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

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Abstract: 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. **Methods:** Healthy white New Zealand rabbits were randomly assigned into three groups with 20 in each group. They accepted trabeculectomy and intraoperational implementation of 4×5 mm bio-amniotic membranes immersed in either physiological saline/water for 10 min, or 25 mg/mL 5-fluorouracil solution for 5 and 10 min, respectively. At 7, 14, 21 and 28 d of postoperation, 5 rabbits from each group were examined with electroretinogram (ERG) and then sacrificed by air embolism. 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 and 75.828 μg 5-fluorouracil after immersed in 5-fluorouracil solution for 5 and 10 min, respectively. Application of these bio-amniotic membranes in trabeculectomy had no effect on ERG and TEM structures of retina. **Conclusion:** The application of improved bio-amniotic membrane by adding anti-metabolism drugs to inhibit fibrosis is an important direction for prevention and treatment of glaucoma.
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Keywords: Biological amniotic membrane; 5-fluorouracil; trabeculectomy; retina

1. Introduction

The aim of penetrating filtrating surgery for glaucoma is to reduce intraocular pressure 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 most often results from fibroblast proliferation-induced scarring of filtration passage (Addicks et al.,1983). 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 (Bruno et al.,2006), thus keeping the filtration pathway smooth and effectively maintaining long-term bleb functioning.

5-fluorouracil can inhibit fibroblast proliferation and help to form and maintain functional filtering bleb (Alvarado et al.,2008). In previous clinical course, anti-proliferative drugs were not applied to the filtration passage after trabeculectomy to avoid drug-induced ophthalmic tissue damage. Application of anti-proliferation drugs immersed bio-amniotic membrane to and bio-amniotic membrane to filtration passage and its effects on eyes, especially on retina have not been reported. In this study, we immersed bio-amniotic membranes in 5-fluorouracil solution, applied the membranes during trabeculectomy and explore their impact on retinal structure and function.

2. Materials and Methods

2.1 Materials

5-fluorouracil standard and 5-fluorouracil injection solution (25 mg/mL) were from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. HPLC grade methanol and ultra-pure water were from J.T. Baker Company (U.S.A). Lyophilized and Co^{60} sterilized bio-amniotic membranes with size of 4 × 5 mm and free of hepatitis B, hepatitis C, syphilis, HIV and other

pathogens were from Jiangxi Rui-Ji Medical Devices Co. Ltd. They were prepared as follows: clean amniotic membranes were soaked in 30% glycerol. After pre-frozen for 10 h at 0~56°C, they were lyophilized at 10~56°C till their water content reached 1~2%. The membranes were then repacked and sterilized by Co^{60}. Healthy adult New Zealand white rabbits (2-3 kg, 30 males and 30 females) were from Jinan Experimental Animal Center and kept under normal conditions. The study has been approved by the Ethics Committee of Jinan Second People's Hospital.

2.2. High pressure liquid chromatography (HPLC)

5-fluorouracil was analyzed on Diamonsil C_{18} 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 standard was first dissolved in methanol and then prepared as 100 μg/mL methanol-water (2:98) solution. 20 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.

2.3 Animal experiments

Male and female rabbits were randomly assigned into three groups with 20 in each group. Rabbits in group A and B were implemented trabeculectomy and intraoperatively transplanted bio-amniotic membranes immersed in 5-fluorouracil solution for 5 or 10 min, or in saline for 5 min followed by rehydration, respectively. Two hours prior to the operation, the operative eye of each rabbit was examined by 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 (1ml/kg) from 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 as setting as13.7 cd • m^{-2} • s 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 intramuscular injecting Ketamine Hydrochoride for Injection 50mg/Kg and Chlorpromazine 10mg/Kg. After washed with sterile saline, the operative eye was routinely disinfected with Iodine preparation disinfectant and covered with sterile towels. Conjunctival flap based on the fornix was created at 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 while a sclera flap with size of about 3 × 4 mm and thickness of l/2 of the sclera was created. Deep scleral tissues equivalent to 1 x 2 mm trabecular along with iris root were excised and pre-immersed 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 1-2 stitches with 10-0 nylon needles to make sure that both Tenon's capsule and the bulbar conjunctiva reaching watertight state. After the operation, 10,000 units of gentamicin and 1mg of dexamethasone were injected immediately underneath the bulbar conjunctiva and dexamethasone/neomycin and 2.5 mg/mL chloramphenicol eye drops were postoperatively applied 3 times a day.

At postoperative day 7, 14, 21, and 28, rabbits in each group were randomly selected, subjected to electroretinogram (ERG), and sacrificed by venous air embolism. Their eyeballs were immediately enucleated and retinas within 4 mm range of optic disc were observed by transmission electron microscopy (TEM).

2.4 Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using multi-factor analysis of variance for completely randomized design in SPSS12.0 software. Pairwise comparisons among groups were analyzed using LSD-t test. $P<0.05$ was considered statistically significant.

3. Results

We first measured the amount of 5-fluorouracil per bio-amniotic membrane after immersed in 25 mg/mL 5-fluorouracil solution by HPLC. Figure 1 shows the representative peaks of the standard and

samples immersed for 5 and 10 min, respectively. Table 1 lists the variation of HPLC analysis of 5-fluorouracil and Table 2 lists the calculated 5-fluorouracil amount absorbed by bio-amniotic membranes. As shown, the absorbed 5-fluorouracil amount per bio-amniotic membrane was between 59-76 μg.

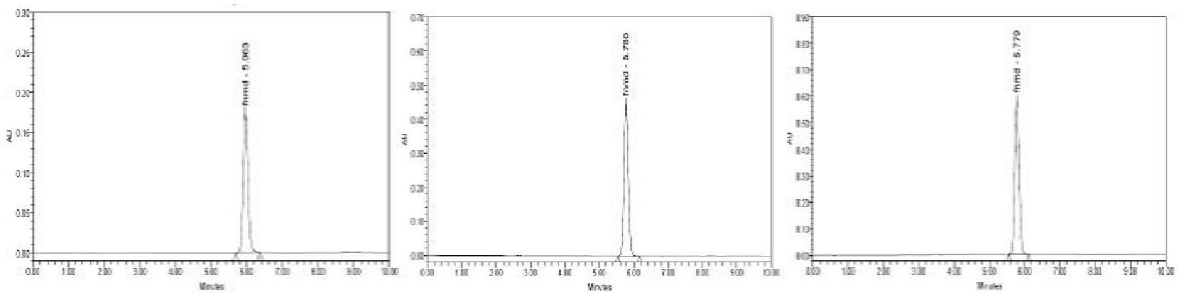

Figure 1. Chromatography of 5-fluorouracil standard and samples. A: 20 μg/mL 5-fluorouracil standard; B: Sample immersed for 5 min; C: Sample immersed for 10 min.

Table 1. Amount of 5-fluorouracil absorbed by bio-amniotic membranes

	5 min (μg/membrane)	10 min (μg/membrane)
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
$\bar{x}$	59.004	75.839

Table 2. 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 vs aA in group C: P＝0.165; bA in group B vs bA in group C: P＝0.052

Table 2 shows the EGR a-wave and b-wave latencies and amplitudes 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).

Figure 2 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.

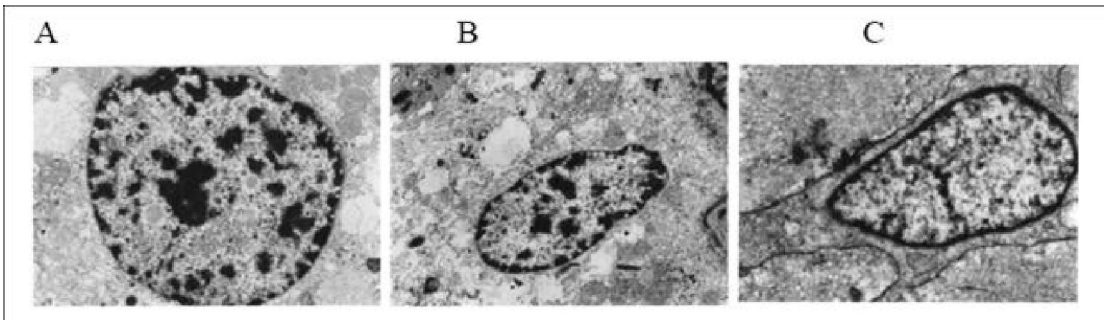

Figure 2 TEM images of retinal ganglion cells in different groups (x1000)
A: without immersed in 5-fluorouracil solution; B: Immersed for 5 min; C: Immersed for 10 min.

4. Discussions

In this study, we first confirmed with HPLC that each bio-amniotic membrane (4×5 mm) could absorb 59.004 and 75.828 μg of 5-fluorouracil after immersing in 25 mg/mL 5-fluorouracil solution for 5 and 10 min, respectively. We further transplanted the membrane beneath scleral flap of the filtration pathway and observed its effect on retinal structure and function. We found that application of 5-fluorouracil absorbed by bio-amniotic membrane did not significantly affect retinal structure and function. To our knowledge, this is the first report on application of drug-improved biological amniotic membrane in trabeculectomy.

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 into deoxythymidine by inhibiting thymidylate synthase and consequently inhibit DNA biosynthesis, eventually causing cell death (Li et al.,2010). As a commonly used clinical anti-metabolic drug, 5-fluorouracil has very low toxicity to normal eye tissues, but the strongest killing effect on proliferating cells, especially those in S phase, and obvious anti-contraction effect on membrane-like substance. Moreover, 5-fluorouracil has been shown to inhibit fibroblast activity and significantly increase the success rate of filtration surgery (Heuer et al.,1986). In glaucoma treatment, it is primarily applied by frequent subconjunctival injection (Lattanzio et al.,2005). However, the drug is hardly directly impact the filtration pathway and high local drug concentration beneath conjunctiva is toxic to corneal and conjunctiva (Knapp et al.,1987; Ticho et al.,1993).

Bio-amniotic membranes prepared from fresh amniotic membranes using lyophilizing techniques and Co^{60} sterilization are transparent. They do not have nerves, blood vessels and lymphatic tissues, but maintain the original amniotic membranes' structure, basic chemical compositions, clinically required toughness and physiological pH. Studies on their biological ultrastructures found that they were 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 the porous size of amniotic membrane (Kubo et al.,2001). Because collagen degradation is slow, 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. Thus it is reasonable to believe that bio-amniotic membrane functions as a "natural slow-release device".

Borhani reported that the highest toxic 5-fluorouracil 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 (Borhani et al.,1995; Mannis et al.,1988). In this study, each bio-amniotic membrane can absorb maximum 75.828 μg of 5-fluorouracil, far below its toxic concentration to eye. Previous studies also found that application of bio-amniotic membrane covalently cross-linked with 12 μg 5-fluorouracil to the filtration pathway beneath scleral flap was safe and could significantly improve success rate of filtration surgery (Wu et al.,2009). We also found that supplementing 5-fluorouracil to bio-amniotic membrane had no significant effects on retinal structure and function. Therefore, we believe that improved surgical techniques had no significant toxicity to retina, thus providing a foundation to further study its role in maintaining the patency of filtration pathway and inhibiting scar formation.

In conclusion, application of bio-amniotic membrane immersed in 25 mg/mL 5-fluorouracil solution for 5 to 10 min to trabeculectomy has no significant side effect on retina. However, its complications and long-term clinical effects (>12 months) need to be further study. In addition, larger scale study is also needed to further confirm out results. Moreover, whether its application could improve the

success rate of trabeculectomy also needs to be investigated. In short, the application of improved bio-amniotic membrane by adding anti-metabolism drugs to inhibit fibrosis is an important direction for prevention and treatment of glaucoma.

Conflict of Interest:

None of the authors has conflict of interest with the submission.

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Running title:

Amniotic and 5-fluorouracil effect on retina

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