·Basic Research·

Effect of amniotic membrane transplantation on rabbit conjunctival surface reconstruction at the recovering stage of alkali burn

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Abstract

• AIM: (1) To investigate the effect of amniotic membrane transplantation (AMT) on rabbit conjunctival surface reconstruction with severe alkali burns. (2) To evaluate the possibility of AMT treatment for ocular alkali burns during recovering stage.

• METHODS: Animal models were established on 30 eyes of rabbits by creating severe alkali burns on the conjunctiva from the upper corneal limbus to the upper conjunctival fornix. Preserved human amniotic membrane transplantations and reconstruction of conjunctival fornix were performed at one week after injury (recovering stage). Epithelium growth of burned area after transplantation was observed using light microscope at 1, 2, 3, 4, and 8 weeks. Conjunctival tissue in transplantation area was collected at 1, 4 and 8 weeks. The ultrastructure of the collected tissue was studied by electron microscope. The results were compared with control group, which received only vitamin C subconjunctival injection and antibiotic eye drops as treatment for alkali burn. Exterior eye pictures were also taken at the end of the observation, the width from upper corneal limbus to the edge of upper fornix was measured. Data were analyzed statistically.

• RESULTS: (1) In the transplant group, conjunctival epithelium growth was observed in the area of AMT under both light and electron microscope 1 week after surgery. At the 4th week, conjunctival epithelium with goblet cells that resembled normal conjunctival tissues was observed in the whole amniotic membrane area. At the 12th week, the conjunctival epithelium on the amniotic membrane was well formed, and the connective tissue under the epithelium was loose at the fornix. No fibrosis was identified. In contrast, conjunctival epithelium necrosis was observed in the control group at 2 weeks after alkali burns. Reepithelization did not occur through the 12-week observation. Severe fibrosis with inflammatory cells infiltration was observed between 4 to 8 weeks. At the 12th week, fibrosis of the connective tissue at the fornix developed and there were no conjunctival epithelium covering the burned area. (2) In the transplant group, the conjunctiva in transplanted area had no scarring and appeared smooth at the 12th week. Upper fornix was reconstructed. The depth of fornix was 7.9 ± 0.3mm (7.6-8.2mm), which was approximate to the normal depth 8.2 ± 0.2mm (8.0-8.4mm, $P>0.05$). While in the control group, the burned area appeared rough with granuloma formation and severe scarring. Upper fornix became shallow. The depth of fornix was 3.1± 1.7mm (1.0 - 4.5mm.), and significant difference was found between control and transplant group ($P<0.01$).

• CONCLUSION: Human amniotic membrane preserved in glycerin can promote cell adhering, migrating and differentiating of normal conjunctival epithelium. Reconstruction of conjunctival surface in early stage of alkali burn can be achieved by AMT. AMT can effectively prevent symblepharon formation.

• KEYWORDS: conjunctiva; chemical burn; amniotic membrane transplantation; rabbit

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INTRODUCTION

Chemical ocular burns, especially by alkali, may result in severe outcomes. For example, conjunctival

epithelium necrosis after alkali burns may lead to conjunctival fibrosis and symblepharon. Reconstruction of the ocular surface and prevention of symblepharon development at the early stage of alkali burn may change the clinical prognosis. To date, no medications are proven to have such therapeutic effects. Several operations, such as oral mucosal graft transplantation, conjunctival transplantation, have been tried, but the therapeutic effects are limited due to the risk of rejection and infection, limited availability of the material, and cosmetic issues. In 1995, Kim *et al* [1] first reported successful reconstruction of ocular surface by preserved amniotic membrane transplantation (AMT) on injured rabbit eyes. The clinical application of AMT on ocular diseases has gained tremendous attention since then [1-5]. The thick basement membrane of amniotic membrane is thought to play an important role in restore ocular surface through promoting epithelial cell migration, proliferation and differentiation. This study is designed to investigate the effectiveness of AMT in restoration of ocular surface and prevention of symblepharon.

MATERIALS AND METHODS

Materials Thirty-four healthy male and female white rabbits, each weighing 2.0-2.5kg, were provided by Experimental Animal Center (EAC) of China Medical University (CMU). All rabbits were free of ocular diseases. Human placentas were obtained immediately after elective Cesarean section delivery from mothers who were seronegative for human immunodeficiency virus (HIV), hepatitis B and C, and syphilis at the Second Affiliated Hospital of CMU. Other materials used in this study included surgical grade microscope (Suzhou, China), JSM-T300 scanning transmission electron microscope (JEOL, Japan), surgical instruments (Suzhou, China), vernier caliper (Suzhou, China), 846 compounded anesthesia injection (CMU), 900mL/L glycerin, 1mol/L NaOH solution, BSS (Alcon, USA), chloramphenicol eye drops(Freda, USA), gentamycin injection, chlortetracycline hydrochloride eye ointment (Freda, USA), 4g/L benoxine anesthesia solution (Santan, Japan), and nitrocellulose paper (Suzhou, China).

Methods

Animal model After anesthetization with intramuscular injection of 0.3mL/kg 846 compounded solution, the right eyelid was lifted and the tertiary eyelid was opened. After applying 4g/L benoxine topical anesthesia solution, filter paper in size of 13mm by 8mm soaked in 1mol/L NaOH solution was placed on exposed conjunctiva between upper corneal limbus and conjunctival fornix for 60 seconds. Then the eye was washed with normal saline. Severe conjunctival alkaline burn was created. Necrotic conjunctiva was seen pale and opaque in burned area.

Intervention after alkali burn AMT were performed 1 week after burn in surgery group. Post-operative treatments included chloramphenicol eye drops twice a day and chlortetracycline hydrochloride ointment once a day. Vitamin C subconjunctival injection was given once a day for 4 days to the control group. Chloramphenicol eye drops and chlortetracycline hydrochloride eye ointment were applied at the same time and were continued after vitamin C injections were stopped.

Amniotic membrane preparation Human placenta obtained immediately after elective Cesarean section delivery under sterile procedure from mothers who were seronegative for human immunodeficiency virus (HIV), hepatitis B and C, and syphilis. The placenta was washed in normal saline and bathed in BSS containing 4MU/L gentamycin for 10 minutes. The amniotic membrane was then separated from the rest of the chorion by blunt dissection through potential spaces with forceps and washed several times in normal saline. Amniotic membrane was placed evenly on top of nitrocellulose paper with epithelium layer up and cut into fragments of 1.5cm by 2.0cm. The fragments were put in 900mL/L glycerin for dehydration for 24 hours. Then all the fragments were removed into another bottle of 900mL/L glycerin, sealed and kept in refrigerator at 4 Celsius degree. Amniotic membrane used in this study was stored between 28 to 44 days. It was washed with normal saline and rehydrated for 30 minutes in BSS containing 4MU/L gentamycin before using.

Amniotic membrane transplantation After anesthetization with intramuscular injection of 0.3mL/kg 846 compounded solution, the right eyelids were opened by sutures, and topical anesthesia solution was applied onto the exposed eyes. After the necrotic tissue was removed from traumatized conjunctiva surface under a microscope, a conjunctival defect 7-8mm by 13-15mm in size was created. Saline containing gentamycin was used to irrigate the area. A layer of prepared amniotic membrane was then placed over the defected area with epithelium side up and trimmed to the appropriate size and shape. The membrane was then secured in place using 10-0nylon interrupted sutures to the surrounding conjunctiva. All sutures went through the episclera to anchor amniotic membrane onto the ocular surface. The amniotic membrane covering the palpebral conjunctiva was attached to the fornix with two bedding

sutures. Sutures went through whole thickness of eyelid ending at the outside of the skin close to orbital margin. The two sutures were then secured on a roll of gauze to hold conjunctiva to fornix and to maintain the depth of conjunctival fornix. Amniotic membrane covering the palpebral conjunctiva was secured in place with 9-0nylon interrupted sutures 2mm away from the edge of eyelid.

Observation

General observation After AMT, each eye was examined each day for any dislocation, discoloration, and any dissolving of amniotic membrane. Pictures of eyes in both transplant and control groups were taken at the 12th week. The depth of the upper conjunctival fornix was measured with a bow compass at 12 o'clock. The distance between corneal limbus and the apex of fornix was recorded. For eyes with symblepharon, the distance between 12 o'clock of corneal limbus and the margin of scar closest to the corneal limbus was recorded.

Light microscope study Two rabbits from each transplant and control group at a time were executed at 1, 2, 3, 4, 8, and 12 weeks after transplantation. A coronal incision was performed on the grafted area in transplant group and on the burned area in control group. At the 12th week, the remaining three rabbits in each transplant and control group were executed. Eyeballs with their fornix and eyelids together were removed and cut in half along the medial sagittal plane. Halved eye tissue was fixed in 40g/L formaldehyde solution, and then embedded in paraffin. Slices prepared from embedded tissue were stained with HE staining and studied under light microscope.

Electron transmission microscope study Tissue of transplanted area was collected at the 1st, 4th and 8th weeks in transplant group, and fixed in 25g/L glutaraldehyde. Samples were prepared routinely and studied under electron microscope. Electron microscope study on the tissue samples from the burned area in control group was prepared with the same procedures. Normal conjunctival tissue and preserved amniotic membrane were also studied using electron microscope for comparison.

RESULTS

Thirty-four rabbits were divided into 3 groups. Alkali burn animal models were created on the right eyes of 30 rabbits. A half of these rabbits were randomly selected for transplant and received AMT at 1 week after injury. The other 15 rabbits became the control group. Vitamin C subconjunctival injection was given once a day for 4 days to the control group. Chloramphenicol eye drops and chlortetracycline hydrochloride eye ointment were applied in both groups. The remaining four rabbits served as the blank group.

Table 1 Depth of the upper conjunctival fornix at the 12th week in rabbit ocular alkaline burn models (mean±SD, mm)

Groups	Depth of the upper conjunctival fornix
Transplant group	7.9±0.3[b]
Control group	3.1±1.7
Blank group	8.2±0.2

[b]P<0.01 *vs* control group

General Observation One week after AMT, the graft remained transparent with slight discharge, and blood vessels were present at its margin. At 2 weeks, most portion of the graft had blood supply. At 4 weeks, vascular supply was well established on the graft. The surface was smooth without scarring. At 8 to 12 weeks, the grafted area resembled a normal conjunctiva (Figure 1). At 12 weeks, the depth of the upper conjunctival fornix was 7.9 ±0.3mm (7.6-8.2mm), approximated normal depth 8.2 ±0.2mm (8.0-8.4mm). There was no significant difference between two groups. Eyeball movements were not limited. In control group, the conjunctiva in the burned area was pale, the surrounding conjunctiva was congested and swelling was present during week one; at the 2nd week, burned conjunctiva became thickened and pale and upper conjunctival fornix became shallow; at the 4th weeks, the conjunctiva appeared congested, and upper conjunctival fornix became furthered narrowed, fibrotic cords adhering upper palpebral and ocular surface together were also observed; at the 8th to 12th weeks, scar tissue on the surface of conjunctiva, as well as cicatricial pterygium and symblepharon were present (Figure 1). At the 12th weeks, the depth of the upper conjunctival fornix was 3.1±1.7mm (1.0- 4.5mm). There were significant difference between control and transplant groups (P<0.01, Table 1). The movements of eyeball were significantly limited.

Light Microscope Study Results

Blank group Normal conjunctival epithelial lining comprises non-keratinized stratified squamous epithelial cells with scattered goblet cells were shown with a layer of connective tissue underneath (Figure 2).

Transplant group Conjunctival epithelium including scattered goblet cells began covering the transplanted amniotic membrane at one week after AMT. Three to six layers of stratified squamous epithelium covered the red stained amniotic basement membrane. The epithelial cells were smaller in size and higher in density comparing to those in blank group. While goblet cells were larger and also were higher in density. Few neutrophil cells infiltrated

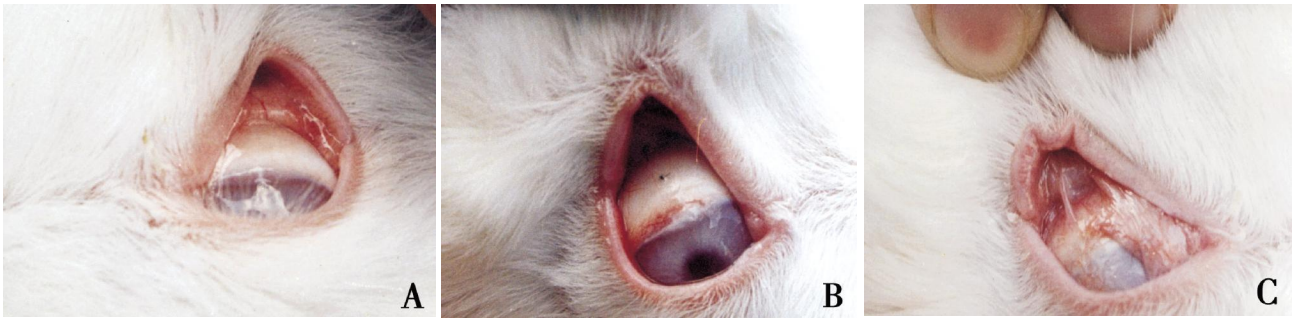

Figure 1 Pictures of eyes in different experimental groups of rabbits at the 12th week A: blank group; B: transplant group; C: control group

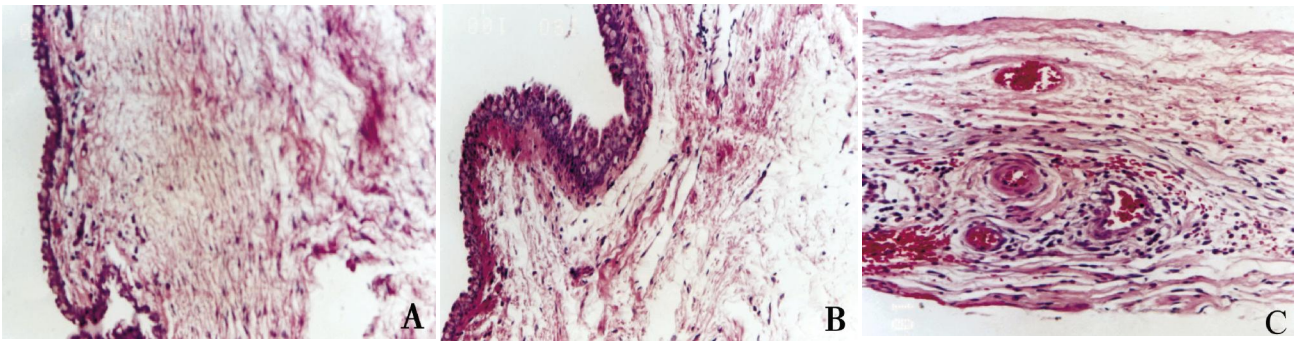

Figure 2 Light microscope study results in different experimental groups of rabbits at one week after transplantation (HE×100) A: blank group; B: transplant group; C: control group

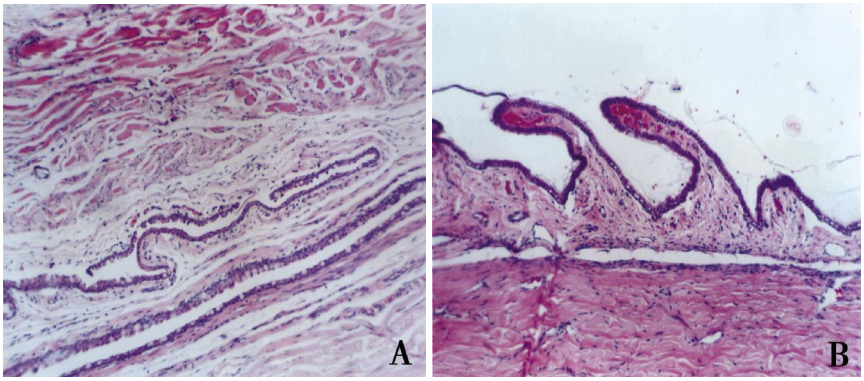

Figure 3 Light microscope study results in different experimental groups of rabbits at the 12th weeks after transplantation (HE×100) A: transplant group; B: control group

beneath epithelial layer (Figure 2). At the 2nd week, conjunctival epithelium covered almost the whole transplanted amniotic membrane. At 4 to 12 weeks, conjunctival epithelium covered the entire amniotic membrane and their structure was similar to normal conjunctival epithelium. At the 12th week, slices from the sagittal plane of the eyeball containing part of the palpebral and bulbar conjunctiva showed that the epithelium on the amniotic membrane was arranged orderly, and the connective tissue under epithelium at fornix was loose without hyperplasia (Figure 3).

Control group At week 1, necrosis of epithelial cells was observed. No epithelial or goblet cells regeneration occurred. Neutrophil infiltration was apparent with blood vessels dilated and congested (Figure 2). At the 2nd week, both collagen fiber proliferation, neutrophils and lymphocytes infiltration were observed. No epithelial cells present. At 4 to 12 weeks, many fibroblasts appeared; the collagen fibers became thicker and more condensed, and were arranged irregularly. At the 12th weeks, slices from the sagittal plane of eyeball showed significant hyperplasia of connective tissue and collagen fibers at fornix. Epithelial layer remained absent (Figure 3).

Electron Microscope Study Results

Blank group Normal conjunctival epithelial cells with microvilli projected from their flat surface. The cells were in polygonal shape. Intracellular junctions were tight. Cellular membrane was continuous and intact (Figure 4).

Preserved human amniotic membrane Under electron

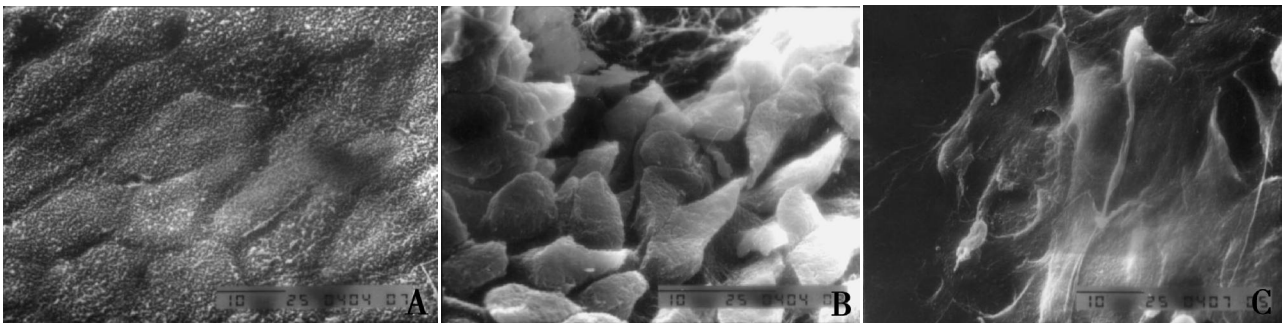

Figure 4 Electron microscope study results in different groups of rabbits at one week after transplantation (×2 000) A: blank group; B: transplant group; C: control group

microscope, abundant collagen fibrils were present in the amniotic membrane. Collagen fibrils arranged reticularly. Some of fibrils bundled together. They are the major component of amniotic basement membrane (Figure 5).

Transplant group At one week after transplantation, regenerated epithelial cells were observed on the surface of the collagen fibrils of amniotic basement membrane. The epithelial cells were in polygonal shape with abundant microvilli projection. Cells were joined by many foot processes. Epithelial cells formed a clear border that can be easily distinguished from the collagen fibrils (Figure 4). At the 4th week, conjunctival epithelial cells covered the majority of the amniotic basement membrane. The cells were connected by projected foot processes. At week 8, microvilli were presented, epithelial cells were arranged in order and the intracellular junctions were tight (Figure 6).

Control group At week 1, collagen fibrils thickened and became distorted. No regenerated epithelial cells were seen. There were some damaged epithelial cells present in irregular shape, and no microvilli projections on their surface. Cells were not joined together. Neutrophils and lymphocytes infiltration were observed (Figure 4). At weeks 4 and 8, collagen fibrils became thicker and more condensed. No regenerated epithelial cells were observed (Figure 6).

DISCUSSION

Mechanism of AMT for Conjunctival Surface Reconstruction The amniotic membrane 0.02-0.05mm in thickness comprises the innermost layer of the placenta. It is transparent, avascular, and with good tenacity. Human amniotic membrane consists of five layers: amniotic epithelium, basement membrane, membrane, compact layer, fibroblast layer, and spongiosis layer. Basement membrane is about 0.1μm thick and is considered the thickest one in human body[1].

Recent studies suggest that several different mechanisms are involved in the process of AMT. (1) Amniotic membrane is a proper carrier facilitating epithelium regeneration. It has been found that amniotic membrane can prolong epithelium life span and maintain the ability of regenesis of epithelial progenitor cells[2]. (2) Amniotic membrane is rich in type IV collagen, fibronectin and laminin [3], which may promote epithelium adhering, proliferation and differentiation and maintain the normal epithelial phenotype [3]. Through immunohistochemical study, Fukuda *et al* [3], reported that the distribution of type IV collagen subunits alpha-2 and alpha-5 in amniotic basement membrane was very similar to that of conjunctival basement membrane. He concluded that amniotic membrane could be used to substitute conjunctival basement membrane and as a carrier for epithelialization. (3) Amniotic membrane exhibits anti-inflammation action due to its anti-protease activities[6-9]. (4) Amniotic membrane has been shown to have the ability of reducing scar tissue formation. It can suppress the inflammatory process as well as collagen fiber hyperplasia induced by inflammatory cells. The amniotic basement membrane may modulate the levels of cytokines expressed by fibroblasts, suppress TGF-beta 1-signalling system, and suppress differentiation of normal fibroblasts to myofibroblast[6-9]. In summary, the transparency

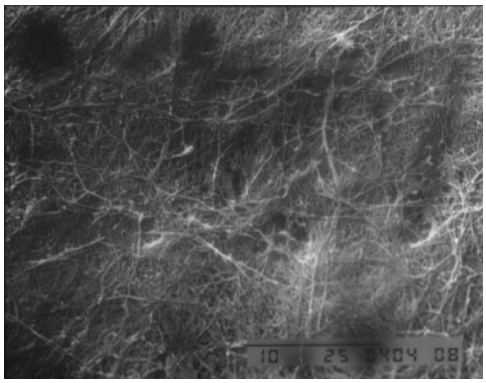

Figure 5 Electron microscope result of preserved human amniotic membrane (×35 000)

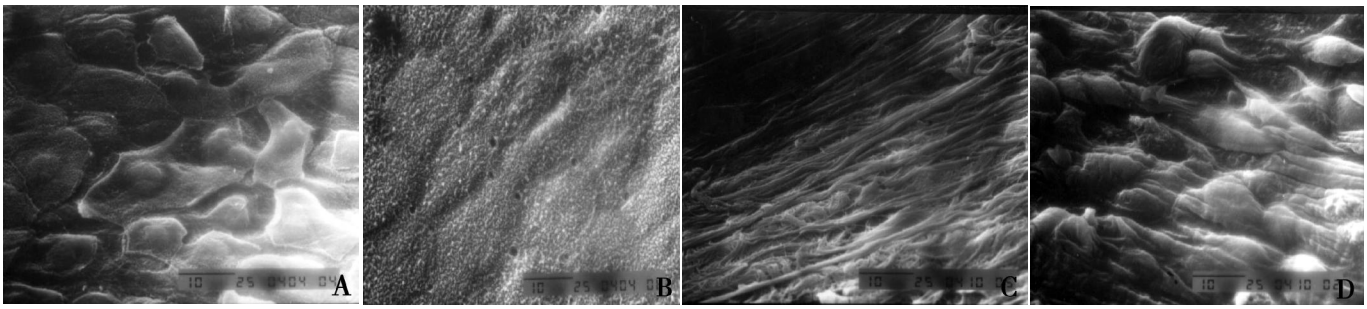

Figure 6 Electron microscope study results in different groups of rabbits at the 4th and 8th weeks after transplantation (×2 000)
A: transplant group at the 4th week; B: transplant group at the 8th week ; C: control group at the 4th week; D: control group at the 8th week

and the avascular properties of amniotic membrane make it a superior biological material for ocular surface transplantation [1]. The amniotic membrane contains abundant collagen to prevent tearing after surgery [9]. Features of amniotic basement membrane surface also favor epithelial cells adherence and regeneration [9]. The histological characteristics of amniotic membrane account for its clinical effectiveness in ocular surface reconstruction.

Effects of AMT for Conjunctival Surface Reconstruction in Rabbit Eyes with Acute Alkali Burns Conjunctiva alkali burn can results in necrosis and breakdown of conjunctival surface because the inflammation process may suppress and even stop epithelium migration, the damaged basement membrane can not facilitate epithelium regeneration. Thus, traumatized conjunctiva often heals with fibrotic tissue and develops symblepharon.

The human amniotic membrane is found to be non-immunogenic [1]. Antibodies or cell-mediated immune response to amniotic membrane has not been demonstrated. In this study, AMT was used for rabbit conjunctival surface reconstruction in recovering stage of alkali burn. Our study demonstrated the following valuable characteristics of amniotic membrane application in treating conjunctival alkali burn: (1) Low antigenicity: in transplant group, only mild conjunctiva congestion and slight discharge were observed. Graft tissue were viable; (2) Inflammatory response suppression: At week 1, few inflammatory cells infiltrated conjunctiva in transplant group was shown, in contrast to a large amount of infiltration and blood vessel dilation in the control group. At week 8, large amounts of inflammatory cells still persisted in the control group; (3) Rapid conjunctival epithelium regeneration and early normal phenotype appearance: at week 1, conjunctival epithelium presented on the surface of graft, and at week 4, they covered the entire graft. The structure of regenerated conjunctival epithelium was similar to normal conjunctival epithelium. (4) Reducing fibroplasias and neovascularization, minimizing tissue adherence: at the 12th week, surface of conjunctiva was smooth and no scarring in transplant group; in contrast, in the control group, neovascularization and fibroplasias were severe, scarring and symblepharon developed. The results of our study were similar to that of the experimental study conducted by Li *et al* [10], and further approved by clinical observation by Chen *et al* [11]. Despite various tissues such as conjunctiva tissue, oral and nasal mucosa have been used as substitute for conjunctiva, human amniotic membrane has its own advantage because neither of the other materials is abundant in source. The preservative technique is relatively easy. Preserved amniotic membrane is immune inert, so immune repelling response is avoided. Human amniotic membrane inhibits inflammation and has lower infection incidence comparing to oral and nasal mucosa. Human amniotic membrane also has anti-adherence function and can reduce possibilities of symblepharon and shallow conjunctiva fornix development. It is an ideal biological material that can be used effectively in treating ocular burns. The conclusions of our study were summarized as following: Before performing AMT, the necrotic conjunctival tissue in burned area must be removed, and blood supply from surrounding tissue to the lesion should be available. The transplanted amniotic membrane should be large enough to cover the entire traumatized area. The edge of amniotic membrane should be embedded under surrounding normal conjunctival tissue and sutured together. To achieve ideal clinical outcome, no space should be present between transplanted amniotic membrane and its underlying sclera surface to avoid unwanted loosening of transplant from tearing at sutures. The results of our study suggested that amniotic membrane was not used to replace damaged conjunctiva. It was used as the carrier for conjunctival reepithialization to reconstruct burned ocular surface. Because of its unique biological characteristics, amniotic membrane can facilitate differentiation, proliferation and migration of conjunctival epithelium. Based on above

features, amniotic membrane is very likely to be used as the carrier for various epitheliums culturing, such as conjunctival epithelium, corneal epithelium, and limbal epithelium, *etc.* Several studies have been conducted[12, 13].

Feasibility and Therapeutic Value of AMT in Recovering Stage of Alkali Burn Traditional treatments for ocular alkali burned eyes include vitamin C subconjunctival injection, application of antibiotics, and self-blood injection (a controversial treatment). The treatments mentioned above cannot prevent dissolving, necrosis, and opacification of cornea or conjunctival from happening. As a result, symblepharon would develop. Furthermore, complementary surgery for symblepharon can only be performed during stable stage after injury. That means patients have to wait for 6 months to receive surgery.

In our study, AMT were performed during early stage (recovering stage, one week after burn). Amniotic basement membrane was used as a carrier to promote surrounding normal conjunctival epithelium differentiation, regeneration and migration. No obvious immune response was observed. At the 4th week, conjunctival epithelial layer covers entire transplanted amniotic membrane. Fibrosis was inhibited, and symblepharon was effectively prevented. In our study, AMT was performed during recovering stage instead of acute stage, because AMT in acute stage (within one week after burn) can result in severe inflammation and increased hemorrhaging in burned area. Whereas during recovering stage, traumatized tissue start healing process, transplanted amniotic membrane can facilitate epithelium regeneration, inhibit inflammatory process and fibrosis formation. This animal experiment suggests feasibility and effectiveness of AMT in treating patients with ocular alkali burn during their recovering stage. AMT can help in reducing pain of patients and decrease incident of symblepharon. Patients are able to receive early intervention instead of waiting passively for surgery which can only be performed during stable stage. Possible complications including extraocular muscles and sclera damage caused by symblepharon surgery can also be avoided at the same time. We provides a successful experimental model for clinical application of AMT in patients with ocular alkali burned.

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