

Amniotic Membrane Transplantation for the Treatment of *Pseudomonas* Keratitis in Experimental Rabbits

M Nejabat^{1*}, MB Masoumpour¹, M Eghtedari¹, N Azarpira², MJ Ashraf³, AR Aстане¹

¹Department of Ophthalmology, ²Transplant Research Center, ³Department of Pathology, Poostchi Ophthalmology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background: Amniotic membrane transplantation (AMT) has been considered in combination with medical treatment in progressive infective keratitis. The purpose of this study was to evaluate the efficacy of AMT as an adjunctive treatment in the management of experimental pseudomonas keratitis.

Methods: Cryopreserved AMT was performed on 12 pseudomonas rabbit corneal ulcers. After one week in one group amniotic membrane transplantation combined Poostchi Eye Research Center with topical medical treatment was done (AMT group). At the end of the second week, the clinical and pathological findings were compared with those obtained from eight corneal ulcers in another group which had only been treated with topical medication (control group).

Results: There was not any significant difference in clinical signs between the two groups at the end of the second week. Corneal perforation was found in three cases of the control group but in none of the cases in the AMT group. Amniotic membranes were melted in four eyes, retracted in three eyes and intact in five eyes. Pathologic examination showed no significant difference in cellular infiltration or density of organism between the two groups.

Conclusion: AMT is effective in preventing corneal perforation in the early stage of experimental pseudomonas keratitis.

Keywords: Amniotic membrane; Corneal ulcer; Keratitis; *Pseudomonas*; Transplantation; Rabbit

Introduction

Keratitis caused by *Pseudomonas aeruginosa* often results in severe corneal ulcers and perforation, leading to loss of vision.^{1,2} *Pseudomonas* virulence factors are important contributors to tissue destruction, giving rise to corneal ulceration. These factors include a number of proteases, a heat-labile and heat-stabile hemolysin, phospholipase C, and toxins which are produced by the organism.³⁻⁸ Recent reports indicate that host-derived pathogenic factors are also responsible for corneal lesions during *P. aeruginosa* infection.

In addition, corneal cells have been shown to produce a series of matrix metalloproteinases (MMPs), which are a family of zinc dependent neutral endopeptidases and participate in tissue degradation and remodeling under physiological and pathological conditions.^{9,10}

Despite topical antibiotic treatment in *Pseudomonas* corneal ulcer, the lesion may progress, and eventually, perforation may occur. In these cases, surgical management might be required.¹¹ Recently, amniotic membrane transplantation (AMT) has been considered in combination with medical treatment in progressive cases. AMT facilitates epithelialization and reduces inflammation and scarring.¹² AMT is thought to have antimicrobial properties and some potential to act as an effective drug delivery system.^{11,13} Several studies have described the usage of AMT for the

*Correspondence: Mahmood Nejabat, MD, Associate Professor of Department of Ophthalmology, Poostchi Eye Research Center, Poostchi Street, Shiraz, Iran. Tel: +98-711-2302830, Fax: +98-711-2302830, e-mail: nejabatm@sums.ac.ir
Received: August 8, 2008 Accepted: February 2, 2009

management of deep corneal ulcers,¹² desmatocoles and corneal perforation,³⁻¹⁶ infectious scleral ulcers after prolonged antibiotic treatment,¹⁷ herpetic keratitis,¹⁸ acantamobal,¹⁹ and fungal keratitis.²⁰ Also, some evidence exists on the success of AMT in *Pseudomonas* keratitis.^{21,22}

This study investigates whether AMT can be used as an adjunctive treatment to promote wound healing and prevent perforation in experimental *pseudomonas* keratitis.

Materials and Methods

Human placenta was obtained shortly after an elective caesarean section in a woman who was seronegative for human immunodeficiency virus, hepatitis B, C and syphilis. Under a lamellar flow hood, the placenta was first washed free of blood clots with sterile saline. The inner amniotic membrane was separated from the rest of the chorion by blunt dissection and flattened onto a nitrocellulose membrane (Whatman, Schleicher and Schuell optitaran BA-S 85) with the epithelial surface up. The membrane with the filter was then washed three times with phosphate buffered saline (PBS) containing 50 µg/ml penicillin, 50 µg/ml streptomycin and 2.5 µg/ml amphotericin B. Then, 1.5 × 1.5 cm pieces were made. Each of them was placed in 4%, 8% and 12% Dimethyl Sulphoxide (DMSO) phosphate buffered saline for 5 minutes and finally placed in a sterile vial containing 10% DMSO medium (Cina gen, Iran). Vials were frozen at -80°C. Microbiological tests were performed in the original placenta, in the final membrane preparation as well as in the fluids used for washing and storing the amniotic membrane.

Twenty female, Dutch, albino rabbits weighting 2-3 Kg were enrolled. The rabbits were housed in accordance with the National Institutes of health guidelines, and all procedures in this study conformed to the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Ethics Committee of Shiraz University of Medical Sciences.

Stock cultures of *P. aeruginosa* strain 27853(ATCC) were stored at 4°C on tryptase agar slants. The bacterial cells in log phase were collected by centrifugation at 8000×g for 20 min at 4°C. The bacteria were washed three times with sterile physiological saline and the concentration was adjusted to 1×10⁴cfu/ml, using a standard curve correlating viable cell number to optical density at 600 nm. The

sensitivity of bacteria to ciprofloxacin was proved.

The rabbits were anesthetized with intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg) and then a drop of tetracaine HCL 0.5% was applied to the rabbits' right eye. About 0.05 cc suspension of *pseudomonas* was injected into corneal stroma with a sterile 30 G needle connected to a microsyringe, using an operating microscope. The experimental keratitis was allowed to proceed untreated for 20 hours. Treatment with topical ciprofloxacin (every one hour) was then initiated on the inoculated eyes after examination of the cornea for presence of keratitis by hand light. After 3 days, the frequency of antibiotic therapy decreased to every 2 hours. On the 7th day, after recording the clinical features, the eyes were randomly divided into two groups. AMT was done in one group (AMT group) and the others were considered as controls.

The preserved amniotic membrane was defrosted at room temperature in a sterile condition. After anesthesia, using the same anesthetics as described above, lid speculum was placed, 360° peritomy was done and the membrane was transferred to the recipient eye epithelial side up and secured to the entire corneal surface by eight interrupted 10.0 nylon sutures. After the operation, the use of eye drops was continued similar to that in the control group for the following week.

Four parameters (conjunctival injection, stromal involvement, stromal edema, and formation of hypopyon) on a scale of 0 (none) to 4 (severe) were used as the scoring method to record the clinical examination by biomicroscope. The sum of the scores was used to produce a single score in each eye. Two weeks after infection, the globes were enucleated under general anesthesia. The globes were fixed in 10% formaldehyde, dehydrated with alcohol and embedded in paraffin. The corneal sections (5 µm thick) were stained with hematoxylin and eosin as well as tissue gram stain and prepared for light microscopic studies. The slides were reviewed by two pathologists and the findings were recorded and scaled.

The data were analyzed by Chi square test, Mann-Whitney U test and Fisher exact test. SPSS software (version 13, Chicago, IL, USA) was used. P value of less than 0.05 was considered significant.

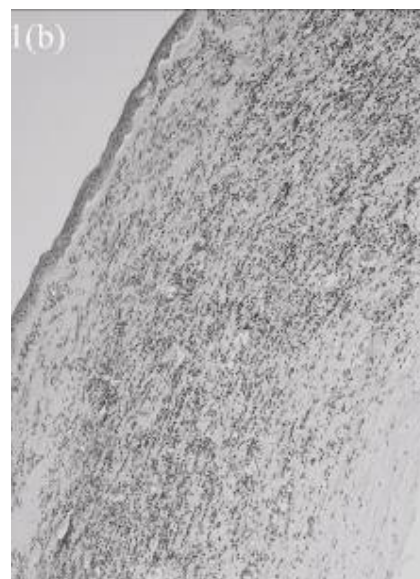
Results

All the rabbit corneas showed an opaque white stromal infiltrate as a ring after 20 hours post-intrastromal

injection of pseudomonas. The infiltration progressively expanded beyond the ring and masked it completely. By the end of the first week, all the corneas developed fulminant ulcers. 12 eyes were in the AMT group and 8 in the control group. After one week, the conjunctiva was markedly hyperemic in both groups. Hypopyon formation was noticed in two eyes in the AMT group and in five eyes in the control group ($p=0.230$). There was not any significant difference in the sum of clinical scores between the two groups at the end of the second week (median score 8.5 (range 5-12) for the AMT group versus 10.5 (range 7-13) for the control group ($p=0.135$).

Corneal perforation was noticed in three cases in the control group but in no case in the AMT group ($p=0.049$). The eyes in the AMT group showed three appearances in the final exam: melted AM in four eyes, retracted in three eyes, and intact in five eyes. Histological examination of the corneas revealed an extensive infiltrate of acute inflammatory cells throughout the entire corneal stroma, without a significant difference between the two groups ($p=0.620$). Figure 1(a & b)

Although the corneas in the control group showed more necrosis and melting, (Figure 2) the pathologic findings are shown in table 1 histological examination showed minute epithelial cell implantation (or even cyst formation) in the corneal stroma in five cases in the AMT group. (Figure 3)



Figs 1a 1b: Integration of epithelium, acute inflammatory cell infiltration and vascularization in stroma. (H&E×100)

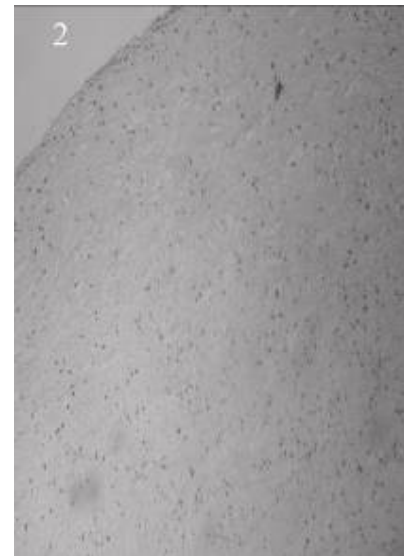


Fig 2: Denudation of epithelium, stromal melting and infiltration of acute inflammatory cells in group 1. (H&E×100)

Discussion

In our study, AMT in the early stage of experimental *Pseudomonas* keratitis was useful in prevention of corneal perforation. However, it developed stromal vascularization and epithelial implantation in the stroma. The bacterial load and the severity of

Table 1: Pathologic findings in AMT group and control group in experimental pseudomonal keratitis.

Finding		AMT group NO. (%)	Control group NO. (%)	P value
Infiltration of inflammatory cell	mild	1 (8.3)	0 (0)	0.620
	moderate	5 (41.6)	6 (75)	
	severe	5 (41.6)	1 (12.5)	
	abscess formation	1 (8.3)	1 (12.5)	
Hypopyon formation		3 (25)	4 (50)	0.250
Vascularization	10 (83.3)	3 (37.5)		

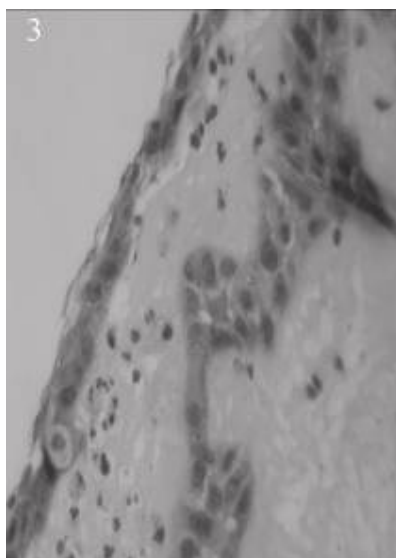


Fig 3: Epithelial cyst formation in the stroma. (H&E×400)

inflammatory cell infiltration in stroma seemed not to be affected.

Pseudomonas keratitis requires immediate aggressive (incisive) treatment to prevent sight threatening complications. Pathogenesis of rapidly progressing corneal disease is multifactorial and depends on both the host-derived and bacterial factors.²³ One of the objectives of treatment in this situation is prevention of tissue destruction and irreversible structural alterations, which may be accomplished with the use of AMT.²⁴

In the early stage of the disease (while the epithelial healing has not been achieved yet), administration of high doses of topical eye drops may lead to epithelial toxicity. So, the AM can be served as a bandage.²² The AM acts as a substrate for epithelial growth in vivo in cases of neurotrophic keratopathy, persistent epithelial defects, and chemical injuries.^{12,25,26} It has also been reported that the amniotic basement membrane facilitates migration of epithelial cells,²⁷ strengthens adhesion of basal epithelial cells, promotes

epithelial differentiation, and prevents apoptosis.²⁸

Also, both AM and amniotic fluid have antimicrobial properties in wound healing.^{13,29-31} Antibacterial effects of amnion have been demonstrated against a wide range of bacteria including hemolytic streptococcus group A, *staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.^{32,33}

In addition, the AM may act as a long term drug delivery system.²⁴ It becomes soaked with antibiotics after the instillation of eye drops. So, it may be useful in reducing the frequency of the standard treatment with topical eye drops.²² Kim *et al.* reported that AMT enhances ofloxacin penetration in the corneas with epithelial defects. It has been shown that ofloxacin level in tears was higher in eyes with AMT up to 1 hour after the topical use.¹¹

The anti-inflammatory and ant-scarring effects of AMT have been described in detail.^{28,34} The AM contains tissue inhibitors of matrix metalloproteinase (MMP)-1 and MMP-2, and thus inhibits the destruction

of collagen in the case of alkali burn.¹² These data suggest that the potential therapeutic effects of AMT for infective keratitis may be mediated in part by the inhibitory effect of AM on proteinase activity including MMP of endogenous and infectious origin. It may also act as a barrier against the infiltration of polymorphonuclear cells from the tear film.³⁵ In addition, the sequestration of infiltrating lymphocytes seems to play a part in the anti-inflammatory effects of the AM.^{36,37} The AM stromal matrix was found to suppress the expression of certain inflammatory cytokines that originate from the ocular surface epithelia including IL-1 α , IL-1 β , IL-2, IL-8, INF- γ and TNF α .¹² Although the ability of preserved membranes to influence wound healing by changing the local milieu of growth factors and cytokines must be very limited,¹³ Koizumi *et al.*,³⁸ found that various growth factors present within preserved AM have the potential to do so.

Resch *et al.* reported that AM can integrate into the host corneal tissue after AMT in intraepithelial, subepithelial or intrastromal patterns, or simply, it may be localized on the corneal surface.³⁹ These findings

are in accordance with our results.

Chen *et al.* reported that AMT may be considered as a method for treating *Pseudomonas* keratitis, especially when stromal melting and loss are extensive. In their case series of six patients, the infection was controlled by using this method.²¹ Gicquol *et al.* reported that early AMT in organism-identified severe bacterial keratitis provides immediate pain relief and allows the use of high doses of topical antibiotics with minimum adverse effects on the epithelium. In addition, the combination of early AMT and early high dose topical application was shown to be safe and did not hinder epithelial healing.²²

Acknowledgment

We wish to thank the Office of Vice Chancellor for Research of Shiraz University of Medical sciences for financial support.

Conflict of interest: None declared.

References

- Hyndiuk RA. Experimental *Pseudomonas* keratitis. *Trans Am Ophthalmol Soc* 1981;**79**:540-624. [6805124]
- Laibson PR. Cornea and sclera. *Arch Ophthalmol* 1972;**88**:553-74. [4343997]
- Miyajima S, Akaie T, Matsumoto K, Okamoto T, Yoshitake J, Hayashida K, Negi A, Maeda H. Metalloproteinases induction by *Pseudomonas* virulence factors and inflammatory cytokines in vitro. *Microb Pathog* 2001;**31**:271-81. [11747375] [doi:10.1006/impat.2001.0470]
- Engel LS, Hill JM, Moreau JM, Green LC, Hobden JA, O'Callaghan RJ. *Pseudomonas aeruginosa* protease IV produces corneal damage and contributes to bacterial virulence. *Invest Ophthalmol Vis Sci* 1998;**39**:662-5. [9501882]
- Hazlett LD, Berk RS, Iglewski BH. Microscopic characterization of ocular damage produced by *Pseudomonas aeruginosa* toxin A. *Infect Immun* 1981;**34**:1025-35. [6800950]
- Kreger AS, Gray LD. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of *Pseudomonas* protease-induced rabbit corneal damage. *Infect Immun* 1978;**19**:630-48. [415981]
- Pillar CM, Hobden JA. *Pseudomonas aeruginosa* exotoxin A and keratitis in mice. *Invest Ophthalmol Vis Sci* 2002;**43**:1437-44. [11980858]
- Manafi A, Kohanteb J, Mehrabani D, Japoni A, Amiri M, Naghmachi M, Hosseinzadeh Zaghi A, Khalili N. Active immunization using exotoxin A confers protection against *Pseudomonas aeruginosa* infection in a mouse burn model. *BMC Microbiology* 2009;**9**:23-27.
- Fini ME, Girard MT. Expression of collagenolytic/gelatinolytic metalloproteinases by normal cornea. *Invest Ophthalmol Vis Sci* 1990;**31**:1779-88. [2170294]
- Fini ME, Yue BY, Sugar J. Collagenolytic/gelatinolytic metalloproteinases in normal and keratoconus corneas. *Curr Eye Res* 1992;**11**:849-62. [1424728] [doi:10.3109/02713689209033483]
- Kim HS, Sah WJ, Kim YJ, Kim JC, Hahn TW. Amniotic membrane, tear film, corneal, and aqueous levels of ofloxacin in rabbit eyes after amniotic membrane transplantation. *Cornea* 2001;**20**:628-34. [11473165] [doi:10.1097/00003226-200108000-00014]
- Solomon A, Meller D, Prabhawat P, John T, Espana EM, Steuhl KP, Tseng SC. Amniotic membrane grafts for nontraumatic corneal perforations, descemetocelles, and deep ulcers. *Ophthalmology* 2002;**109**:694-703. [11927426] [doi:10.1016/S0161-6420(01)01032-6]
- Dua HS, Gomes JA, King AJ, Mahajan VS. The amniotic membrane in ophthalmology. *Surv Ophthalmol* 2004;**49**:51-77. [14711440] [doi:10.1016/j.survophthal.2003.10.004]
- Hanada K, Shimazaki J, Shimamura S, Tsubota K. Multilayered amniotic membrane transplantation for severe ulceration of the cornea and sclera. *Am J Ophthalmol* 2001;**131**:324-31. [11239864] [doi:10.1016/S0002-9394(00)00825-4]
- Su CY, Lin CP. Combined use of an amniotic membrane and tissue adhesive in treating corneal perforation: a case report. *Ophthalmic Surg Lasers* 2000;**31**:151-4. [10743929]
- Duchesne B, Tahi H, Galand A. Use of human fibrin glue and amniotic membrane transplant in corneal perforation. *Cornea* 2001;**20**:230-2. [11248838] [doi:10.1097/00003226-200103000-00027]
- Ma DH, Wang SF, Su WY, Tsai RJ. Amniotic membrane graft for the management of scleral melting and corneal perforation in recalcitrant infectious scleral and corneoscleral ulcers. *Cornea* 2002;**21**:275-83. [11917176] [doi:10.1097/00003226-200204000-00008]

- 18 Heiligenhaus A, Bauer D, Meller D, Steuhl KP, Tseng SC. Improvement of HSV-1 necrotizing keratitis with amniotic membrane transplantation. *Invest Ophthalmol Vis Sci* 2001;**42**:1969-74. [11481259]
- 19 TanHY, Tsai RJ. Amniotic Membrane Transplantation for Acanthamoeba Keratitis. *Invest Ophthalmol Vis Sci* 2004;**45**:E-Abstract 110.
- 20 Chen HC, Ma DH, Huang SC. Amniotic Membrane Transplantation for the Treatment of Acute Fungal Keratitis. *Invest Ophthalmol Vis Sci* 2004;**45**:E-Abstract 4950.
- 21 Chen JH, Ma DH, Tsai RJ. Amniotic membrane transplantation for Pseudomonas keratitis with impending perforation. *Chang Gung Med J* 2002;**25**:144-52. [12022734]
- 22 Gicquel JJ, Bejjani RA, Ellies P, Mercie M, Dighiero P. Amniotic membrane transplantation in severe bacterial keratitis. *Cornea* 2007;**26**:27-33. [17198010] [doi:10.1097/ICO.0b013e31802b28df]
- 23 Kernacki KA, Barrett RP, McClellan S, Hazlett LD. MIP-1alpha regulates CD4+ T cell chemotaxis and indirectly enhances PMN persistence in Pseudomonas aeruginosa corneal infection. *J Leukoc Biol* 2001;**70**:911-9. [11739554]
- 24 Kim JS, Kim JC, Hahn TW, Park WC. Amniotic membrane transplantation in infectious corneal ulcer. *Cornea* 2001;**20**:720-6. [11588424] [doi:10.1097/00003226-200110000-00010]
- 25 Joseph A, Dua HS, King AJ. Failure of amniotic membrane transplantation in the treatment of acute ocular burns. *Br J Ophthalmol* 2001;**85**:1065-9. [11520758] [doi:10.1136/bjo.85.9.1065]
- 26 Kobayashi A, Shirao Y, Yoshita T, Yagami K, Segawa Y, Kawasaki K, Shozu M, Tseng SC. Temporary amniotic membrane patching for acute chemical burns. *Eye* 2003;**17**:149-58. [12640400] [doi:10.1038/sj.eye.6700316]
- 27 Tseng SC. Amniotic membrane transplantation for persistent corneal epithelial defect. *Br J Ophthalmol* 2001;**85**:1400-1401. [11734507] [doi:10.1136/bjo.85.12.1400]
- 28 Wang MX, Gray TB, Park WC, Prabhasawat P, Culbertson W, Forster R, Hanna K, Tseng SC. Reduction in corneal haze and apoptosis by amniotic membrane matrix in excimer laser photoablation in rabbits. *J Cataract Refract Surg* 2001;**27**:310-9. [11226800] [doi:10.1016/S0886-3350(00)00467-3]
- 29 Rao TV, Chandrasekharam V. Use of dry human and bovine amnion as a biological dressing. *Arch Surg* 1981;**116**:891-6. [7259490]
- 30 Robson MC, Krizek TJ. Clinical experiences with amniotic membranes as a temporary biologic dressing. *Conn Med* 1974;**38**:449-51. [4412471]
- 31 Robson MC, Samburg JL, Krizek TJ. Quantitative comparison of biological dressings. *J Surg Res* 1973;**14**:431-4. [4196152] [doi:10.1016/0022-4804(73)90050-4]
- 32 Kjaergaard N, Hein M, Hyttel L, Helmig RB, Schønheyder HC, Ulbjerg N, Madsen H. Antibacterial properties of human amnion and chorion in vitro. *Eur J Obstet Gynecol Reprod Biol* 2001;**94**:224-9. [11165729] [doi:10.1016/S0301-2115(00)00345-6]
- 33 Kjaergaard N, Helmig RB, Schønheyder HC, Ulbjerg N, Hansen ES, Madsen H. Chorioamniotic membranes constitute a competent barrier to group b streptococcus in vitro. *Eur J Obstet Gynecol Reprod Biol* 1999;**83**:165-9. [10391527] [doi:10.1016/S0301-2115(99)00009-3]
- 34 Lambiase A, Sacchetti M, Sgrulletta R, Coassin M, Bonini S. Amniotic membrane transplantation associated with conjunctival peritomy in the management of Mooren's ulcer: a case report. *Eur J Ophthalmol* 2005;**15**:274-6. [15812773]
- 35 Kim JS, Kim JC, Na BK, Jeong JM, Song CY. Amniotic membrane patching promotes healing and inhibits proteinase activity on wound healing following acute corneal alkali burn. *Exp Eye Res* 2000;**70**:329-37. [10712819] [doi:10.1006/exer.1999.0794]
- 36 Shimmura S, Shimazaki J, Ohashi Y, Tsubota K. Antiinflammatory effects of amniotic membrane transplantation in ocular surface disorders. *Cornea* 2001;**20**:408-13. [11333331] [doi:10.1097/00003226-200105000-00015]
- 37 Bauer D, Wasmuth S, Li J, Li H, Steuhl KP, Heiligenhaus A. Improvement of Herpetic Stromal Keratitis by Amniotic Membrane Transplantation: Study on the Influence on Lymphocytes. *Invest Ophthalmol Vis Sci* 2005;**46**:E-Abstract 2788.
- 38 Koizumi NJ, Inatomi TJ, Sotozono CJ, Fullwood NJ, Quantock AJ, Kinoshita S. Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res* 2000;**20**:173-7. [10694891] [doi:10.1076/0271-3683(200003)20:3;1-9;FT173]
- 39 Resch MD, Schlötzer-Schrehardt U, Hofmann-Rummelt C, Sauer R, Kruse FE, Beckmann MW, Seitz B. Integration patterns of cryopreserved amniotic membranes into the human cornea. *Ophthalmology* 2006;**113**:1927-35. [17074561] [doi:10.1016/j.ophtha.2006.03.065]