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BIOCOMPATIBILITY STUDIES

Novel implantable composite biomaterial by fibrin glue and amniotic membrane for ocular surface reconstruction

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Abstract Amniotic membrane transplantation (AMT) is considered a substantial treatment option in the management of ocular surface disorders. However, several inherent drawbacks still remain. The present study devised a novel implantable composite biomaterial of fibrin glue-double layer Amniotic membrane (AM) and evaluated the biomechanical properties and effects on corneal surface reconstruction in alkali-burned rabbit model. Biomechanic parameters were calculated by an electronic universal testing machine. Corneal alkali burning was done in the right eyes of thirty rabbits, which were randomized into three groups of ten animals each. The eyes in group 1 underwent fibrin glue-double layer AMT, the eyes in group 2 underwent ordinary single layer AMT, and the eyes in group 3 (control group) did not undergo any surgical procedure. Healing of corneal epithelial defect, extent of corneal vascularization and corneal clarity were assessed and compared at two time points. One month after surgery, animals were killed and the eyes were processed for histopathology. The fibrin glue-double layer AM composites had more ideal biomechanical properties. In fibrin glue-double layer AM group, the rate of epithelial healing, vascularization inhibition and corneal clarity was significantly better than the other two groups. Novel fibrin glue-double layer AMT with corneal alkali burns is more effective and useful for ocular surface reconstruction and has great potential applications.

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1 Introduction

Amniotic membrane (AM) is the inner membrane of the placenta that surrounds a fetus in utero, consisting of a single layer of epithelium and an avascular stromal rich in extracellular matrix providing heparin sulfate proteoglycans, laminin, and collagens, which are important for basement membrane integrity, and is able to express multiple antiangiogenic factors, anti-inflammatory proteins, growth factors, and protease inhibitors [1–3]. In 1995, Kim and Tseng [4] published their promising results of using AM to encourage corneal epithelium regeneration in a chemical burn model. Since then, the application of single layer AM transplantation (AMT) is ever increasing and gradually become a substantial treatment option in the management of ocular surface disorders [5].

Unique properties such as reduction of scarring and inflammation, promotion of wound healing and epithelialization, inhibition of vascularisation, anti-microbial properties, make the AM ideal for ocular surface reconstruction procedures [6, 7]. These mechanisms have been invoked in relation to its clinical application. Single layer AMT has been used in a wide variety of ocular surface disorders like corneal ulcers [3, 8], persistent epithelial

defects [9], stem cell deficiency [10], chemical burns [8, 11, 12], pterygium [13], symblepharon [14] and so on. Until now, it is considered one of the major new developments in surgery of ocular surface.

However, despite the success application of traditional single layer AMT, several inherent drawbacks still remain. Firstly, single layer AM could stay in the wound only for a few days to 1 week after AMT and finally completely dissolved or falls off. Secondly, it is difficult to fully play its various biological activities and produce the effect of biological treatment in such short period of time. Furthermore, the single layer AM is thin and soft with poor mechanical strength. So it is easy to curl, fold, tear, and is difficult to shear or suture in operation.

In recent years, the application of multiple layers of amniotic tissue has been reported used in patients with persistent epithelial defects, deep corneal ulcers, perforations and severe ocular burns [15–17]. Its curative effect is significantly improved than single layer AM. Nevertheless, all the methods were overlapped the membranes, sutured them to the ocular surface or fill in the wound. After that, patients need to wear a compression bandage or a hydrophilic corneal contact lens for a relatively long time. The double layer or multilayer AM easily layered, hydrops formed between membranes, the surface membrane fell off quickly, and then all the membranes dissolved or fell off. Moreover, overlap of the membranes increased the operation difficulty, large number of suture added new injuries, excessive stitches easily induced inflammation and neovascularization.

To overcome these problems and defects of existing technology, we devised a novel implantable composite biomaterial by fibrin gel and AM. The present study was designed to assess the potential efficacy of fibrin glue-double layer amniotic membrane (FG-AM) transplantation involving thirty rabbits with severe cornea alkali burns and to compare the results with control groups. Although the use of multilayer AMT has been reported previously [15–17], to the best of our knowledge, this is the first report of the use of fibrin glue combined with double-layer AMT for ocular surface reconstruction. The aim of our study was to evaluate feasibility and effects of the new composite biomaterial on corneal surface reconstruction in alkali-burned rabbit model.

2 Materials and methods

2.1 Amniotic membrane Preparation

The study was approved by the Institutional Ethics Committee of Chongqing Medical University and followed the tenets of the Declaration of Helsinki. With proper informed consent, human amniotic membranes were obtained after elective cesarean section from seronegative donors (HIV, hepatitis B surface antigen, hepatitis C virus, syphilis). Under strict asepsis, AM was separated from chorion, cleaned and wrapped onto the nitrocellulose filter paper. The pieces were stored at 4 °C in vials containing pure glycerin and were used within 20 days before transplantation. The preparation and preservation method has been described previously [4, 12].

2.2 Fibrin glue preparation

Commercially available fibrin gel kits were purchased from Beixiu Biotech Co. (Guangzhou, China). The kit consists of two components contained in separate syringes sitting side by side. Component A (fibrinogen and factor XIII) and Component B (thrombin and calcium chloride) were prepared at a 1:1 ratio. Mimicking the final steps of coagulation cascade, the inject system allowed simultaneous injection of fibrinogen and thrombin, conversion of fibrinogen to fibrin, and formation of the fibrin glue.

2.3 Fibrin glue-double layer amniotic membrane preparation

During application, once fresh fibrin adhesive was injected to the bed AM using the double syringe, another AM was pressed rapidly and firmly onto the bed for about 5 min before the adhesive hardened. Then the fibrin glue-double layer AM formation, thickness ranging from 0.1 to 0.3 mm. Airing and curing at 20 °C for 12 h before mechanical testing. The products were cut into 2 cm diameter disks by a circular cutter and used for animal experiment within 4 h once prepared.

2.4 Mechanical testing

To investigate the physical characteristics of FG-AM, mechanical testing were performed on 1 × 3.5-cm samples, using an electronic universal testing machine. Choose twelve films of FG-AM at random, the same quantity of the monolayer AM as control. All the samples were tested at a 5 mm/min extension rate. Results including tensile strength, elongation at break, elastic modulus and the stress–strain curve were calculated directly by the testing machine.

2.5 Animals and corneal alkali burn

New Zealand rabbits were obtained from the animal center at Chongqing Medical University (Chongqing, China). All experiments in this study were conformed to the institutional animal guidelines and according to the ARVO

Statement for Use of Animals in Ophthalmic and Vision Research. Forty-five New Zealand rabbits, of either sex, weighing 2–2.5 kg each, were randomized into three groups of 15 animals each. The eyes in group 1 (FG-AM) underwent fibrin glue-double layer AMT, the eyes in group 2 (AM) underwent ordinary single layer amniotc membrane transplantation, and the eyes in group 3 (control group) did not undergo any surgical procedure. Animals were anesthetized using sumianxin II (0.2 ml/kg body weight) by intramuscular injection. One eye of each rabbit was subjected to an alkaline burn by placing a 9-mm-diameter circular piece of filter paper soaked in 1 N NaOH on the central cornea. After 60 s, The cornea was rinsed with 50 ml of physiological saline immediately after alkali exposure.

2.6 Surgical procedure

Fresh fibrin glue-double layer AM was secured onto the corneal surface of eyes in group 1. Perilimbal, interrupted 12 8-0 nylon sutures were applied to anchor the membrane to the underlying conjunctiva and episclera. Hence, the FG-AM was spread fully on the cornea, serve as a patch for the entire ocular surface.

The AM was thawed before proceeding to transplantation in eyes with ocular. In a similar way to group 1, AM was maneuvered into the rinsed cornea with the basement membrane side facing down, and was secured onto the ocular surface of eyes in group 2 using 8-0 nylon sutures.

During the postoperative period, topical eyedrops containing a combination of dexamethasone and antibiotics were applied three times daily in each group for 7 days.

2.7 Follow-up evaluation

Detailed ophthalmic examination was performed by masked observers with the aid of a slit lamp, noting the extent of the burn, dissolution of transplanted membrane, ocular surface and the like. Documentary external photographs were obtained at 14 and 28 days after surgery. Cornea was assessed for extent of opacity, vascularisation, and size of epithelial defect. The scores used for opacity was based on the classification described by Sonoda [18] as shown in Table 1. Corneal photographs of vascularisation and fluorescein staining were stored, digitized and imported to the computer for measurement of the vascularisation and epithelial defect area. To minimize observer bias, photographs were analyzed randomly by two double-blinded investigators.

2.8 Histopathology

For Histopathologic evaluation, fresh fibrin glue-double layer AM was under hematoxylin-eosin (HE) stain and light microscope observation. Rabbits were killed 28 days after surgery by air embolism and eyes were randomly selected and excised, processed for histopathology. The corneas were fixed in 4 % Paraformaldehyde solution and embedded in paraffin, sliced and stained with hematoxylin and eosin. Sections were evaluated by light microscopy.

2.9 Statistical analysis

The data were represented as mean ± SD, and comparison was made between groups using ANOVA for repeated measures with the aid of the program SPSS v11.5. *P* values of <0.05 were considered to be significant.

3 Results

3.1 Mechanical testing

Under wet conditions, FG-AM showed an average tensile strength of 0.727 Mpa, elongation at break of 24.130 %, elastic modulus of 1.283 Mpa. The shape of the stress–strain curve of FG-AM was regular, the curve could be repeated well. The tensile strength and the elongation at break of FG-AM were more than those of monolayer AM. The elastic modulus of the FG-AM was smaller than that of the monolayer AM ($P<0.05$). The FG-AM composites had more ideal biomechanical properties than the monolayer amniotic membranes. Table 2 and Fig. 1 showed biomechanic parameters and stress–strain curve of 1 × 3.5-cm membranes.

Table 1 Definition of scores for parameters used for opacity [18]

Score	Description
0	Clear and compact cornea
1	Minimal superficial (nonstromal) opacity
2	Minimal; deep (stromal) opacity; pupil margin and iris vessels visible
3	Moderate stromal opacity; only pupil margin visible
4	Intense stromal opacity; only a portion of pupil margin visible
5	Maximum stromal opacity; anterior chamber not visible

Table 2 Biomechanic parameters of 1 × 3.5-cm membranes (mean ± SD)

Group	n	Tensile strength (Mpa)	Elongation at break (%)	Elastic modulus (Mpa)
FG-AM	12	0.727 ± 0.142[a]	24.130 ± 4.523[a]	1.283 ± 0.482[a]
AM	12	0.552 ± 0.135	12.745 ± 3.198	4.048 ± 1.702

Data are presented as means (m) and standard deviation (sd)

[a] Represent statistically significant differences compared with AM group

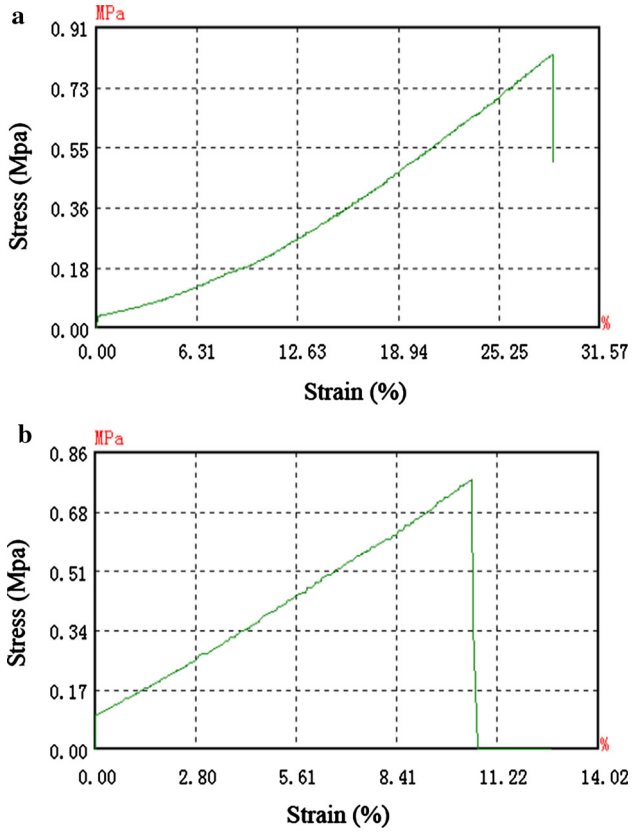

Fig. 1 **a** Stress–strain curve of FG-AM. **b** Stress–strain curve of AM

3.2 Dissolution of transplanted membrane

In the 30 animals initial in this study, six were excluded because of respiratory infections or digestive disorders, resulting in nine in group 1, nine in group 2 and eight in the control group (group 3) that had a follow-up of 28 days. In the five excluded animals, two were followed for 14 days (one from group 1 and one from group 2).

Early after alkali Burn, we noted that the corneas were polluted and porcelain white, the surfaces were rough, and the intraocular structure could not be seen. All the eyes showed moderate to severe corneal and conjunctival damage. For the control group, such problems remained until the end of follow-up. At 5 days after operation, single-layer amniotic membranes in group 2 gradually dissolved or dropped from surface of cornea, while the fibrin glue-double layer AM in group 1 has no obvious change. Untill 14 days after operation, most of the fibrin glue-double layer AM dissolved off. The best clinical outcome were found in group 1 (Fig. 2).

3.3 Corneal opacity, vascularization and fluorescein staining

With respect to corneal opacity, significant difference was found between group 1 and group 3 at 14 days, no statistically significant difference was detected between the other groups ($P > 0.05$). At 28 days after injury, however, we noted significant difference between the three groups ($P < 0.05$), with the control group having the most opacity, group 2 with intermediate opacity ($P < 0.05$), and group 1 with the least opacity ($P < 0.05$). Table 3 shows the scores of corneal opacity among the different groups.

With respect to vascularization, there was significant difference was found between group 1 and group 3 at 14 days, but no statistically significant difference among the others ($P > 0.05$). While at 28 days after injury, we detected clearly significant difference ($P < 0.05$) between group 1 and 2 and the control group. Group 2 and the control group had significantly more vascularization than group 1 ($P < 0.01$). Table 4 shows vascularization in the three groups.

With respect to corneal fluorescein staining, we found significant difference between the three groups ($P < 0.05$). At 14 and 28 days after surgery, corneal fluorescein staining area in group 1 and group 2 was much smaller compared with control group. And there was a significant difference between the two experiment groups ($P < 0.05$), group 1 had the smallest fluorescein staining area. In other words, compared with control group, the group 2 had better epithelial healing ($P < 0.05$), and group 1 had the best epithelial healing ($P < 0.01$). Table 5 shows fluorescein staining in the three groups.

3.4 Histopathologic analysis

Histopathologic analysis revealed that, amniotic basement membrane closely adhering to fibrin glue in the middle, without membrane shrinkage (Fig. 3a). The fibrin glue-double layer AM was obviously thicker than single layer

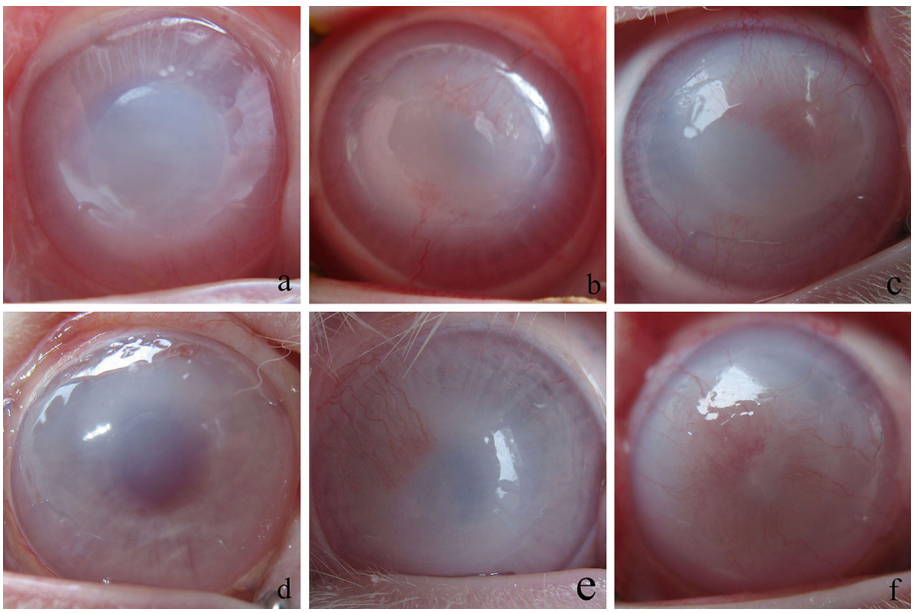

Fig. 2 Representative series of slit-lamp photographs. Case from FG-AM group, at 14 (**a**), 28 (**d**) days postoperative. Case from AM group, at 14 (**b**), 28 (**e**) days postoperative. Case from control group, at 14 (**c**), 28 (**f**) days postoperative

Table 3 Corneal opacity scores in each group (mean ± SD)

Group	14 (days)		28 (days)	
	m ± sd	n	m ± sd	n
FG-AM	2.600 ± 0.966[a]	10	2.111 ± 0.928[a]	9
AM	3.333 ± 1.000	9	3.250 ± 1.035[a]	8
Control	4.125 ± 0.835	8	4.250 ± 0.707	8
P	0.009		<0.001	

Data are presented as means (m) and standard deviation (sd)

[a] Represent statistically significant differences compared with control group

Table 4 Vascularization in each group (mm^2, mean ± SD)

Group	14 (days)		28 (days)	
	m ± sd	n	m ± sd	n
FG-AM	33.908 ± 11.410[a]	10	34.472 ± 8.663[a]	9
AM	45.442 ± 12.638	9	55.718 ± 10.951[a]	8
Control	56.545 ± 12.167	8	84.822 ± 9.816	8
P	0.002		0.000	

Data are presented as means (m) and standard deviation (sd)

[a] Represent statistically significant differences compared with control group

AM. Simple columnar AM epithelium cells could be seen on the liber AM epithelium. Basal surface of an AM tightly binded to basal surface of another AM by fibrin glue. There was no gap between them. Fibrin glue and AM were structurally-complete as a whole and hardly separable. Thickness of the fibrin glue in the middle was uniform and no obvious delamination and degeneration was found.

Table 5 Fluorescein staining in each group (mm^2, m ± sd)

Group	14 (days)		28 (days)	
	m ± sd	n	m ± sd	n
FG-AM	4.330 ± 3.039[a]	10	2.700 ± 2.014[a]	9
AM	11.011 ± 5.026[a]	9	7.875 ± 4.162[a]	8
Control	24.938 ± 6.703	8	12.575 ± 5.851[a]	8
P	<0.001		<0.001	

Data are presented as means (m) and standard deviation (sd)

[a] Represent statistically significant differences compared with control group

28 days after injury, a large amount of neovasculars formed in control group. Fresh epithelial cells juncted loosely to the corneal stroma, arranged irregularly with some of them fell off. Many inflammatory cells was found infiltrating in the corneal stroma. However, in FG-AM group, 5–7 layer intact corneal epithelial cells connected tightly with corneal stroma, collagen fibers arranged regularly. Membranes showed a stronger, healthier, and more stratified epithelial layer. The least stromal edema, neovasculars and inflammatory cell reaction were detected. Results of AM group was better than control group and worse than FG-AM group (Fig. 3b–d).

4 Discussion

Here we describe a method of fibrin glue combined with double-layer AM. With this method, the biological and physics characteristics such as flexible of AM were maintained, the thickness of the AM was increased and it is

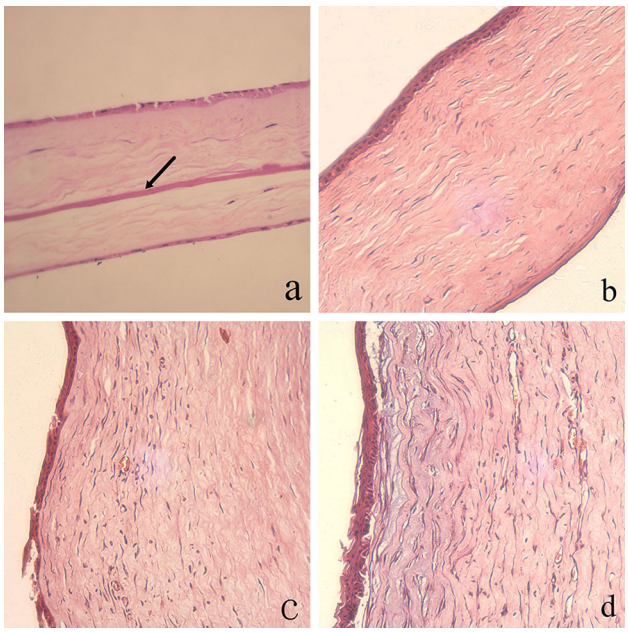

Fig. 3 Hematoxylin and eosin staining photographs. (Original magnification × 200) HE staining of fibrin glue-double layer amniotic membrane (**a**), amniotic basement membrane closely adhering to fibrin glue (*arrow*). HE staining from FG-AM group, at 28 (**b**) days postoperative. HE staining from AM group, at 28 (**c**) days postoperative. HE staining from control group, at 28 (**d**) days postoperative

conducive to the surgical operation. It can be easily obtained, prepared and its availability is almost unlimited. Collaborative therapeutic effect was come into play as fibrin glue combined with AM.

Preparation of fresh fibrin glue-double layer AM consists of five stages: match, injection, adhesion, airing and temperature curing. Each of these stages closely affects the quality of the composite membranes. To avoid fibrin gel premature solidified, injection should be quick. During preparation, strict aseptic technique and appropriate pressure should be applied, do not move back and forth when pressing. The advisable volume of fresh fibrin adhesive injected from the double syringe at once is 0.5–1 ml. The appropriate thickness of FG-AM is about 0.2–0.5 mm and the suitable temperature is 15–30 °C.

The biomechanic parameters and stress–strain curve of mechanical testing showed that the FG-AM composites had more ideal biomechanical properties than the monolayer amniotic membranes. The properties consists of good rigidity, flexibility, clot formation properties and rheological properties including elasticity, tensile strength, adhesiveness. Our research indicated that the FG-AM composites had sufficient strength to withstand suture force and ocular surface tension, a certain degree of flexibility to bend and expand with ocular movement and to prevent oppression on the eye.

In this study, the outcome of animal experiment was measured in several ways. With respect to corneal neovascularization and opacity, group 1 receiving fibrin glue-double layer AMT resulted in fewer new vessels than group 2 receiving single layer AMT and the control group. This difference did not reach statistical significance between operated groups 1 and 2 at 14 days ($P > 0.05$). But we detected a clearly significant difference between operated groups 1 and 2 and the control group at 28 days. Both group 1 and group 2 had less vascularization and opacity than the control group ($P < 0.05$). With regard to epithelial healing, we found that corneal epithelial healing area was significantly larger in group 1 than in group 2 at 14 days and 28 days. There was a significant difference between the three groups ($P < 0.05$).

In the FG-AM group, the procedure has been satisfactory and there have been no major complications. We observed no clinical or histopathologic evidence of adverse effects to epithelium, keratocytes, or endothelium. Our study demonstrate that the use of fibrin glue-double layer AMT might be an optimal alternative for traditional single layer AMT because of the following advantages: it saves suture time, it's easy to use, it's associated with less corneal opacity, neovascularization, inflammation and better epithelial healing.

Fibrin glue is a natural, biologically compatible, resorbable biomaterial that that imitates the final step of coagulation. It is a two-component system consisting of fibrinogen and thrombin. When the two components are mixed and fibrinogen is activated by thrombin, an adhesive fibrin network is formed. Fibrin glue is mainly used to control bleeding, to adhere tissues together, and to seal tissue defects [19]. Fibrin glue can allow sufficient working time before totally "setting" in situ, have adequate tensile strength to maintain corneal integrity, be clear to permit vision, be permeable to fluids and metabolites to prevent necrosis, not cause inflammation, and eventually disappear to permit healing at the adhesive interface [20]. For these reasons, fibrin glue is regarded as best reaching the ideal ocular adhesive in recent years. It has been applied in a variety of ophthalmic surgeries, such as strabismus surgery [21], corneal surgery [22], glaucoma surgery [23], and cataract surgery [24].

Ocular Alkali injury represent one of the most serious forms of eye trauma and may cause extensive injury to the anterior ocular segment [25, 26]. Multiple facets of ocular alkali injury interfere with the proper healing process, especially persistent inflammation and release of collagenase, result in formation of scar tissue, recurrent corneal erosions and non-healing defects [25, 26]. Moreover, since the exact mechanisms of this devastating series of local events are incompletely understood, the management of ocular alkali injury is usually not satisfactory [27]. A substantial variety of medical techniques and surgical procedures generated, but with only limited success and long-standing challenge [28].

The main aim of treatment of ocular alkali injury is to promote epithelial healing, reduce inflammation and prevent progressive tissue melting to minimise scarring sequelae and severe visual loss with medical and surgical therapy [29]. AMT has been used for ocular surface reconstruction in acute chemical injury and reported to be effective in a number of studies [8, 30]. The biological properties attributed to AM include lack of immunogenicity; promotion of epithelialization; and inhibition of fibrosis, angiogenesis, and inflammation [31, 32]. The fundamentals of AMT onto the corneal surface is based on the aforementioned mechanisms [33].

Recent years fibrin glue has been reported used as AM tissue adhesive for several ophthalmic applications [6, 34, 35]. In these studies, single layer AM was fixed directly on ocular surface only by fibrin glue without suture. However, this is still not an idea method for AM fixation. The corneal surface is rather repellent and eye lid movements exert tangential shear forces on the membrane [36]. In addition, fibrin glue is known to rapidly disintegrate when exposed to the tear film [37]. Aforementioned Factors lead to insufficient adhesion and postoperative graft loss. Therefore, the use of fibrin glue for reliable adherence of AM onto the ocular surface has been limited so far [36].

The present study indicate that the use of fibrin glue in conjunction with double layer AM has significantly enhanced the effectiveness of AMT in ocular surface reconstruction. The transplantation of fibrin glue-double layer AM presents several advantages compare with single layer AM. Firstly, FG-AM is flexible and stable. It can cover the ocular surface for a relatively longer time, thus significantly prolong the action time of AM and fibrin glue. One might speculate that AM inhibits protease activity, slow hydration and rapid lysis of clotted fibrin by the tear film [37, 38]. Meanwhile, fibrin glue might delay AM dissolution or loss. Yet, the traditional single layer AM generally can only stay on the ocular surface for about 1 week, difficult to fully play its therapeutical effect. Secondly, FG-AM decreased the suture difficulty and membrane shrinkage. It has been known that once shrinkage takes place, the AM lying above will invariably dissolve due to collagenases present on the ocular surface [39]. Fibrin glue exert steady adhesion forces on the AM, thus prevented the shrinkage of the AM in the FG-AM group [36]. Furthermore, some other significant advantageous properties associate with could be demonstrated regarding well biocompatible and biodegradable, promoting local tissue growth and repair, without inducing inflammation, foreign body reactions, tissue necrosis, or extensive fibrosis [20]. Our study confirmed the above advantages and we believe that these are key points for the successful use of FG-AM.

Currently, we are investigating this new technique in clinical. A consecutive study has been started in humans and preliminary shows satisfactory curative effect. Although we report our experience with animal experiment and only a small number of patients, follow-up is not extensive, procedure is not mature enough and much work remains, use of fibrin glue-double layer AM transplants has led to a novel approach to ocular surface repair. Better preservation and preparation technology, a larger number of patients as well as a longer follow-up time would be preferable in order to draw final conclusions. Further studies to resolve this issue are needed in the future.

5 Conclusion

Fibrin glue-double layer AMT represents a viable approach for the treatment of ocular alkali injury. Moreover, this study demonstrates the advantages of the fibrin glue in association with double layer AM in the management of serious eye trauma. Good stability of composite biomaterial graft, short operating time, a comfortable postoperative period, and lack of complications with the use of fibrin glue-double layer AM was achieved. This technique promotes a stable and rapid reconstruction of the ocular surface.

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