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Gelatin methacryloyl hydrogel eye pad loaded with amniotic extract prevents symblepharon in rabbit eyes

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Abstract. – OBJECTIVE: The aim was to evaluate the ability of gelatin methacryloyl (GelMA) hydrogel eye pads loaded with amniotic extract to prevent symblepharon in rabbits.

MATERIALS AND METHODS: Forty-eight rabbits were divided into 3 groups. After ocular alkali burn, Group A (n=16) was treated with amniotic extract-loaded hydrogel eye pads placed in the conjunctival sac, Group B (n=16) was treated with amniotic membrane transplantation, and Group C (n=16) received no treatment. At 1, 2, 3, and 4 weeks post-injury, 4 rabbits from each group were selected to evaluate for symblepharon, determine epithelial healing rate and corneal neovascularization, conduct histopathology, and to quantify the expression of TGF-β1.

RESULTS: At 1 week post-injury, the epithelial healing rate in Groups A and B was higher than Group C (p=0.002, 0.001, respectively). At 2 weeks, corneal neovascularization in Group B was less than Group C (p=0.004). At 3 and 4 weeks, no symblepharon has been found in Group A, but it was found in some eyes in Group B and C (p=0.009, 0.013). Further, the expression of TGF-β1 in Group A was lower than in Group B and C (p<0.001). H&E staining showed that the controls in Group C had more edema and inflammatory cell infiltration in the first 2 weeks, relative to Groups A and B. At 4 weeks, Masson's Trichrome staining showed that fibers were most regularly aligned in Group A and that immuno-histochemical staining found that proliferating cell nuclear antigen was highest expressed in Group C.

CONCLUSIONS: Treatment with GelMA hydrogel eye pads loaded with amniotic extract shortly after chemical injury prevented symblepharon in rabbits.

Key Words:
Amniotic extract, Gelatin methacryloyl (GelMA), Amniotic membrane, Alkali burn, Symblepharon.

Introduction

Ocular chemical injuries are common and can be associated with a diverse set of complications. Among these, severe symblepharon not only affects a patient's appearance, but also restricts eye movement and can decrease the efficacy of vision enhancement surgeries. Symblepharon depends on the degree of initial injury, but early and effective management is vital in reducing the extent of symptoms. Preventive measures include medical treatment, such as glucocorticoids, but prolonged treatment with glucocorticoids may lead to corneal perforation, inhibition of corneal epithelial growth[1,2], secondary glaucoma, or cataract[3]. Mechanical separation treatments exist, such as the use of a symblepharon ring[4], gelatin sponge[5], or glued-on contact lens[6], but have not been the subject of many clinical studies. Surgical treatments such as amniotic membrane transplantation (AMT) [7] are the most common choice following symblepharon. AMT is completed using both suture-based and non-suture-based methods. Suturing has many drawbacks; it is time consuming, requires repeated operations, and can cause additional pain or even endophthalmitis[8]. Non-suture methods can include treatment with amniotic membrane fixed on symblepharon rings[9], ocular

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biomembrane-fixed equipment[10], or a temporary amniotic membrane patch[11]. Yet, after treatment with either suture or non-suture methods, there is always a risk of viral infection. However, amniotic extract (AE) prepared at high temperatures can prevent virus transmission and promote repair, reduce inflammation, and decrease scarring[12]. The effect of using amniotic extract has been shown to be non-inferior to AMT[12-15].

Gelatin methacryloyl (GelMA) is gelatin that has been modified with methacrylic anhydride (MA)[16,17]. Shima et al[18] have suggested that biomaterials consisting of gelatin as the main component are better than amniotic membranes for alleviating scars, reducing inflammation, and promoting epithelialization. GelMA can be stabilized by photocrosslinking and promotes cell adhesion, migration, proliferation, and accelerates wound healing[19]. Particularly, GelMA can be rationally designed to resemble the native extracellular matrix (ECM) and to provide three-dimensional (3D) scaffolds that promote cellular growth and tissue formation[20].

Based on the prior findings that AE and GelMA hydrogel can each independently promote healing and prevent fibrosis and that GelMA hydrogel can form a stable scaffold, we sought to combine the two technologies in this work. We used GelMA hydrogel as carrier which we loaded with AE, and then, formed eye pads to be used as a treatment in early stage of ocular chemical injury. We hypothesized that these eye pads would prevent symblepharon by accelerating healing and separating the conjunctiva and cornea. In addition, the GelMA hydrogel was designed to slowly release the AE over the course of its degradation.

Materials and Methods

Preparation of Amniotic Membrane and Amniotic Extraction

We collected human placenta after selective cesarean delivery with a negative diagnosis of human immunodeficiency virus (HIV), human hepatitis B and C virus (HBV and HCV), and syphilis. Each placenta was rinsed with normal saline solution containing 50 UI/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml neomycin, and 2.5 μg/ml amphotericin B. The amnion and chorion were separated. Part of the amniotic membrane (AM) was laid on surgical adhesive paper (with the epithelial surface facing up) and cut into 3×2 cm sections, then, soaked in glycerin: DMEM (1:1) and stored at -80°C. The remaining AM was cut into pieces and submerged in liquid nitrogen for 48 hours. Then, it was ground into a powder and dissolved in normal saline. Next, the solution was boiled for 30 minutes. After that, the homogenate was centrifuged at 10,000 rpm for 30 minutes. The supernatant was collected and adjusted to pH 7.2. Finally, the sample was sterile-filtered through a 0.22 μm filter (Millipore, Burlington, MA, USA) and stored in a sterile container at 4°C.

Preparation of Eye Pads

Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP; StemEasy Biology Co., Ltd., Jiangyin, China) was added to AE until the concentration of LAP was 0.05% (w/v). Then, GelMA powder with 75% MA substitution (Youmo Biotechnology Co., Ltd., Wenzhou, China) was added to the 0.05% LAP solution to produce a mixture with 20% (w/v) GelMA. This was fully dissolved at 60°C and sterile-filtered through a 0.22 μm filter, resulting in a 20% concentration of GelMA hydrogel precursor that contained AE. This solution was added to an eye pad mold and photo-crosslinked at 405 nm (Q-LINE, China) to obtain the final pads (Figure 1). Pads were stored in a sterile container at -20°C.

Animal Experiments

All protocols were approved by the Animal Laboratory Center of Hangzhou Normal University and were in line with the ARVO Statement. Forty-eight New Zealand white rabbits (male, 2 to 2.5 kg) were purchased and housed at the Animal Laboratory Center of Hangzhou Normal University. Using a portable direct ophthalmoscope (yz11d ophthalmoscope, Suzhou 66 Vision Technology Co., Ltd., Suzhou, China), animals were

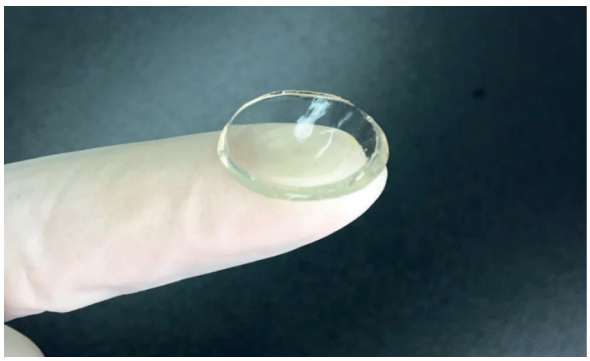

Figure 1. The eye pad made of GelMA hydrogel with amniotic extract was soft, smooth, and transparent, with a vertical diameter of 18 mm, horizontal diameter of 15 mm, and a thickness of 1 mm.

confirmed to have no disease in either eye. The left eye was used for operation (n=48). Rabbits were anesthetized by intramuscular injection of ketamine (15 mg/kg) and xylazine (5 mg/kg), and topical anesthesia was applied using 0.5% proparacaine hydrochloride eye drops. Semicircle filter paper with a diameter of 16 mm was immersed in 2 mol/L NaOH solution for 1 min, and then, placed in the superior fornix to cover one-third of the cornea for 60 s. Then, filter paper was removed and the conjunctival sac was washed with normal saline for 5 min. Next, 0.1% sodium fluorescein was added drop-wise into the conjunctival sac and photographed under cobalt blue light. Approximately, 0.1-0.2 ml aqueous humor was extracted using a 1 ml syringe from transparent limbus cornea at a position of 3 o'clock and then stored in a sterile EP tube at -80°C. After NaOH chemical injury, the rabbits were assigned to 3 groups: Group A: Received one piece of eye pad in the conjunctival sac, followed by temporary blepharoplasty (n=16); Group B: Received amniotic membrane transplantation, where the amniotic membrane covered the whole cornea and bulbar conjunctiva with epithelial surface facing down, and was fixed by 10-0 nylon suturing at the upper and lower fornix and the limbus of cornea (8-10 stitches in total). Group C: Received no treatment except 0.3% ofloxacin ointment applied to the conjunctival sac. All animals received 0.3% ofloxacin ointment twice a day for 5 days after the alkali burn. Rabbits in Group A were given new eye pads every 7 days. At 1, 2, 3, and 4 weeks, 4 rabbits in each group were selected randomly, anesthetized as described above, clinically observed, and 0.1-0.2 ml aqueous humor was collected. After that, the rabbits were euthanized by intravenous injection of pentobarbital (100 mg/kg). The upper eyelid, together with the conjunctiva and cornea, were fixed with 10% neutral buffered formaldehyde for 24 hours and dehydrated with an increasing alcohol gradient. We obtained a 5 mm-wide section of the middle of the upper eyelid together with the conjunctiva and cornea, which was then fixed in paraffin and sectioned in 5 μm slices.

Clinical Observation

After alkali burn, at 1, 2, 3, and 4 weeks, we graded the symblepharon (Grade I: no symblepharon and fornix constriction; Grade II: symblepharon area is less than 1/3 of fornix; Grade III: symblepharon area is 1/3-2/3 of fornix; Grade IV: symblepharon area is 2/3-1 of fornix). The corneal neovascularization were analyzed with Image Pro Plus 6.0 using the formula: $A=C/12\times3.1416\times[R^2-(R-L)^2]$; where A is the area of corneal neovascularization (mm^2); C: the clocks of neovascularization involving the cornea; R is rabbit corneal radius 7 mm; L is the length of neovascularization from limbus to cornea (mm). The corneal epithelial healing rate was analyzed with Image Pro Plus 6.0 and using the formula: $R=(S_0-S_d)/S_0\times100\%$; where R is the rate of corneal epithelial healing; S_0 is the staining area on the day of the alkali burn (mm^2); S_d is the staining area on the day of observation (mm^2).

TGF-β1 in Aqueous Humor

The expression of TGF-β_1 in the aqueous humor immediately after alkali burn and after 1, 2, 3, or 4 weeks was detected by ELISA (Qincheng Biological Company, China) according to the kit's instructions.

Histological Examination

The morphology of conjunctiva and cornea, inflammatory cell infiltration, and fibroblast presence were observed by H&E staining. The arrangement of collagen fibers was visualized by Masson's Trichrome staining. The proliferation of fibroblasts was evaluated by Immuno-histochemical staining for proliferating cell nuclear antigen (PCNA).

Statistical Analysis

All statistical analyses were performed with SPSS 25.0 statistical software. Data are shown as mean ± SD. The rank data of multiple groups were compared using the Kruskal Wallis test of multiple groups of ordered multi classification data, and a one-way ANOVA test was used for those who satisfied the normal test and the homogeneity of variance. LSD-t test was used for comparisons. $p < 0.05$ was considered to be statistically significant.

Results

Clinical Observation

Information of the eye pads in Group A and the amniotic membrane in Group B are listed in Table I. At 3 and 4 weeks, no symblepharon was observed in Group A, however, it was found in some eyes in Group B and C. The differences were statistically significant (Figure 2).

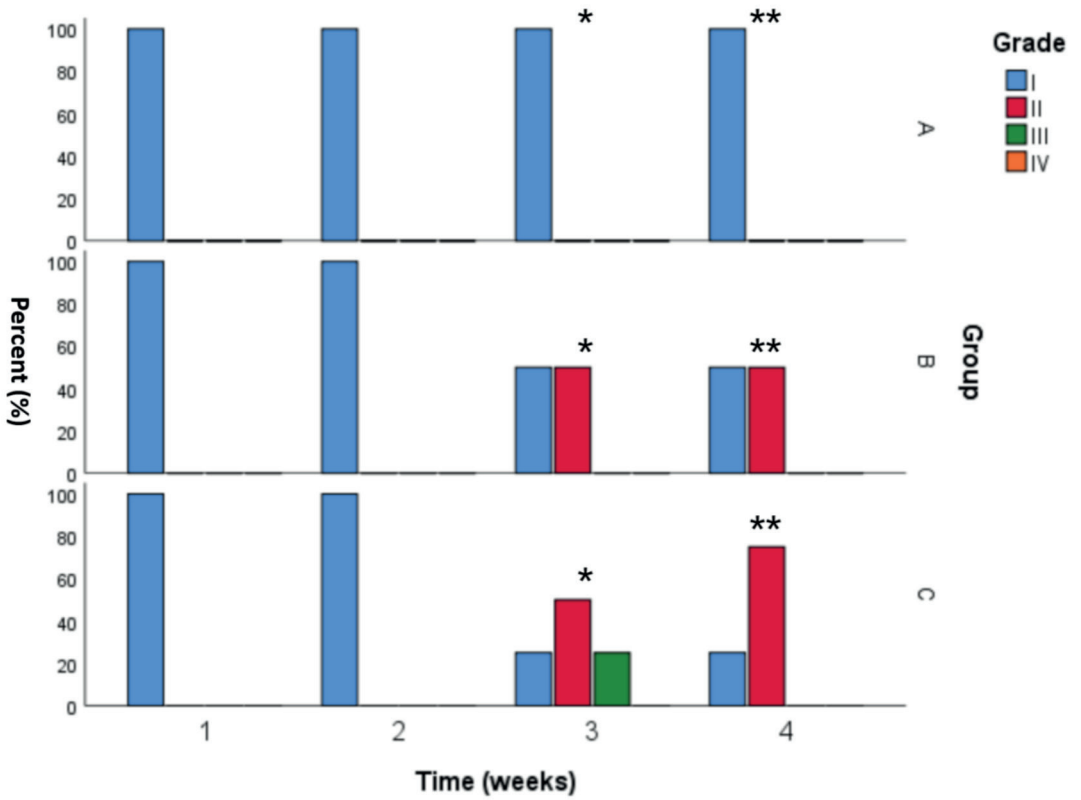

Figure 2. At 1 and 2 weeks, 4 eyes (100%) were in Grade I in Group A, B, and C. At 3 and 4 weeks, 4 eyes (100%) were in Grade I in Group A, 2 eyes (50%) in Grade II and 2 eyes (50%) in Grade I were observed in Group B. In Group C, at week 3, 1 eye (25%), 2 eyes (50%), and 1 eye (25%) were in Grade I, II, and III, respectively; at week 4, 1 eye (25%) and 3 eyes (75%) were in Grade I and Grade II, respectively. The differences between the three groups were significant at 3 weeks (H=9.533; p=0.009) (*) and 4 weeks (H=8.733; p=0.013)(**).

Corneal Epithelial Healing Rate

The corneal epithelial healing rates in Group A (0.67±0.05) and B (0.74±0.15) were higher than Group C (0.19±0.09) at 1 week (p=0.002, p=0.001) with no significant difference between Group A and B (p=0.494). No significant differences were observed in the epithelial healing rate of any groups during week 2 (F=3.200, p=0.113), 3 (F=3.563, p=0.095), or 4 (F=1.377, p=0.322). In the three groups, the corneal epithelial healing rates at 1 week were lower than the rates at week 2, 3, and 4 (p=0.002-0.037), but no statistical differences between the week of 2, 3, and 4 (p=0.244-0.832) in Group A and B were found. However,

Table I. Information of eye pads in Group A and amniotic membrane in Group B.

Groups	Conditions	1 week	2 week	3 week	4 week
The eye pads in Group A	In conjunctival sac without degradation nor rupture	0	0	3	3
	In conjunctival sac with slight degradation or rupture	2	2	1	0
	In conjunctival sac with obvious degradation or rupture	2	1**	0	0
	Not in conjunctival sac	0	1	0	1
The amniotic membrane in Group B	Attached to ocular surface without melting or falling off	2	1	0	0
	Partial melting	1	2	2	0
	Obvious melting or falling off	1*	1	2	4

1*, at 1-week post-operation, one subject in Group B had infection and the amniotic membrane had dissolved and fell off. This animal had conjunctival congestion, swelling, and purulent secretion; 1**, at 2 weeks, the eye pad in one subject in Group A was mostly dissolved and ruptured. This animal had corneal perforation and hypopyon.

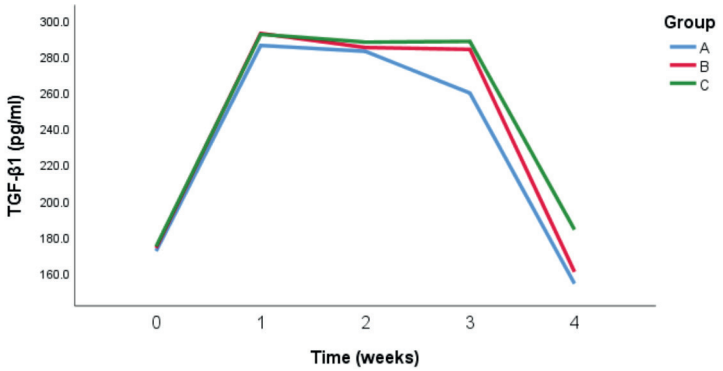

Figure 3. At 1 week, the expression of TGF-β1 increased rapidly to reach a peak, then decreased mildly in the following two weeks, after that, decreased dramatically at week 4. No significant difference of TGF-β1 across groups immediately following the alkali burn (0 week), and no difference at 1 and 2 weeks (p>0.05). But at 3 and 4 weeks, TGF-β1 in Group A was significantly lower than Group B and C (p>0.001).

in Group C, the corneal epithelial healing rates at week 2 (0.67±0.01) were also lower than week 3 (0.82±0.02) and 4 (0.94±0.07) (p=0.004, 0.017) with no significant difference between the rates at week 3 and 4 (p=0.227).

Corneal Neovascularization

The extent of corneal neovascularization was reduced in Group B compared with that Group C at week 2 (p=0.004). At this time, there was no significant difference between Group A and B (p = 0.054). No difference was observed between the 3 groups at week 1 (F=4.938, p=0.054), 3 (F=3.414, p=0.102), or 4 (F=0.638, p=0.561).

TGF-Ð1 in Aqueous Humor

Following alkali burn immediately, no difference of the expression of TGF-β_1 across Group A, B, and C (F=0.007, p=0.993), as expected, and no statistical difference at 1 week (F=0.990, p=0.409) and 2 weeks (F=0.642, p=0.549) was detected. At 3 and 4 weeks, TGF-β_1 in Group A (259.6 ± 2.4 pg/ml and 154.0 ± 1.7 pg/ml) was significantly lower than Group B (283.8 ± 0.7 pg/ml and 160.6 ± 1.2 pg/ml, $p < 0.001$) and Group C (288.2 ± 0.2 pg/ml and 183.9 ± 0.8 pg/ml, $p < 0.001$) (Figure 3).

Pathological Examination

Analysis of H&E staining revealed that the conjunctiva and corneal were swollen and disorganized and that there was a presence of infiltrating inflammatory cells, an increased number of fibroblasts, and increased neovascularization in all 3 groups at week 1 and 2 following the alkali burn, especially in Group C. They gradually alleviated over the final 2 weeks. After Masson's staining, there was evident edema, wide fiber spacing, and high infiltration of inflammatory cells in all subjects in the first two weeks, followed by hyperplasia and irregular arrangement of fibers over the last two weeks. However, the fibrous hyperplasia was lighter and more regularly arranged in Group A than in Group B and C. The subjects in Group C appeared to have the most serious fibroplasia and disorganization of fibers, especially in the 4th week. Immuno-histochemical staining for proliferating cell nuclear antigen (PCNA) revealed that, in the 3rd and 4th week, fibroblasts expressing PCNA were more evident in Group C than in Groups A and B (Figure 4).

Discussion

Symblepharon is a common complication of ocular chemical injury, however, there is currently no standard for effective treatment. It has been shown that early management can help prevent or reduce the degree of symblepharon. Promoting epithelial growth, controlling inflammation, and preventing tissue dissolution are the main principles and objectives for treating ocular burns[21]. The amniotic membrane is the innermost membrane of the placenta. Previous reports[7,22-26] have shown that treatment with AMT can promote corneal epithelial repair, reduce scars, inhibit inflammation, and decrease neovascularization. In this study, we produced hydrogel eye pads that contained amniotic extracts isolated from amniotic membrane homogenate. This homogenate was filtered to re-

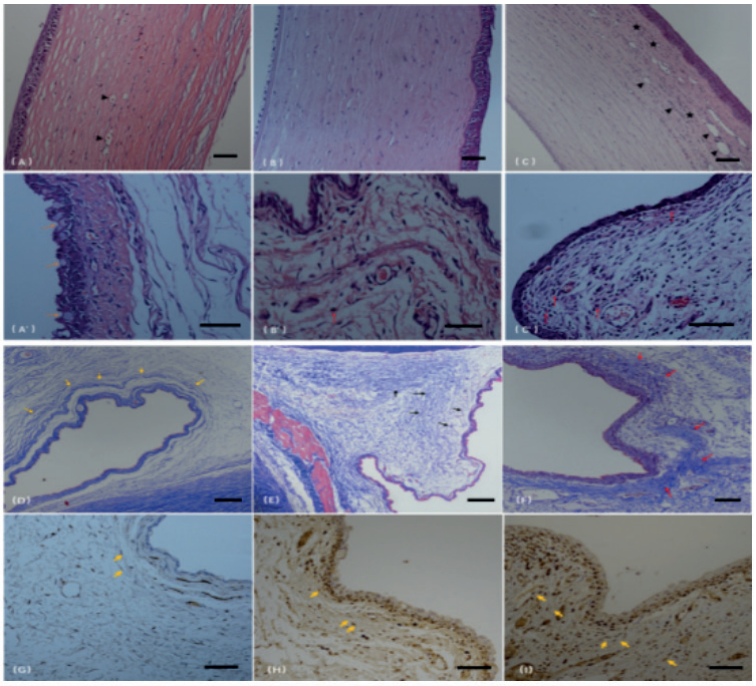

Figure 4. At 2 weeks following the surgery, H&E staining of the cornea in Group A (**A**) showed less neovascularization than Group C (**C**) (black triangle), and there were significantly more infiltrating inflammatory cells in Group C (**C**) (black star). Neovascularization and inflammatory infiltration were not evident in Group B (**B**); From H&E staining, the fornical conjunctiva in Group A (**A'**) showed more conjunctival goblet cells (yellow arrow), and there were fewer inflammatory cell infiltrates (red arrow) relative to Group B (**B'**) and C (**C'**). At week 4, the fibers were arranged regularly in Group A (**D**) without obvious fiber proliferation (yellow arrow). However, fibers were arranged less regularly in Group B (**E**) and with slight fibrous hyperplasia (black arrow). In Group C (**F**), fiber proliferation was evident, and the arrangement was disordered (red arrow). There were fewer fibroblasts expressing proliferating cell nuclear antigen (yellow arrow) in Group A (**G**) and Group B (**H**) relative to Group C (**I**). Bar represents 100 μm.

move impurities, and expected to be associated with a low degree immunogenicity. Additionally, it provides some growth factors that may promote corneal epithelial repair and inhibit corneal neovascularization[27-30]. The eye pads were made of a GelMA hydrogel. The main component of GelMA is gelatin, which comes from collagen. It is a mixture of polydisperse peptides produced by partial irreversible hydrolysis of collagen and has been shown to have lower immunogenicity than collagen. GelMA possesses a cell-binding arginine glycine aspartate motif (RGD) and a matrix metalloproteinase peptide motif that promote epithelial repair and wound healing[31]. The findings in our study are in good agreement with these previously demonstrated properties of GelMA. In this work, we have shown that placing this eye pad in the conjunctival sac or providing treatment with an amniotic membrane, transplant promoted increased corneal epithelial repair in the 1st week compared with untreated controls and that AMT effectively inhibited corneal neovascularization in the 2nd week compared with the controls. Further, there was no significant difference between treatment with the hydrogel eye pads and performing AMT in terms of corneal epithelial healing rate and corneal neovascularization. The extent of inflammation in animals that received eye pads or AMT was less than controls in the first 2 weeks. Further, these treated groups reduced fibroblast proliferation and prevented the occurrence of symblepharon. It has been reported that amniotic extracts can reduce inflammation and corneal scar formation in alkali burns of the cornea in rats[32] and inhibit the formation of haze after Epi-LASIK and PRK[33,34]. In our research, clinical and pathological examinations indicated that treatment with the eye pads provided superior prevention of symblepharon relative to AMT. This is potentially because the eye pads used in this study were designed to exactly fit the depth of the normal rabbit conjunctival sac. After the initial alkali burn, we inserted the eye pads into rabbits' conjunctival sac and replaced them once

per week. This treatment was easy to carry out. Most of eye pads were relatively stable for the first week. Of interest, they were actually more stable in the late stage (the last two weeks) after alkali burn than in the early stage (the first two weeks). We believe this may be related to the release of more collagenase in the early physiologic response to alkali burn, because collagenase can promote the degradation of GelMA. Based on the eye pads' stability and ease of use and replacement, they provided a more persistent effect than AMT. AMT requires suturing, which is difficult on sensitive, inflamed tissue. Additionally, the amniotic membrane itself is unstable and may fall off. Repeated treatment with AMT will cause trauma and pain.

Symblepharon, as a form of scar hyperplasia, occurs on the ocular surface tissue, and to date, the specific mechanism of its development has not been fully understood. Scar formation is related to extracellular matrix (ECM) remodeling and abnormal deposition, and TGF-β is an important factor that regulates ECM synthesis and degradation. TGF-β_1 has been confirmed to be a representative cytokine and an important fibrogenic growth factor, and is closely related to scar formation. TGF-β_1 leads to scar formation by downregulating ECM enzyme expression, which in turn reduces ECM degradation and promotes ECM deposition. This upsets the careful balance of ECM synthesis and degradation, leading to proliferation of scar tissue[35]. In this study, the expression of TGF-β_1 in the aqueous humor in the 3 groups increased significantly 1 week after the initial alkali burn. Whereas platelets and macrophages release TGF-β_1 and other factors to promote hemostasis and anti-inflammation in the early stages of wound healing, in the late stage, fibroblasts migrate in to deposit new extracellular matrix[36].We found that the expression of TGF-β_1 in eye pad-treated group was significantly lower than in animals in the AMT and control groups at 3 and 4 weeks post-injury, and no symblepharon was observed in the eye pad-treated group. This may be related to the addition of the hydrogel as a mechanical barrier that separated the wound surface and thus reduced mechanical stress. Mechanical stress is an important factor in scar formation that promotes the formation of fibroblasts through TGF-β/Smad and other signaling pathways, causing scar-forming hyperplasia[37].

In this study, amniotic extracts were loaded into a GelMA hydrogel from which they were gradually released with the degradation of hydrogel. Previous investigations[12,29,38,39] have reported that the anti-inflammatory effects of amniotic fluid extract are concentration dependent, but that there were no apparent differences between high and medium concentrations[39]. Therefore, we used the amniotic extraction method of Jiang et al[12] and used a high concentration of amniotic extract.

In our study, we observed one animal with hyphemia and corneal perforation in the eye pad-treated group. In that animal, we found that the eye pad in the conjunctival sac was broken and irregular, potentially due to the scratching of eyes by the rabbit itself or because of unequal degradation of the pad. The broken eye pad likely caused irritation and damage. We noticed several other instances of broken eye pads in other animals. When the eye pad is broken and not in its desired shape, it cannot perform its therapeutic function. Indeed, on the contrary, it may pose a secondary source of damage to the eye. We suspect this may be partially because the eye pad model was designed based on a disposable artificial eye piece commonly for human ocular prostheses. However, the curvature of the cornea and eyeball is different between the human and the rabbit eye. In addition, uneven blue light irradiation treatment may have caused uneven photo-crosslinking. The eye pads would be expected to degrade early at sites that received a reduced level of crosslinking. These sites would have unstable shape and may begin to rupture. GelMA hydrogel is currently one popular biological regeneration material. It can carry cells and be printed as part of a three-dimensional (3D) scaffold[19,40]. If we were to create a digital model of the surface morphology of a rabbit eye, 3D printing could be used to create eye pads suitable for rabbits. During the 3D printing process, the blue light irradiation is uniform, so the eye pieces would be more stable and better fit the curvature of the eye, potentially resulting in improved prevention of symblepharon.

Generally, we found that the GelMA hydrogel eye pad was transparent, soft, comfortable, and easily attached to the ocular surface. However, the structural stability needs further improvement as it was relatively brittle and easy to damage. In these experiments, we used rabbits, which significantly do not cooperate with therapeutic manipulations, as well as human subjects, so the eye pads were more likely to fall off.

There were several limitations to this work. We did not differentiate between the effects of the GelMA hydrogel and amniotic extract. Indeed, although we believe that the combination of these

two components likely provides synergistic therapeutic benefit, it is entirely possible that either component alone would have yielded similarly beneficial results. Determining the relative contribution of GelMA and the amniotic extracts could only be determined by testing each separately as a treatment. Further, the experiments conducted in this study had a relatively small sample size and follow-up time was limited to 4 weeks.

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Conflict of Interest

The authors have no conflicts of interest to disclose. All listed authors contributed to the planning, performing, and reporting of this work.

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