



## Microscopic corneal epithelial changes and clinical outcomes in simple limbal epithelial transplantation surgery after treatment with amniotic membrane eye drops (AMED): A case report

Erika Bonacci<sup>a</sup>, Raphael Kilian<sup>a,\*</sup>, Clara Rizzo<sup>a</sup>, Alessandra De Gregorio<sup>b</sup>, Francesca Bosello<sup>a</sup>, Adriano Fasolo<sup>a,c</sup>, Diego Ponzin<sup>c</sup>, Giorgio Marchini<sup>a,1</sup>, Emilio Pedrotti<sup>a,1</sup>

<sup>a</sup> Ophthalmic Unit, Department of Neurosciences, Biomedicine, and Movement Sciences, University of Verona, Verona, Italy

<sup>b</sup> Ophthalmic Unit, San Bassiano Hospital, Bassano del Grappa (VI), Italy

<sup>c</sup> The Veneto Eye Bank Foundation, Venice, Italy

### ARTICLE INFO

#### Keywords:

Amniotic membrane  
Amniotic membrane eye drops  
AMED  
AMEED  
Limbal stem cell deficiency  
Simple limbal epithelial transplantation

### ABSTRACT

**Purpose:** To describe the microscopic epithelial changes and the clinical outcomes of a patient treated with amniotic membrane eye drops (AMED) because of a persistent epithelial defect (PED) and a partial limbal stem cell deficiency (LSCD) after simple limbal epithelial transplantation (SLET) and deep anterior lamellar keratoplasty (DALK).

**Observations:** A 72-year-old patient, who had previously undergone SLET and DALK due to a total LSCD, presented with a PED related to a partial LSCD, and was treated with AMED for one month. We evaluated the patient's visual acuity, the Oxford grading scale, the Wong-Baker Pain Rating Scale, and in vivo confocal microscopy, both at baseline and 3 months after the end of treatment. Visual acuity improved from 0.5 to 0.4 LogMAR, the Oxford grading scale changed from grade III to grade I and the Wong-Baker Pain Rating Scale from grade 4 to grade 1. The corneal surface, which initially showed conjunctival characteristics over approximately 50% of the whole area, consisted mainly (75%) of mature corneal epithelium 3 months after the end of treatment.

**Conclusions and importance:** While improving symptoms and clinical characteristics, AMED was also able to restore the normal corneal epithelium's morphology in a case of partial LSCD after SLET and DALK.

### 1. Introduction

The regenerative, anti-inflammatory, anti-angiogenic, and antimicrobial properties of human amniotic membranes (h-AM) have been known for a long time and lately, since the processing techniques have been constantly evolving, their indications as well as their form of administration, have also expanded. Particularly, in recent years amniotic membrane eye drops (AMED), in the form of an homogenate suspension, have been used in the treatment of severe dry eye disease, persistent epithelial defects (PED), limbal stem cell deficiencies (LSCD), neurotrophic keratitis and chemical burns, especially when these conditions were refractory to conventional treatments.<sup>1–3</sup> Even though many studies have shown the effectiveness of these applications, to our knowledge there is no published evidence of the actual microscopic

effects of AMED on the cornea's ultra-structure.

In this study we report on both the clinical and morphological changes that AMED caused in a patient with partial LSCD following a chemical burn that had previously been treated with a simple limbal epithelial transplantation (SLET) and a deep anterior lamellar keratoplasty (DALK).

### 2. Case report

In June 2021, a 72-year-old patient was referred to the Eye Clinic of the University of Verona due to visual loss in his right eye. The patient had previously undergone a successful autologous SLET<sup>4</sup> (September 2017) followed by a DALK (March 2019), for the management of a complete LSCD secondary to a chemical burn. Also, in March 2020 he

\* Corresponding author.

E-mail address: [raphaelkilian8@yahoo.it](mailto:raphaelkilian8@yahoo.it) (R. Kilian).

<sup>1</sup> Prof. Marchini and Prof. Pedrotti contributed equally to this study.

had undergone cataract surgery, achieving a final uncorrected visual acuity (UCVA) of 0.3 LogMAR. All surgeries had been performed at our institution, by the same surgeon (EP).

In June 2021, the UCVA was found to be dropped to 0.5 LogMAR and the biomicroscopic examination revealed two epithelial defects surrounded by zones of late fluorescein staining in the inferior quadrants (Fig. 1). On in vivo confocal microscopy (IVCM-HRT3 RCM, Heidelberg Engineering GmbH), the corneal surface showed areas of inflammation with Langerhans cells and leucocytes, and conjunctival epithelium-like features (i.e., cuboidal or polygonal cells of increased dimensions, visible nuclei, loss of cellular borders, higher cytoplasmic reflectivity compared to the normal corneal epithelium, presence of goblet cells)<sup>5,6</sup> over approximately 50% of the whole surface (Fig. 1). The remaining corneal surface showed a healthy corneal epithelium with a regular basal epithelium.

Also, while no transition zone between the corneal and conjunctival epithelium was noted in 2 out of 4 quadrants (i.e., inferior-nasal and inferior-temporal),<sup>6</sup> the sub-basal nerve plexus showed a reduced corneal nerve density in all the analyzed sectors.

The IVCM was carried out on the central cornea and on four main corneal quadrants (i.e., superior-temporal, inferior-temporal, inferior-nasal and superior-nasal). The exam was performed under topical anesthesia (oxybuprocaine 0.4%) and polyacrylic gel (0.2%) was used as a coupling medium between the cap of the objective lens and the cornea.

After initial evaluation, the patient was given a preservative free topical lubricant q.2h., and a bandage contact lens was put on the eye to allow for a faster epithelial recovery. At the same time, despite the risk of toxicity on the corneal epithelium and possibly on the components of the amniotic tissue themselves, we also administered topical ofloxacin q.i.d., to prevent corneal infection of the non epithelialized areas, until the PED was closed. Despite the patient feeling more comfortable, six months thereafter, slit lamp and IVCM findings were unchanged, still displaying a PED and alterations of the normal epithelial structure. Approximately half of the corneal surface was covered by mature corneal epithelium, whereas the other half had a conjunctival epithelium overlay. At this point, AMED was added for 1 month (q.2h. for 1 week, then q.i.d. for 3 weeks).<sup>7</sup> This eye drop was prepared at the Veneto

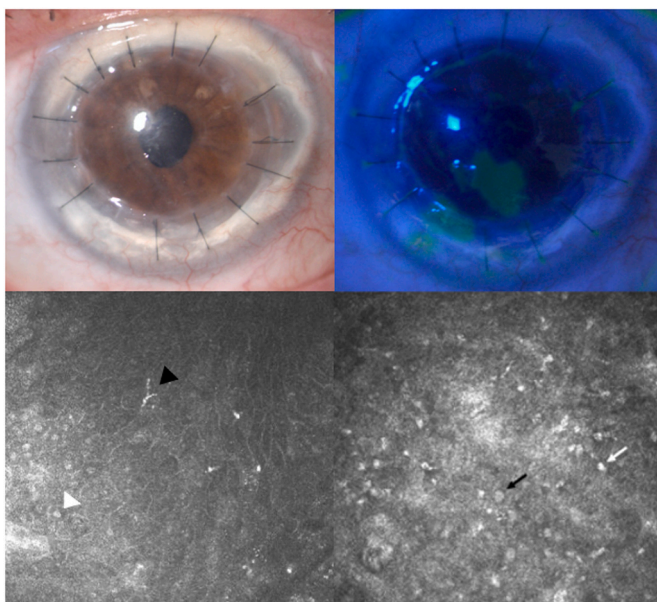
Eye Bank Foundation (Fondazione Banca degli Occhi del Veneto, FBOV, Venice, IT) as described by Castiglia et al.,<sup>8</sup> and contained a minimally manipulated amniotic membrane homogenate to preserve the AM's biological characteristics. Briefly, to prepare the eye drops, the bank selects placentas of donors undergoing elective caesarean delivery at least at 35 weeks of gestation. The AM is then separated from the underlying chorion, rinsed with 0.9% NaCl, immersed in a solution containing vancomycin, meropenem, and gentamicin, and then dissolved in BASE medium (Alchimia, Pordenone, Italy) for one night, at +4 °C. After that, the tissue is cut into fragments and ground in sterile balanced salt solution.

The patient was followed up for 3 months after the end of treatment (i.e., every two weeks for one month, then monthly for two months). The UCVA, biomicroscopic findings (i.e., Oxford grading scale)<sup>9</sup> and the Wong-Baker Pain Rating Scale were recorded at each visit,<sup>10</sup> whereas IVCM was performed at the end of the treatment and at the last follow-up.

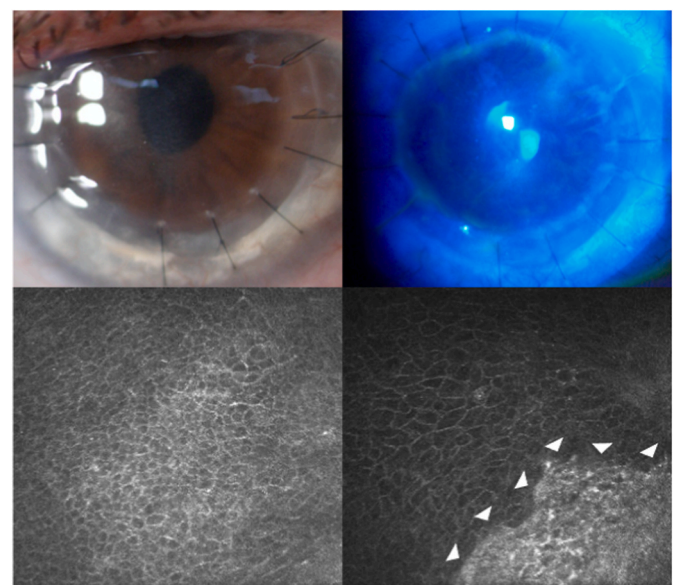
At the end of treatment, the patient's UCVA improved to 0.4 LogMAR (i.e., one line better than before the treatment was started), the PED was healed, the Oxford grading scale for corneal staining shifted from grade III to grade I and the late staining area was reduced in the inferior nasal quadrant (Fig. 2). These parameters remained stable until the last follow-up. The Wong-Baker Pain Rating Scale, on the other hand, changed from grade 4 at baseline, to grade 2 and grade 1, respectively one month and three months after the treatment was ended. Also, at both timepoints, IVCM showed a multilayered mature corneal epithelium with regular basal epithelial cells over approximately 75% of the corneal surface. The transition zone on the other hand, underwent a more gradual recovery. While still being absent in 2 out of 4 quadrants at the end of treatment, at the last follow up visit it showed to be recovered in the inferior-nasal quadrant (Fig. 2). The sub-basal nerve plexus density did not show any sign of improvement either at the end of treatment or at the last follow-up.

### 3. Discussion

The known beneficial effects of h-AMs have been attributed to the rich molecular milieu they are able to express. Indeed, h-AMs are a source of molecules such as fibronectin, hepatocyte growth factor, epidermal growth factor, basic fibroblast growth factor, transforming



**Fig. 1.** Slit lamp view (upper two images) and IVCM (images at the bottom) of the affected eye before starting the treatment. Confocal microscopy showed nucleated cells with high cytoplasmic reflectivity (white arrowhead), Langerhans cells (black arrowhead), goblet cells (black arrow) and leucocytes (white arrow).



**Fig. 2.** Slit lamp view (upper two images) and IVCM (images at the bottom) of the affected eye 3 months after the end of treatment in the inferior-nasal quadrant (transition zone – arrowheads).

growth factor, and collagen types I, III, IV, and V, all of which are important elements for corneal epithelial proliferation and migration.<sup>11</sup>

There are several ophthalmologic indications for the use of a h-AM, including PED, fornix reconstruction, chemical burns, and Stevens-Johnson's syndrome.<sup>12</sup> Also, due to their ability at inducing limbal stem cell (LSC) migration, inhibiting cellular apoptosis, and at maintaining epithelial progenitor cells within the LSC niche, recent studies have identified h-AMs as useful scaffolds to be applied to the field of corneal tissue engineering.<sup>6,13–15</sup>

In the clinical setting AMs are mostly used in the form of a h-AM transplantation (AMT), which however has several pitfalls. These include the risk of viral infections, difficulties in AM manipulation, long surgical time, risk of complications such as granuloma formation and papillary conjunctivitis, patient discomfort and high costs.<sup>16,17</sup> To overcome all the above, several groups of study have researched on the use of alternative forms of AM-administration, such as homogenates.<sup>18,19</sup>

The ability of AMED to promote LSC proliferation has already been demonstrated, both in vitro and in vivo.<sup>7</sup> As such, in recent years AMED has been shown to be effective in three of the largest categories of ocular surface disease, i.e., dry eye disease, wound healing delay, and LSCD. However, to our knowledge, this is the first study to report on the microscopic changes following the treatment with AMED in a case of partial LSCD. Particularly, AMED was effective both at resolving the PED and at restoring the normal corneal epithelium's morphology and its distribution across most of the corneal surface. Indeed, despite our goal being just to recover the PED, it seems like these eye drops were also able to help in the management of partial LSCD, sustaining the prevalence of normal corneal epithelium over the conjunctival one. This result was surprising since we did not mechanically debride the conjunctival epithelium off the corneal surface before starting the treatment with AMED.<sup>20</sup> We speculate that these eye drops were able to enhance the expansion of LSC in such a way that corneal turnover prevailed against the conjunctival one, leading to a repopulation of the corneal surface by the corneal epithelium itself. Whether the adjacent transition zone, some islands of limbal explant-derived cells within the conjunctival overgrowth, or even corneal stromal stem cells (CSC),<sup>21</sup> were the source of the regenerated corneal epithelium, remains unclear. However, we suppose the conjunctival epithelium growing over corneal surface must have been at an immature state or mixed with limbal explant-derived corneal cells,<sup>20</sup> otherwise it would have been difficult for the remaining LSC to prevail.

The results from the Pain Rating Scale, which got better even after the end of treatment, and the clinical findings (i.e., Oxford grading scale and PED healing), that remained stable over the course of the follow-ups, suggest the effects of AMED on the corneal surface are long standing. This goes in hand with the microscopic evolution of the transition zone, which was found to be expanded between the end of the treatment and the last follow-up. Probably these findings are related to AMED's ability at restoring a healthy molecular microenvironment on the ocular surface.

Interestingly, Baradaran-Rafii et al. showed the treatment with AMED to be helpful for in vivo cultivation of LSCs in cases of SLETs.<sup>22</sup> This is not surprising, since AMT was already known for its beneficial effects in LSCD cases.<sup>23,24</sup> Nonetheless, the true effectiveness of AMED on LSCD has yet to be proven in appropriate clinical settings.<sup>25</sup>

There are studies in the literature, both in vivo and in vitro, that support the use of autologous serum in cases of LSCD.<sup>26,27</sup> Indeed, although literature currently does not agree with the use of an autologous serum eye drop rather than AMED in the management of LSCD,<sup>23,24,28</sup> we feel like this could be a valid alternative to the use of AMED. However, because the amniotic membrane is the actual substrate for in vivo stem cell expansion during SLET, we believe that the AMED might be more specific as first line treatment in this case. Nonetheless, since to our knowledge no comparative study has been performed yet, we strongly support future research in this field.

Despite not having used impression cytology to confirm our results, a high degree of concordance between the findings obtained with that technique and IVCN has already been demonstrated.<sup>5</sup> If AMED truly resulted to have similar effectiveness to AMT, it could become the preferred therapeutic option for many corneal affections where the integrity of the eye is not threatened (e.g., deep corneal ulcers).

#### 4. Conclusion

While being able to improve symptoms and clinical characteristics of a patient with initial corneal conjunctivalization after SLET and DALK, AMED was also able to restore the normal corneal epithelium morphology. The exact clinical role these eye drops have on LSCs needs to be confirmed by further large prospective randomized studies.

#### Patient consent

The patient's informed consent was obtained in order for us to use his data for research purposes.

#### Authorship

All authors attest that they meet the current ICMJE criteria for Authorship.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgements

None.

#### References

- Bonci PO, Bonci PA, Lia A. Suspension made with amniotic membrane: clinical trial. *Eur J Ophthalmol.* 2005 Jul-Aug;15(4):441–445. <https://doi.org/10.5301/EJO.2008.2218>. PMID: 28221412.
- Yeu E, Goldberg DF, Mah FS, et al. Safety and efficacy of amniotic cytokine extract in the treatment of dry eye disease. *Clin Ophthalmol.* 2019 May 27;13:887–894. <https://doi.org/10.2147/OPTH.S203510>. PMID: 31213759; PMCID: PMC6549774.
- Liang L, Li W, Ling S, et al. Amniotic membrane extraction solution for ocular chemical burns. *Clin Exp Ophthalmol.* 2009 Dec;37(9):855–863. <https://doi.org/10.1111/j.1442-9071.2009.02159.x>. PMID: 20092594.
- Sangwan VS, Basu S, MacNeil S, Balasubramanian D. Simple limbal epithelial transplantation (SLET): a novel surgical technique for the treatment of unilateral limbal stem cell deficiency. *Br J Ophthalmol.* 2012 Jul;96(7):931–934. <https://doi.org/10.1136/bjophthalmol-2011-301164>. Epub 2012 Feb 10. PMID: 22328817.
- Nubile M, Lanzini M, Miri A, et al. In vivo confocal microscopy in diagnosis of limbal stem cell deficiency. *Am J Ophthalmol.* 2013 Feb;155(2):220–232. <https://doi.org/10.1016/j.ajo.2012.08.017>. Epub 2012 Nov 3. PMID: 23127748.
- Pedrotti E, Chierigo C, Cozzini T, et al. In vivo confocal microscopy of the corneal-conjunctival transition in the evaluation of epithelial renewal after SLET. *J Clin Med.* 2020 Nov 6;9(11):3574. <https://doi.org/10.3390/jcm9113574>. PMID: 33171960; PMCID: PMC7694659.
- Shayan Asl N, Nejat F, Mohammadi P, et al. Amniotic membrane extract eye drop promotes limbal stem cell proliferation and corneal epithelium healing. *Cell J.* 2019 Jan;20(4):459–468. <https://doi.org/10.22074/cellj.2019.5423>. Epub 2018 Aug 1. PMID: 30123991; PMCID: PMC6099140.
- Castiglia D, Fortugno P, Condorelli AG, et al. A novel phenotype of junctional epidermolysis bullosa with transient skin fragility and predominant ocular involvement responsive to human amniotic membrane eyedrops. *Genes.* 2021 May 11;12(5):716. <https://doi.org/10.3390/genes12050716>. PMID: 34064633; PMCID: PMC8151857.
- Bron AJ, Evans VE, Smith JA. Grading of corneal and conjunctival staining in the context of other dry eye tests. *Cornea.* 2003 Oct;22(7):640–650. <https://doi.org/10.1097/00003226-200310000-00008>. PMID: 14508260.
- Foundation W-BF. *Wong-Baker FACES Foundation.* 2015.
- Dudok DV, Nagdee I, Cheung K, et al. Effects of amniotic membrane extract on primary human corneal epithelial and limbal cells. *Clin Exp Ophthalmol.* 2015 Jul;43(5):443–448. <https://doi.org/10.1111/ceo.12480>. Epub 2015 Jan 15. PMID: 25495256.

12. Suri K, Kosker M, Raber IM, et al. Sutureless amniotic membrane ProKera for ocular surface disorders: short-term results. *Eye Contact Lens*. 2013 Sep;39(5):341–347. <https://doi.org/10.1097/ICL.0b013e3182a2f8fa>. PMID: 23945524.
13. Deihim T, Yazdanpanah G, Niknejad H. Different light transmittance of placental and reflected regions of human amniotic membrane that could be crucial for corneal tissue engineering. *Cornea*. 2016 Jul;35(7):997–1003. <https://doi.org/10.1097/ICO.0000000000000867>. PMID: 27149533.
14. Malhotra C, Jain AK. Human amniotic membrane transplantation: different modalities of its use in ophthalmology. *World J Transplant*. 2014 Jun 24;4(2):111–121. <https://doi.org/10.5500/wjt.v4.i2.111>. PMID: 25032100; PMCID: PMC4094946.
15. Dadkhah Tehrani F, Firouzeh A, Shabani I, Shabani A. A review on modifications of amniotic membrane for biomedical applications. *Front Bioeng Biotechnol*. 2021 Jan 13;8, 606982. <https://doi.org/10.3389/fbioe.2020.606982>. PMID: 33520961; PMCID: PMC7839407.
16. Zhao Y, Ma L. Systematic review and meta-analysis on transplantation of ex vivo cultivated limbal epithelial stem cell on amniotic membrane in limbal stem cell deficiency. *Cornea*. 2015 May;34(5):592–600. <https://doi.org/10.1097/ICO.0000000000000398>. PMID: 25789694.
17. Guo Q, Hao J, Yang Q, Guan L, Ouyang S, Wang J. A comparison of the effectiveness between amniotic membrane homogenate and transplanted amniotic membrane in healing corneal damage in a rabbit model. *Acta Ophthalmol*. 2011 Jun;89(4):e315–e319. <https://doi.org/10.1111/j.1755-3768.2010.02097.x>. Epub 2011 Feb 11. PMID: 21310014.
18. Choi JA, Jin HJ, Jung S, et al. Effects of amniotic membrane suspension in human corneal wound healing in vitro. *Mol Vis*. 2009 Nov 5;15:2230–2238. PMID: 19907665; PMCID: PMC2774451.
19. Jiang A, Li C, Gao Y, et al. In vivo and in vitro inhibitory effect of amniotic extraction on neovascularization. *Cornea*. 2006 Dec;25(10 Suppl 1):S36–S40. <https://doi.org/10.1097/01.ic0.0000247211.78391.af>. PMID: 17001191.
20. Dua HS. Sequential sectoral conjunctival epitheliectomy (SSCE). In: *Ocular Surface Disease Medical and Surgical Management*. New York, NY: Springer; 2002. [https://doi.org/10.1007/0-387-21570-0\\_14](https://doi.org/10.1007/0-387-21570-0_14).
21. Hashmani K, Branch MJ, Sidney LE, et al. Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation. *Stem Cell Res Ther*. 2013 Jun 24;4(3):75. <https://doi.org/10.1186/scrt226>. PMID: 23800436; PMCID: PMC4058700.
22. Baradaran-Rafii A, Asl NS, Ebrahimi M, et al. The role of amniotic membrane extract eye drop (AMEED) in in vivo cultivation of limbal stem cells. *Ocul Surf*. 2018 Jan;16(1):146–153. <https://doi.org/10.1016/j.jtos.2017.11.001>. Epub 2017 Nov 8. PMID: 29104070.
23. Sridhar MS, Bansal AK, Sangwan VS, Rao GN. Amniotic membrane transplantation in acute chemical and thermal injury. *Am J Ophthalmol*. 2000 Jul;130(1):134–137. [https://doi.org/10.1016/s0002-9394\(00\)00500-6](https://doi.org/10.1016/s0002-9394(00)00500-6). PMID: 11004281.
24. Kobayashi A, Shirao Y, Yoshita T, et al. Temporary amniotic membrane patching for acute chemical burns. *Eye*. 2003 Mar;17(2):149–158. <https://doi.org/10.1038/sj.eye.6700316>. PMID: 12640400.
25. Amin S, Jalilian E, Katz E, et al. The limbal niche and regenerative strategies. *Vision*. 2021 Sep 22;5(4):43. <https://doi.org/10.3390/vision5040043>. PMID: 34698278; PMCID: PMC8544688.
26. Yeh SI, Chu TW, Cheng HC, Wu CH, Tsao YP. The use of autologous serum to reverse severe contact lens-induced limbal stem cell deficiency. *Cornea*. 2020 Jun;39(6):736–741. <https://doi.org/10.1097/ICO.0000000000002264>. PMID: 31985518.
27. Wu MF, Stachon T, Seitz B, Langenbacher A, Szentmáry N. Effect of human autologous serum and fetal bovine serum on human corneal epithelial cell viability, migration and proliferation in vitro. *Int J Ophthalmol*. 2017 Jun 18;10(6):908–913. <https://doi.org/10.18240/ijo.2017.06.12>. PMID: 28730081; PMCID: PMC5515161.
28. Lekhanont K, Jongkhajornpong P, Anothaisintawee T, Chuckpaiwong V. Undiluted serum eye drops for the treatment of persistent corneal epithelial defects. *Sci Rep*. 2016 Dec 2;6, 38143. <https://doi.org/10.1038/srep38143>. PMID: 27909310; PMCID: PMC5133461.