

Efficacy of Amniotic Membrane After Short Term Storage

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Purpose: To evaluate the short term viability of amniotic membrane clinically and histopathologically.

Material and Methods: This study was conducted over a period of one year from 1st January to 31st December, 2005 at the Institute of Ophthalmology, King Edward Medical College, Mayo Hospital, Lahore. Amniotic membrane was stored at 4 degrees centigrade in normal saline for 5 days after retrieval and analyzed by a histopathologist before and every day after storage to check for its viability. Amniotic membranes stored in this way were used in 20 patients for various corneal surface disorders and assessed for their clinical efficacy.

Results: Histopathology showed that the basement membrane remained intact for 7 days and the epithelium for 5 days. There were 4 cases of descematocele, 5 cases of corneal ulcers, 2 cases of hypopyon corneal ulcers, one case of perforated corneal melt, 2 cases of bullous keratopathy, one case of chemical burn, 4 cases of pterygia excision and one case of necrosed dermis fat graft. Thirteen cases, including descematocele, corneal ulcers, bullous keratopathy and chemical burn showed improvement of corneal pathology in various ways. Five cases, including pterygia and necrosed dermis fat graft, showed epithelial growth beneath or above the membrane respectively. One case showed failure of the membrane when applied over a perforated corneal melt and another case with hypopyon fungal keratitis failed to show any improvement after amniotic membrane transplantation.

Conclusion: Short term storage of amniotic membrane shows stable clinical and histopathological behavior for the management of corneal surface disorders.

A mniotic membrane is the inner most layer of fetal membranes^{1,2}. It consists of a single layer of epithelial cells that are attached to a thick basement membrane, and an avascular stromal matrix. Its use has been described in persistent epithelial defects, corneal ulcers³, bleb leaks⁴, pterygium excision⁵ and conjunctival reconstruction after tumor removal⁶ or fornix formation⁷. In ophthalmology, it has been used as a graft with its epithelium up when it is expected to become covered

by host conjunctival or corneal epithelium; as a protective patch with its epithelium down, which facilitates trapping of inflammatory cells in the stroma, reducing inflammation; and in a combination of both, one used as a graft and the other as a patch¹. The success of this membrane in all these procedures is due to its anti-inflammatory effect, its antiscarring effect and neurotrophic factors, and its antiangiogenic effect. Amniotic membrane transplant (AMT) is normally stored in eye banks at -70°C and it is

defrosted immediately before use². In Pakistan, where eye banks are still being developed, there is non-availability of frozen AMT. Therefore we developed this method for short term storage of the membrane.

MATERIALS AND METHODS

It was a retrospective interventional case series conducted over a period of one year from 1st January to 31st December, 2005 at the Institute of Ophthalmology, King Edward Medical College, Mayo Hospital, Lahore. Amniotic membrane was stored at 4 degrees centigrade in normal saline for 5 days after retrieval and analyzed by a histopathologist before and every day after storage to check for its viability. Amniotic membranes stored in this way were used in 20 patients for various corneal surface disorders and assessed for their clinical efficacy.

Amniotic membrane was harvested from the placenta of elective caesarean section delivery after taking consent from the patient. The mother was screened for hepatitis B, C and HIV. Under sterile conditions the placenta was washed with normal saline and then a large piece of amniotic membrane was removed. This large piece was then washed with 5% povidone iodine solution and later cut into 8 squares measuring approximately 2.5 cm². Each piece was put in a separate sterile container filled with normal saline and labelled day 0 to day 7. They were then stored in a refrigerator at 4 degree centigrade. Over the next week a specimen of amniotic membrane was sent for histopathology daily from one of the containers according to the labelled day. All the 8 histopathology slides, which had been processed earlier, were reviewed by 2 consultant pathologists at the end of the week.

Amniotic membranes were harvested again in the same fashion over the subsequent months as a large piece measuring approximately 10 cm² and stored in normal saline at 4 degree centigrade for 5 days. The date of retrieval of the membrane was labelled on the container. The necessary amount of the membrane was retrieved when required under aseptic conditions and used for reconstruction of various ocular surface disorders after washing it again in 5% povidone iodine solution. The age of the amniotic membrane on the day of transplantation was noted and recorded in the chart of the patient.

Patients were photographed pre-operatively once and post-operatively daily for the first week and then weekly for the first month. All patients not willing to

receive amniotic membrane were excluded from the study.

RESULTS

Histopathology was done serially every day for 8 days on the stored amniotic membranes (AM) using hematoxylin and eosin (H & E) stains. Examination showed that the epithelium and basement membranes of the first 4 specimens remained intact (fig. 1-3). But the epithelium showed various stages of disintegration in specimens stored for 5 days and more (fig. 4). These changes included, reduction in the size of the cells, loss of the cells, pyknosis of the nuclei and loss of the cytoplasm. The basement membrane remained intact in all the specimens till 7 days (fig. 5).

The amniotic membrane was transplanted in 20 patients for various corneal surface disorders (table 1). The viability of the membrane and its integration with the cornea or conjunctiva was evaluated. There were 4 cases of descematocele, 5 cases of corneal ulcers, 2 cases of hypopyon corneal ulcers, one case of perforated corneal melt, 2 cases of bullous keratopathy, one case of chemical burn, 4 cases of pterygia excision and one case of necrosed dermis fat graft. Fifty percent of the amniotic membranes were 0-1 days old while the rest were 2-5 days old (Table 2). Thirteen cases, including descematocele, corneal ulcers, bullous keratopathy and chemical burn showed improvement of corneal pathology in various ways. Five cases, including pterygia and necrosed dermis fat graft, showed epithelial growth beneath or above the membrane respectively. One case showed failure of the membrane when applied over a perforated corneal melt and another case with hypopyon fungal keratitis failed to show any improvement after amniotic membrane transplantation (Table 1). The AMT remained viable on the ocular surface for 2-3 weeks after grafting. No patient developed any corneal infection due to AMT on follow up.

There were four cases having corneal scarring with central descematocele formation. Three patients had resolved bacterial keratitis and one patient had resolved viral keratitis. Amniotic membrane was used as a single layer epithelial side up graft in such cases after removal of corneal epithelium surrounding the descematocele. Post-operative follow up showed the formation of a thin white membrane over the descematocele.

There were 7 cases having corneal ulcers, out of which 2 had hypopyon. Two cases had fungal

keratitis, 4 cases had bacterial keratitis and one had shield corneal ulcer associated with atopic conjunctivitis. All patients had poor response with topical antibiotics or antifungal. Five patients had localized patches of amniotic membrane applied over the area of corneal ulcer (fig. 6) while two patients had large graft covering the whole of the cornea. All patients were told to continue their topical drops after the amniotic membrane transplantation. The patients with localised bacterial keratitis and shield ulcers responded well (fig. 7) with rapid improvement of the keratitis. Patients with fungal keratitis showed subjective improvement in pain with good response in one case while the other showed no improvement in keratitis in one case. This case also had hypopyon measuring 3 mm.

There was one case having corneal melt with iris prolapse measuring 4 mm in size. Double layered AMT was done but the defect failed to close and needed tectonic graft.

Two cases of bullous keratopathy responded very well to AMT. One patient had inferior bullous keratopathy secondary to insect bite (fig. 8) while the other had bullous keratopathy involving the whole cornea secondary to endothelial decompensation after cataract surgery. Both patients had resolution of symptoms with stabilization of the corneal epithelium (Fig. 9,10).

Four patients underwent amniotic membrane transplant after primary pterygium excision. The amniotic membrane was buried beneath the conjunctiva surrounding the bare sclera left after excision of the pterygium. We found no recurrence after 3 months of follow up.

We used AMT with favourable outcome in one patient with grade III alkali burn as a double layered graft to prevent immediate corneal melt. A case having dermal necrosis following a dermis fat graft was salvaged using AMT. The amniotic membrane gave time to the surrounding conjunctiva to grow beneath the AMT and save the dermis.

DISCUSSION

Amniotic membrane transplant (AMT) is normally stored at -70°C after being placed in a sterile vial containing 10% dimethylsulphoxide (DMSO) medium. The membrane is defrosted immediately before use by warming the container to room temperature for 10 minutes, and rinsed three times in saline². Due to the

non-availability of frozen AMT we developed this method for short term storage of the membrane. It was harvested every week and used as required during the first five days. Thus it was very cost effective and convenient to use.

While developing this technique our main concern was the viability of the membrane during storage. This was confirmed by histology and clinical examination. We found that the epithelium remained viable for 5 days and the basement membrane for 7 days when it was stored at 4 degree C in normal saline. Clinically it was observed that the membrane remained at the transplanted site for 2-3 weeks.

It has been recommended that the donor mother should be screened for hepatitis B, C and HIV at the time of harvesting of the membrane. The membrane should then be stored for at least 6 months after which it should be used only if the donor is still HIV test negative. We could not adhere to this method in our study because the membrane could only be stored for 5 days using our technique. We can only justify this act by comparing it to corneal donations in which the donor is screened only once at the time of retrieval.

Table 1:

Clinical Condition	No. of cases	Clinical Response	Success
Descematocele	4	Formation of thin membrane over it	Y
Corneal Ulcer	5	Promoted healing	Y
Hypopyon Corneal Ulcer	2	Promoted healing in 1 case	50 %
Corneal melt with iris Prolapse	1	Did not stay in place	N
Bullous Keratopathy	2	Adhesion with epithelium	Y
Chemical Burn (Grade III)	1	Prevented corneal melt	Y
Pterygium	4	Epithelial growth above	Y
Dermis Fat Graft	1	Epithelial growth beneath	Y

Table 2:

Age of AM	No. used
0 Day old AM	2 membranes

1 Day old AM	8 membranes
2 Day old AM	1 membrane
3 Day old AM	4 membranes
4 Day old AM	1 membrane
5 Day old AM	4 membranes

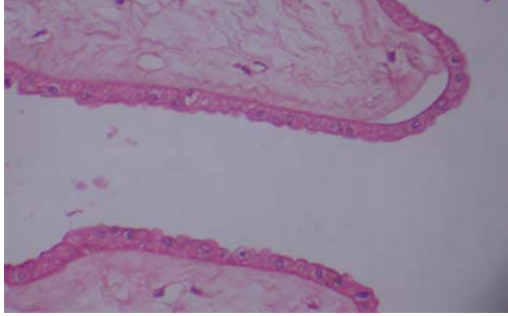


Fig 1. H/E stain of day 1 AMT.

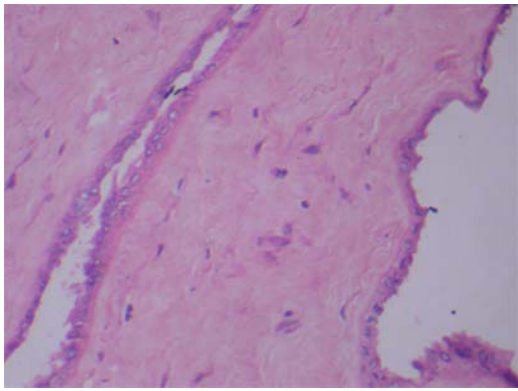


Fig 2. H/E stain of day 2 AMT.

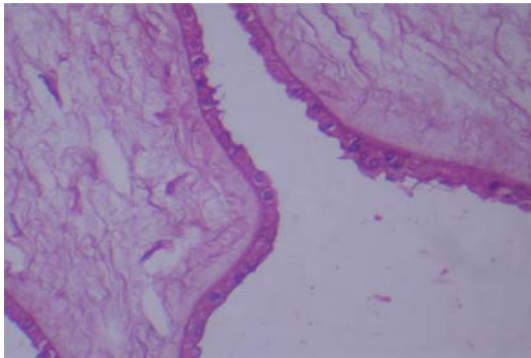


Fig 3. H/E stain day 4 AMT.

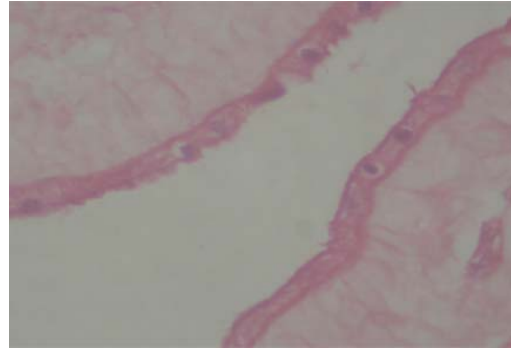
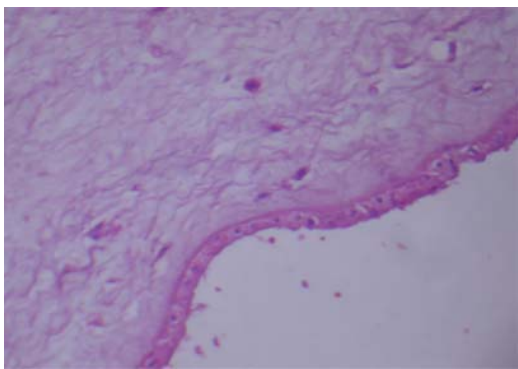


Fig 4. H/E stain of day 5 AMT.

Fig 5. H/E stain of day 7 AMT.

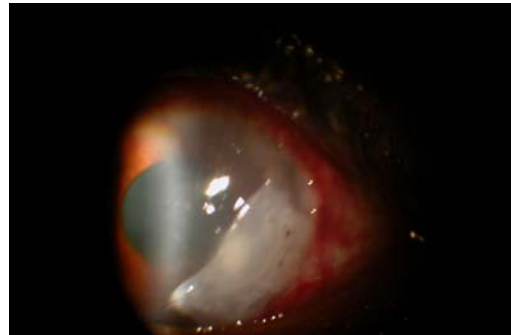
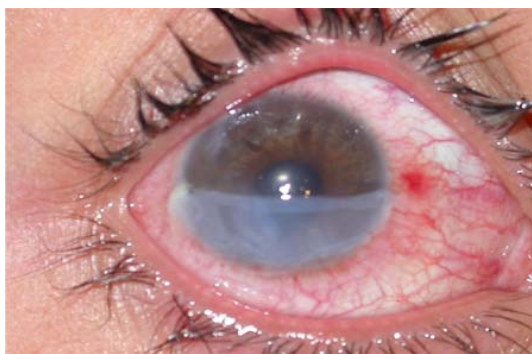
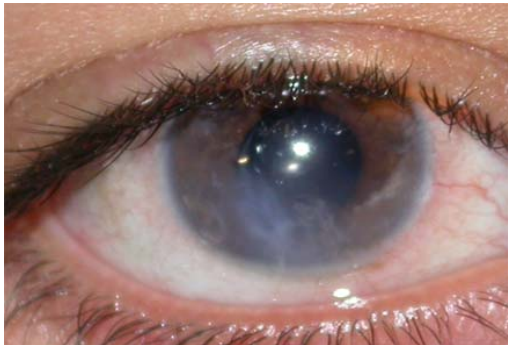


Fig 6. Post op day 1, AMT for corneal ulcer.

Fig 7. Post op 3weeks, AMT for corneal ulcer.



keratopathy (BK) inferiorly.

Fig 9. Post op day 1, AMT for BK.



Fig 8.
Pre op
Bullous

Fig 10. Post op 3 mths, AMT for BK.

Several mechanisms have been proposed to be involved in the selective anti-inflammatory effect^{8,9}. One is the reduction of tissue inflammation by modulation of the production of activin¹⁰. Another mechanism is the presence of lactoferrin and interleukin-1 receptor antagonist on amniotic membrane¹¹. Lactoferrin is an antibacterial protein that exerts an anti-inflammatory effect by serving as an antioxidant and an iron chelator and sequestor¹¹. By contrast, interleukin-1 receptor antagonist is a potent inhibitor of interleukin-1 and thus will suppress the inflammation mediated by interleukin-1¹². Kim et al¹³. reported that the patching of amniotic membrane on epithelial defects of the cornea resulted in rapid epithelialization and decreased infiltration of inflammatory cells by suppressing proteinase and matrix metalloproteinase activation. This evidence may explain some of the clinical effects observed in the use of amniotic membrane for ocular surface reconstruction. We found the anti-inflammatory properties of AMT to be very helpful in treating bacterial and fungal keratitis. All 7 patients except one had marked improvement of keratitis after amniotic membrane transplantation as a patch graft.

A key aspect of the protective function of amniotic membrane in utero is its ability to promote scarless healing^{8,9}. One theory is supported by suppression of transforming growth factor- β signaling in the fetus, therefore inhibiting scar formation¹⁴. Amniotic membrane also supports nerve growth and synthesizes various neurotransmitters, neuropeptides, and neurotrophic factors^{15,16}. The fact that the amniotic membrane contains and produces these neurotrophic factors strongly suggests that it may help the development of the nervous system in the fetus and ensure scarless wound healing.

The fact that the amniotic membrane does not have blood vessels prompts one to hypothesize that the amniotic membrane should hold an antiangiogenic effect¹⁷. Further investigations have shown expression of vascular endothelial growth factor and basic fibroblast growth factor in the amniotic membrane, indicating that it possesses the angiogenic factors like many other vascularized tissues¹⁸⁻²⁰. Apart from these angiogenic factors, a recent study has shown that human amniotic membrane proteins inhibit vascular endothelial cell proliferation while promoting cornea

epithelial cell growth^{8,9}. The latter activity may explain why amniotic membrane transplantation promotes epithelialization and wound healing in the cornea²¹. Amniotic membrane is used for corneal reconstruction, conjunctival reconstruction, and other miscellaneous applications.

In corneal reconstruction, amniotic membrane is used for limbal stem cell deficiency, corneal ulcers and perforations, and bullous keratopathy. Several publications have demonstrated the effects of amniotic membrane in limbal stem cell deficiency^{8,22-27}. Sangwan et al²⁸. reported excellent results with the use of amniotic membrane for partial limbal stem cell deficiency, confirming previous reports by Tseng et al.²³ and Gomes et al²⁶. As for total limbal stem cell deficiency, studies have shown successful outcomes varying between 60% and 70% of the cases²²⁻²⁶. It can also be used as a substrate for expanding limbal epithelial stem cells for subsequent transplantation in the treatment of limbal stem cell deficiency^{8,29-32}.

One or multiple layers of amniotic membrane have been used for the treatment of corneal ulcers^{8,33,34}. Rodríguez-Ares et al³⁵. reported the use of amniotic membrane transplantation in the treatment of corneal perforations of different sizes. The authors found that multilayer amniotic membrane transplantation was successful in 73% of the cases (11/15 cases) and was effective for treating corneal perforations with diameters less than 1.5 mm. Three of the four unsuccessful treatments were of perforations 3 mm or more in diameter. Hick et al.³⁶ proposed the use of amniotic membrane in the management of different types of corneal ulcers by fibrin glue and amniotic membrane and observed an overall success rate of 80% (27/33 eyes). Grafts with fibrin sealant showed a success rate of 92.9% (13/14 eyes) compared with 73.7% (14/19 eyes) for amniotic grafts alone. We had one case with failure of the AMT for a large corneal perforation 4 mm in size. While 4 cases with descematoceles responded quite well to AMT.

Amniotic membrane has been used successfully for the treatment of symptomatic bullous keratopathy^{8,37}. Espana et al.³⁸ evaluated the long-term outcome of epithelial debridement and amniotic membrane transplantation in 18 eyes with symptomatic bullous keratopathy and poor visual potential. Complete corneal epithelial healing occurred in all except one eye. Pain relief was obtained in 88% of patients. The results are similar to those in other series reported in the literature³⁷. Gomes and Dua have used a 9mm trephine to punch out disks of amniotic

membrane and an 8.5mm trephine to mark the area of epithelium to be debrided. With a crescent blade, they make a 360- subepithelial/superficial stromal pocket out of the 8.5mm mark. The amniotic membrane disk is placed on the bed of the de-epithelialized 8.5mm area, and its edge is rolled outward to the subepithelial/superficial stromal pocket and sutured with continuous 10.0 nylon. A bandage contact lens is placed after the procedure³⁹. We used the amniotic membrane as a patch graft after denuding the epithelium in cases of bullous keratopathy and found favorable results.

Amniotic membrane is used for primary and recurrent pterygium, tumors, symblepharon, and other applications. Recent comparative studies have presented controversial results on the use of amniotic membrane for primary and recurrent pterygium^{8,5,40}. Tananuvat and Martin performed a prospective randomized study in Thailand comparing the use of amniotic membrane (39 eyes) with conjunctival autograft (42 eyes) as an adjunctive therapy after surgical excision of primary pterygium. The authors found a much higher rate of recurrence in the amniotic membrane group (40.9%) than in the conjunctival transplantation group (4.76%). This is not the experience reported by most of the other authors^{5,40,39,41}. Interestingly, the recurrences occurred within 3 months in eyes that underwent conjunctival transplantation and as long as 1 year postoperatively in eyes with amniotic membrane transplantation. We did not find any recurrence till 3 months in our cases. To further improve the surgical results for pterygium and decrease its recurrence rate, many authors are trying different combinations of amniotic membrane and other adjunctive treatments. Shimazaki et al.⁴¹ performed a retrospective study of recurrent pterygia that underwent amniotic membrane transplantation combined with either limbal autograft (15 eyes) or conjunctival autograft transplantation (12 eyes). The authors observed a lower recurrence rate with the latter procedure (8.3% compared with 20%), but there was no statistically significant difference. Ma et al.⁴² compared the excision of recurrent pterygia followed by amniotic membrane alone (48 eyes) with amniotic membrane combined with intraoperative 0.025% mitomycin C for 3 minutes (47 eyes). The authors found 12.5% and 12.5% of conjunctival and corneal recurrences for amniotic membrane, respectively, and 8.5% and 12.8% of conjunctival and corneal recurrences for the combination of amniotic membrane with mitomycin C (MMCD), respectively. No

significant difference was found in the conjunctival and corneal recurrence rate between the two groups.

Amniotic membrane transplantation has been used successfully in the treatment of ocular surface neoplasia^{8,6}. Espana et al.⁶ described a series of 16 eyes that underwent excision of large (>20 mm square) ocular surface neoplasia, including conjunctival intraepithelial neoplasia, primary acquired melanosis, and malignant melanoma, that was followed by adjunctive cryotherapy and amniotic membrane transplantation. Complete ocular surface healing occurred in all cases. Tumor recurrence occurred in 1 of 10 cases of conjunctival intraepithelial neoplasia (10%), and no recurrences were observed in the patients with melanotic lesions. More recently, Gunduz et al.⁴³ reported the use of nonpreserved human amniotic membrane for conjunctival reconstruction after excision of 10 ocular surface neoplasias. Over a mean follow-up time of 10 months, all but one eye remained free of tumor recurrence. Treatment complications included partial limbal stem cell deficiency in two eyes and symblepharon formation in one eye.

Solomon et al.⁴⁴ described a success rate of 70.6% (12/17 eyes) with the use of amniotic membrane transplantation for fornix reconstruction in a variety of ocular surface disorders. In cases in which the surrounding host tissue was associated with inflammatory activity, subconjunctival injections of long-acting triamcinolone acetate were given along the edges of the excised conjunctiva. The authors also observed that the underlying cause of a lack of success was either recurrent pterygia or an autoimmune disorder. Jain and Rastogi⁴⁵ reported a recurrence rate in 40% (8/20) of eyes that underwent amniotic membrane transplantation for symblepharon, but in two of these the cicatrization was focal and did not induce functional impairment. Preoperative dry eye and previous conjunctival surgery were important risk factors identified. Besides eyes with severe dry eye, recurrent pterygia seems to present an extremely high potential for the recurrence of symblepharon. More recently, Tseng et al.⁷ reported excellent results with the intraoperative use of mitomycin C and amniotic membrane transplantation for fornix reconstruction in 16 patients with severe cicatricial ocular surface diseases. After a mean follow-up time of 14 months, the authors observed deeper fornix and noninflamed ocular surface in all eyes. Partial motility restriction recurred in 10% of the cases (2 recurrent pterygia and 1 chemical burn). Amniotic membrane transplantation

alone is not sufficient to obtain a successful reconstruction of the conjunctival fornices in cases with active inflammation, recurrent pterygia, and autoimmune diseases. The use of intraoperative long-acting steroids and mitomycin C may improve these results.

Amniotic membrane can be used as an anti-inflammatory patch in the acute phase of chemical and thermal burns and in Stevens-Johnson syndrome^{8,46}. It is reabsorbed after a few days, depending on the degree of the inflammatory process. With its properties, it seems to facilitate corneal surface recovery by reducing corneal and limbal inflammation and restoring the conjunctival surface, which in turn limits symblepharon formation⁴⁶. The amniotic membrane patch, however, may not be enough to treat more severe chemical burns (grades III and IV) with an important ischemic component⁴⁷. We found that amniotic membrane prevented early melting of the cornea and gave more time for the growth of blood vessels from the fornices.

Anderson et al.⁴⁸ successfully used amniotic membrane graft for the treatment of 16 eyes with band keratopathy after removing the calcium deposits; however, this method does not prevent new calcium deposits from forming. By contrast, the stromal side of amniotic membrane seems to attract new deposits of calcium. Amniotic membrane transplantation also can be used successfully to cover large conjunctival defects after resection of the conjunctiva in conjunctivochalasis. Meller et al.⁴⁹ reported good results in 47 eyes with improvement of the symptoms and avoidance of conjunctival cicatricial complications such as symblepharon and motility restriction.

In severe vernal conjunctivitis, amniotic membrane transplantation can be used successfully as a patch or a graft, in the treatment of shield ulcer, or as a tarsal conjunctival substitute after resection of giant papillae⁵⁰. We found AMT to be very successful in treating one case of shield ulcer in our series.

Amniotic membrane also may be used in glaucoma as an adjunct to reduce scarring or to treat conjunctival complications after glaucomatous filtering surgery, such as leaking blebs, or to cover valve implants and scleral or pericardium patches.

We recently reported a case in which amniotic membrane was used successfully to save a dermis fat graft. The patient had developed necrosis of the dermis one week after surgery thereby exposing the

fat which was protected by the amniotic membrane. The surrounding conjunctiva grew beneath the amniotic membrane to resurface the fat⁵¹.

CONCLUSION

Short term storage of amniotic membrane shows stable clinical and histopathological behaviour for the management of corneal surface disorders.

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