the sclera flap with epithelium upward. The scleral flap was then sutured one stitch each at the two corners and the bulbar conjunctiva was sutured one to two stitches with 10-0 nylon needles to make sure that both Tenon's capsule and bulbar conjunctiva reached watertight state. After the operation, 10,000 units of gentamicin and 1 mg of dexamethasone were immediately injected underneath the bulbar conjunctiva and dexamethasone/neomycin and 2.5 mg/mL chloramphenicol eye drops were postoperatively applied three times a day.

Observation of bleb

The dispersion degree, bubble wall thickness and color, degree of vascular filling, neovascularization, and bleb height were observed. Filtering blebs at late stage (2 weeks after operation) were divided into four types based on their appearance and function as proposed by Krofeld.^[6] Type I blebs are thin-walled, polycystic, and avascular. Type II blebs are flat, diffusive, pale, and thicker. Type III blebs are scar-like ones with blood-filling, slightly raised conjunctival hyperemia and adhesive sub-conjunctival tissues, and vessel-rich scleral surface. Type IV blebs are capped with partial dome-shaped bulge, and have cystic hyperplasia and a dense ball fascia cavity. The former two types are functioning and the latter two types are nonfunctioning [Table 1].

Intraocular pressure observation

Baseline IOP was defined as IOP before being prepared as hypertension model. Preoperative IOP was defined as IOP before operation. Postoperative IOP was defined as IOP observed with Schiøtz at 14, 21, and 28 days postoperation [Table 2].

Microscopical observation

At 7, 14, 21, and 28 days postoperation, rabbits in each group were randomly selected. After subjecting them to ERG, they were sacrificed by venous air embolism. Their eyeballs were immediately enucleated, and their retinas within 4 mm range of optic disc were observed by transmission electron microscopy (TEM).

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using multi-factor analysis of variance for completely randomized design in SPSS 12.0 software. Pairwise comparisons among groups were analyzed using least significant difference (LSD) *t*-test. *P* ≤ 0.05 was considered statistically significant.

Results

We first measured the amount of 5-fluorouracil per bio-amniotic membrane after being immersed in 25 mg/mL 5-fluorouracil solution by HPLC. Fig. 1 shows the representative peaks of the standard and samples immersed for 5 and 10 min, respectively. Table 3 lists the variation of HPLC analysis of 5-fluorouracil and Table 4 lists the calculated 5-fluorouracil amount absorbed by bio-amniotic membranes. As shown, the absorbed 5-fluorouracil amount per bio-amniotic membrane was 59-76 µg.

Table 4 shows the a-wave and b-wave latencies and amplitudes of EGR of rabbits in different groups. As shown, there were no significant differences in a-wave and b-wave latencies and amplitudes among the three groups (*P* = 0.165 and *P* = 0.052, respectively). Figs. 2 and 3 shows the TEM images of retinal ganglion cells of rabbits in different groups. As shown, the nuclei of retinal ganglion cells of rabbits in all three groups were normal.

Discussions

In this study, we confirmed with HPLC that each bio-amniotic membrane (4 × 5 mm) could absorb 59.004 and 75.828 µg of 5-fluorouracil after remaining immersed in 25 mg/mL

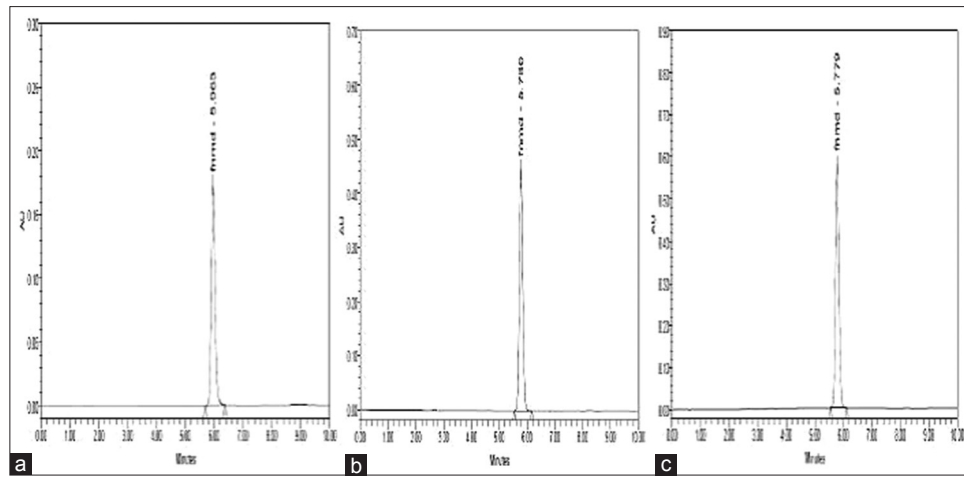


Figure 1: Chromatography of 5-fluorouracil standard and samples. (a) 20 µg/mL 5-fluorouracil standard. (b) Sample immersed for 5 min. (c) Sample immersed for 10 min

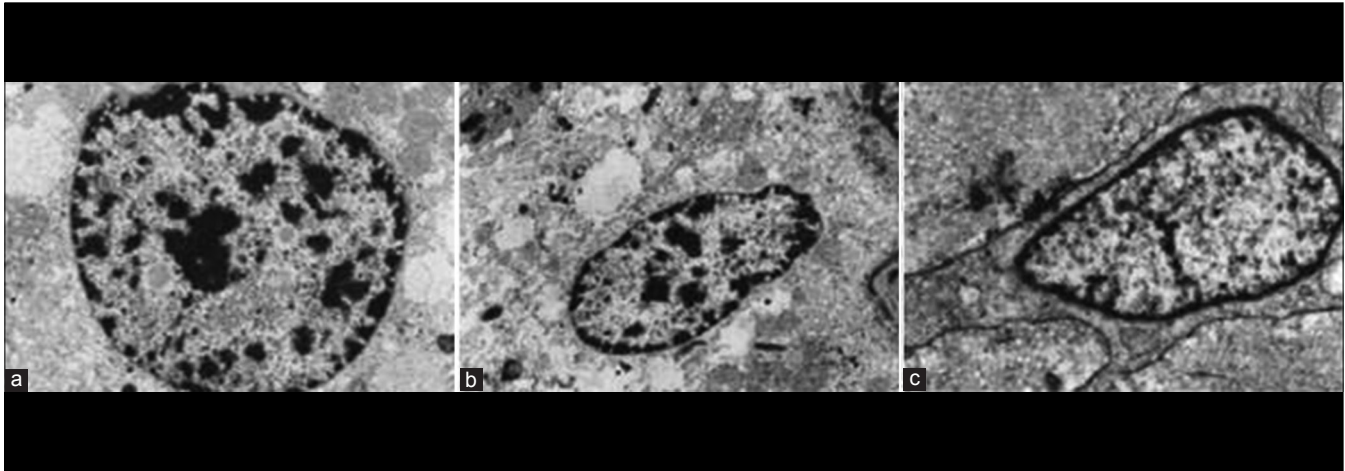


Figure 2: TEM images of retinal ganglion cells in different groups (x1000). (a) Without immersed in 5-fluorouracil solution. (b) Immersed for 5 min. (c) Immersed for 10 min

5-fluorouracil solution for 5 and 10 min, respectively. We further implanted the 5-FU containing amniotic membrane under the scleral flap of filtration channel of rabbit eyes in trabeculectomy to observe postoperative IOP changes and bleb morphology, and to preliminarily assess the effect of the surgical approach on rabbit eyes with focuses on retinal function and morphology using ERG and electron microscopy. The experiment indicated that application of this surgical approach in rabbit eyes could promote the formation of a well-functioning bleb and maintain a good and relatively low IOP within a certain time, while having no significant effect on retinal structure and function.

Inhibiting fibroblast proliferation, angiogenesis and collagen synthesis within 14 days of surgery is the key to control postoperative scar formation at early stage. 5-fluorouracil is a chemotherapeutic drug. Its *in vivo* metabolite 2'-deoxy-5-fluoro-uridin can block conversion of deoxyuridine to deoxythymidine by inhibiting thymidylate synthase and consequently inhibit DNA synthesis, eventually causing cell death,^[7] especially those in S phase. In addition, 5-fluorouracil has obvious anticontraction effect

on membrane-like substance. Moreover, 5-fluorouracil has been shown to inhibit fibroblast activity and increase the success rate of filtration surgery.^[8] In glaucoma treatment, it is primarily applied by frequent subconjunctival injection.^[9] However, the drug hardly directly impacts the filtration pathway, and high local drug concentration beneath conjunctiva is toxic to the corneal and conjunctiva.^[10,11]

Bio-amniotic membranes prepared from fresh amniotic membranes using lyophilizing techniques and Co⁶⁰ sterilization are transparent. Studies on their biological ultrastructures found that they are rich in collagen fibers and reticular fibers arranged in woven status with mesh gap about 0.5-15 µm and could absorb a large amount of drugs with size less than their porous size.^[12] Because collagen degradation is slow, the absorbed drugs can be gradually released over an extended period to maintain local drug concentration at certain level for a long period. Thus, bio-amniotic membrane is a good, semi-quantitative, membrane-controlled drug delivery system.

Borhani reported that the highest toxic 5-fluorouracil

Table 1: Bleb formation of rabbits in different groups before sacrifice

Groups	A	B	C
Eye number	20	20	20
Type I	10	10	5
Type II	5	6	9
Type III	2	2	4
Type IV	3	2	1
Functional blebs	15	16	14
Non-functional blebs	5	4	5

Note: $P>0.05$ for functional blebs among different groups; $P<0.05$ for Type I blebs in groups A and B compared to that in Group C

Table 2: Intraocular pressure of rabbits before and after operation at different times

Group	Basal pressure (mmHg)	Post-operation (day)			
		7	14	21	28
A	21.64±6.37	9.36±4.32	13.54±5.17	16.21±6.21	18.01±6.92
B	21.83±7.28	7.22±4.26	11.45±5.02	14.43±5.45	16.51±6.24
C	21.66±6.88	13.53±5.39	15.34±5.85	17.23±6.78	18.86±6.43

Intraocular pressure of rabbits in group B at 7, 14, 21, and 28 days of post-operation is significantly lower than that of rabbits in groups A and C ($P<0.05$)

Table 3: Amount of 5-fluorouracil absorbed by bio-amniotic membranes

	5 min (µg/membrane)		10 min (µg/membrane)	
	1	2	3	4
1	59.085		75.811	
2	58.969		75.845	
3	58.958		75.861	
4	58.839		75.893	
5	59.012		75.827	
6	58.898		76.032	
7	58.893		75.874	
8	58.909		74.169	
9	59.159		75.867	
10	58.954		75.796	
χ	59.004		75.839	

Table 4: EGR b-wave latency and amplitude of rabbits in different groups (µV, n=20)

	Group A		Group B		Group C	
	aA	bA	aA	bA	aA	bA
Average	79.01	180.38	78.32	179.24	78.48	181.35
Standard deviation	13.55	18.57	13.43	18.33	16.83	19.12
Minimum	47.82	151.39	45.96	154.51	53.65	148.42
Maximum	118.20	219.61	119.26	220.28	120.09	212.39
Range	56.29	63.33	51.24	63.26	64.37	63.99

Note: aA in group B versus aA in group C: $P=0.165$; bA in group B vs bA in group C: $P=0.052$

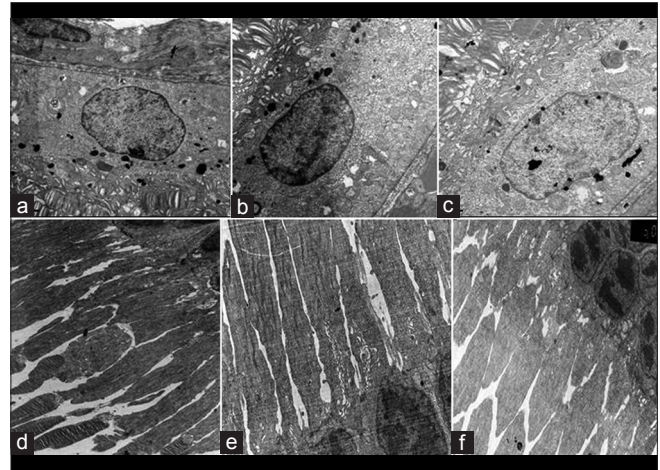


Figure 3: TEM images of retina pigment epithelial cells. (a) Showing clear microvilli structure and intact and smooth glass membrane (x5000). (b) Showing clear microvilli structure and intact and smooth glass membrane (x7500). (c) Showing clear microvilli structure and intact and smooth glass membrane (x7500). (d) Showing neatly arranged retinal outer segment membrane disc (x7500). (e) Showing neatly arranged retinal outer segment membrane disc, but abnormal structures (x7500). (f) Showing neatly arranged, intact retinal outer segment membrane disc (x5000)

concentration is 0.25 mg/mL for retina and Mannis reported that the highest toxic 5-fluorouracil concentration is 1-10 mg/mL for corneal endothelium.^[13,14] In this study, each bio-amniotic membrane can absorb maximum 75.828 µg of 5-fluorouracil, far below its toxic concentration to eye. We also found that supplementing 5-fluorouracil to bio-amniotic membrane had no significant effects on retinal structure and function. Because our sample size is small, its efficacy and safety need further in-depth, repeated investigation using a large sample size. In addition, we used 5-FU instead of mitomycin C to preliminarily explore the safety of the method because the latter has 100-fold stronger antifibroblast proliferation function.^[15] In short, the experiment provided a basis for further exploration.

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Cite this article as: Zhang C, Wu X, Liu W, Wang J, Guo B, Sun Y, Zhang Y, Zhang H, Wang Y, Zhao J. Impact of application of bio-amniotic membrane immersed in 5-fluorouracil solution in trabeculectomy on rabbit retina. *Indian J Ophthalmol* 2013;61:734-8.

Source of Support: The study was sponsored by the Jinan Health Technology Fund Project (No: 2008013). **Conflict of Interest:** None declared.