Molecular Vision 2024; 30:188-199 <http://www.molvis.org/molvis/v30/188>
Received 22 May 2023 | Accepted 26 March 2024 | Published 28 March 2024

Clinically correlated dose of the amniotic membrane extract is superior to its transplantation in corneal wound healing

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Purpose: This study investigates the superiority of sterile lyophilized amniotic membrane extract (AME) prepared at a clinically correlated dose over amniotic membrane transplantation (AMT) in an experimental corneal wound model.
Methods: AME was prepared from a pool of five amniotic membranes. After homogenizing the membranes, they were lyophilized and sterilized by gamma radiation to obtain sterile, lyophilized AME powder. The total protein amount and growth factor levels were measured in the AME samples. AME eye drops were prepared considering the protein concentration of the standard-size amniotic membrane weight used for transplantation, and this total amount was used as the daily dose. For the experimental animal corneal wound model, a full-thickness mechanical corneal epithelial defect was created in 15 eyes of 15 New Zealand rabbits. The rabbits were divided into four groups: Group 1: AME eye drop (n = 4 eyes), Group 2: AMT (n = 4 eyes), Group 3: preservation-free artificial tear (n = 4 eyes), and Group 4: control (n = 3 eyes). Daily anterior segment evaluation and photography were performed to determine the clinical efficacy of the AME. The rabbits were euthanized on day 7, and wound healing was examined histopathologically.
Results: The total protein amount in the AME was 0.149 ± 0.01 mg/ml. The growth factor levels were as follows: EGF = 41.19, FGF = 43.11, HGF = 203.67, KGF = 328.03, NGF = 207.92, and TGF-β = 506.93 pg/ml AME. On clinical examination, the mean wound closure times in Groups 1, 3, and 4 were 2.75 ± 0.50 (2–3), 3.5 ± 1.0 (3–5), and 3.33 ± 1.52 (2–5) days, respectively ($p > 0.05$). Histopathological examination revealed Group 1 corneal epithelium with full thickness, regular healing pattern, and normal anterior stromal keratocytes. In the remaining three groups, there were interruptions in epithelial healing, and loss of anterior stromal keratocytes was evident. Inflammation was more prominent in Group 2.
Conclusions: AME is a liquid product that contains the essence of the amniotic membrane after homogenization and centrifugation. AME has the potential to overcome the disadvantages of AMT, such as surgery requirement and the limitation of postoperative objective clinical observation due to the semi-opaque nature of the amniotic membrane. Although, there are studies showing the advantages of AME over AMT in the literature, the preparation, preservation and sterilization of AME are still controversial. This study is specifically addressing the shortcomings of acquiring AME in the literature, such as minimizing inter-donor variability in AME by pooling amniotic membranes from different donors, lyophilizing AME to preserve its biochemical composition, and preventing infection transmission by using gamma sterilization. Herein, we observed that the AME prepared with this method contains high concentrations of growth factors. In the present study, the dose of AME was correlated with clinical use for the first time, and for the first time, the superiority of sterile lyophilized AME over AMT was clinically demonstrated in a corneal wound model. Furthermore, histopathological findings confirmed that AME seems to not only promote epithelial proliferation during wound healing but also prevent stromal keratocyte loss, inhibit inflammation and accelerate collagen remodeling.

The human amniotic membrane (HAM), the inner layer of the placenta, has anti-inflammatory, anti-fibrotic, and anti-angiogenic activities and stimulates epithelial cell proliferation. HAM has been used for decades in a variety of ocular surface diseases to promote corneal wound healing [1,2]. These therapeutic effects have been largely attributed to the biochemical composition of HAM, which is rich in growth factors [3,4].

Amniotic membrane transplantation (AMT) is one of the most common methods used in ophthalmology practice, and cryopreserved HAM is routinely used for this technique [5-7]. However, AMT has limitations, such as the risk of infection transmission, inter- and intra-donor variations, the requirement of surgical procedure, premature degradation or dislocation of the membrane requiring repeated transplantations, and residual membrane in the visual axis. Further, HAM obscures

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anterior segment details due to its semi-opaque nature, and the clinical response of the tissue underneath cannot be evaluated optimally during treatment [3,8,9].

Amniotic membrane extract (AME) and amniotic membrane extract eye drop (AMEED) have emerged as amniotic membrane-derived alternatives that could potentially overcome the aforementioned disadvantages of AMT [10]. In recent years, numerous studies have demonstrated the preparation, preservation, and biochemical analysis of AMEED [11,12]. Some studies have focused on improving the optimal preservation and sterilization methodologies of AMEED [10,13,14]. AMEED has been clinically shown to promote corneal epithelial healing and suppress inflammation. Although its clinical efficacy has been investigated, strong data on the effects of AMEED on the corneal epithelium and stroma during wound healing are lacking [15,16]. Further, there is no consensus on the application dose of AMEED in clinical use, and dose adjustment is generally made without an evidence-based procedure of protein content amount.

However, modifying the AMEED preparation method can significantly affect its biochemical composition and thus its clinical efficacy. To the best of our knowledge, there are no published data demonstrating the clinical efficacy of lyophilized and gamma-sterilized AMEED in a corneal wound model. The aim of this study was to demonstrate the clinical and histopathological superiority of sterile lyophilized AME over AMT in an experimental corneal wound model.

METHODS

The study was performed at the Ege University Department of Ophthalmology, with contributions from Medical Biochemistry, Medical Pathology, and Pharmaceutical Technology Departments. Ethical approval was obtained from the Ege University Medical Faculty Medical Research Ethics Committee (18–12T/27) and the Ege University Animal Experiments Local Ethics Committee (2021–004). This experimental study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practices Guideline, relevant legislation provisions, and the "Principles of Laboratory Animal Care" by the National Institute of Health. The placenta donors were informed about the study, and written informed consent was provided by all of them. During the experiment, the animals were individually housed under standard, controlled conditions and freely accessed food and water.

Procurement and preparation of amniotic membrane extract: Five placentas from five healthy pregnant women who were seronegative for hepatitis B, hepatitis C, HIV-1, HIV-2, and syphilis were obtained during elective cesarean delivery. Amniotic membranes were stripped from the placentas and separated from the chorion by blunt dissection under sterile conditions. They were washed 3 times for 15 min with phosphate buffered saline (PBS) solution containing 50 µg/ml of penicillin, 50 µg/ml of streptomycin, 100 µg/ml of neomycin and 2.5 µg/ml of amphotericin B to remove blood clots. These five amniotic membranes were collected in a pool to minimize donor-induced variability in the biochemical composition of the amniotic membranes and to obtain an extract with standard content. All procedures were performed under sterile conditions at neutral pH, and the amniotic membranes were protected from direct light. The amniotic membranes were cut into small pieces of 2 × 2 cm^2 and immersed in liquid nitrogen. The membranes were then ground into powder with a mortar and pestle manually at 4 °C. PBS was added at a ratio of 1:1, the mixture was homogenized with a probe sonicator (Bandelin Sonopuls HD 2070; Bandelin Electronic; Berlin, Germany), and the homogenate was centrifuged at 5250 ×g for 30 min at 4 °C using a benchtop centrifuge (Allegra X-15R, SX4750A rotor; Beckman Coulter, Inc., Brea, Canada).

The supernatant (extract) was collected, aliquoted into 0.5 ml vials, and frozen by immersion in liquid nitrogen. Supernatants were lyophilized for the long-term preservation of their biologic activity. The lyophilized AME was sterilized by 25 kGy gamma radiation and stored at 4 °C until further use. The lyophilized AME was reconstituted with PBS before use to obtain a liquid eye drop (Figure 1).

Biochemical analysis of the amniotic membrane extract: The concentration of growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), keratinocyte growth factor (KGF), nerve growth factor (NGF) and transforming growth factor-beta (TGF-β) was measured by enzyme-linked immunosorbent assay (ELISA) method. The proportion of each growth factor in the AME was calculated as pg/ml.

Corneal wound healing in a rabbit model: Sixteen adult (male or female) New Zealand rabbits weighing 1.5–2.0 kg were included in the study. During the pre-experimental care period, one rabbit was excluded due to unexplained death. All rabbits were anesthetized with a combination of intramuscular 35–50 mg/kg ketamine hydrochloride (Ketalar®, Pfizer, Turkey) and 5–10 mg/kg xylazine hydrochloride (Xylazin Bio 2%, Bioveta, Czech Republic). To ensure that the rabbits met their physiologic needs during the experimental period, an experimental corneal wound model was created in only one eye (the right eye for each) of the remaining 15 rabbits. Following topical application of proparacain hydrochloride (Alcaine®, Alcon, USA), a 6.5 mm diameter trephine was

Figure 1. Lyophilized AME (A) and AME eye drops reconstituted with PBS (B). Abbreviations: AME, amniotic membrane extract; PBS, phosphate buffered saline.

used to outline a circular area in the central cornea. A corneal wound was created by mechanically removing the corneal epithelium in this circular area of the right eye of each rabbit with the aid of a sponge (Figure 2).

The 15 rabbits with corneal wounds in their right eyes were randomly grouped into three treatment groups (Group 1, n = 4 eyes: AME eye drop; Group 2, n = 4 eyes: AMT; and Group 3, n = 4 eyes: preservative-free artificial tear) and one control group (Group 4, n = 3 eyes). Group 1 received topical AME eye drops 4 times a day (at 08:00, 12:00, 16:00, and 20:00). For the daily dose of AME eye drops, the amount was adjusted according to the protein concentration in the 2 × 2 cm^2 amniotic membrane, which is used for amniotic membrane transplantation in clinical practice. AMT surgery was performed in Group 2. Cryopreserved amniotic

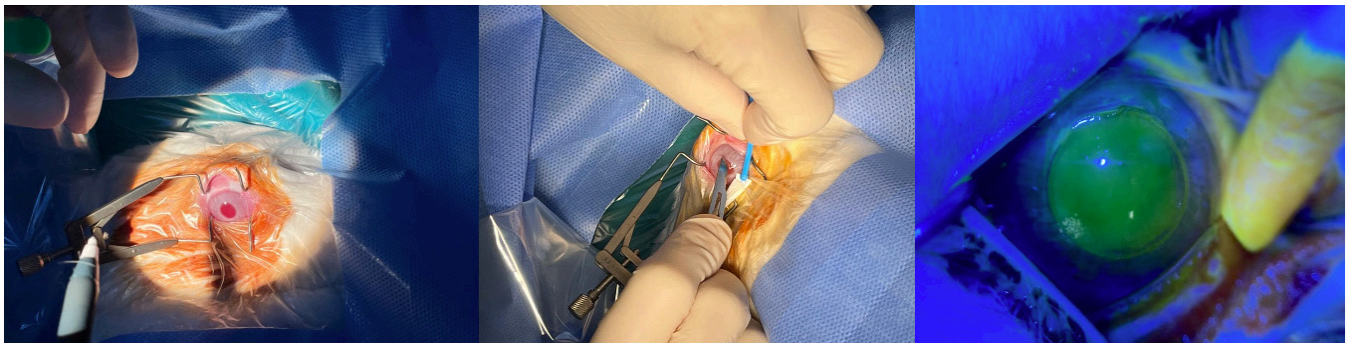

Figure 2. Creation of the corneal wound model by mechanically removing the corneal epithelium.

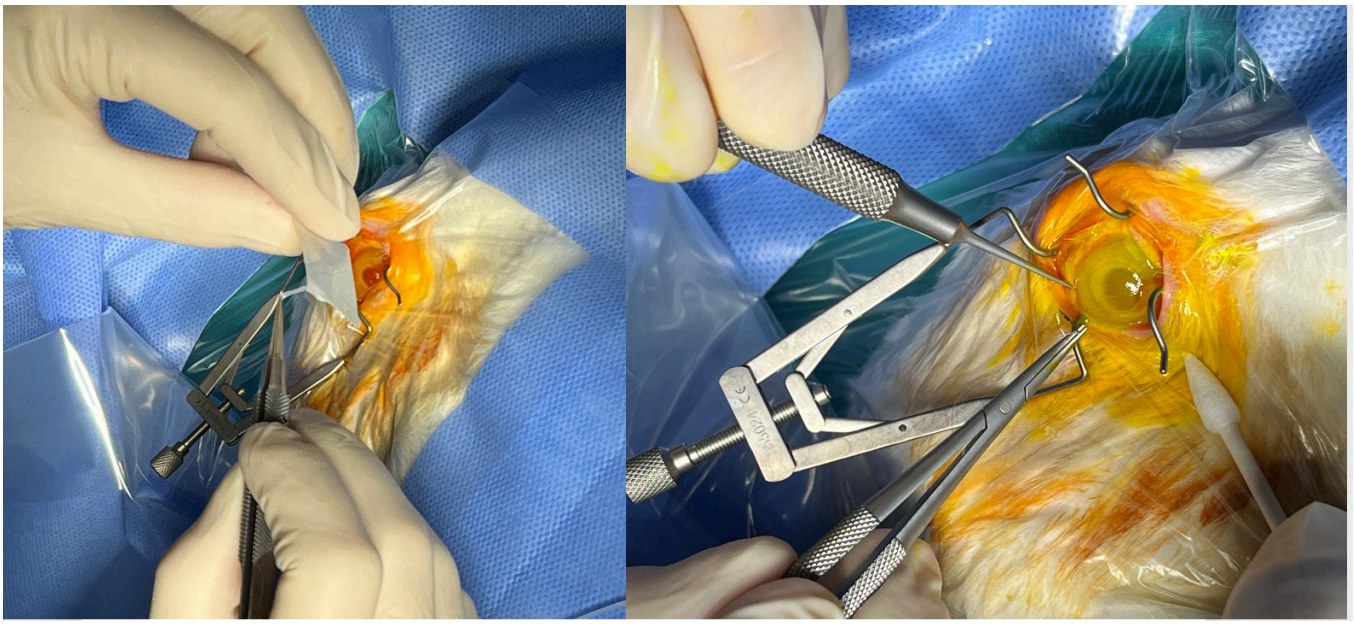

Figure 3. Amniotic membrane transplantation.

membranes were prepared using the technique previously described by Kim and Tseng [2]. A 2 × 2 cm^2 sized cryopreserved amniotic membrane was sutured to the ocular surface with 8/0 vicryl, covering the entire corneal surface with the epithelial side up (Figure 3). Group 3 was treated with one drop of preservative-free artificial tears (Eyestil 0.15% single dose, Sifi, Italy) 4 times a day. Preservative-free topical antibiotics (Netilmicin, Netira single dose, Sifi, Italy) were administered to all groups 4 times a day to prevent secondary infections. Group 4, the control group, received only topical antibiotics.

Clinical outcome measurements: The anterior segments of all eyes were evaluated daily for 7 days and were photographed at ×20 magnification using the smartphone-integrated Smart Eye Camera (SEC, Qui Inc., Japan). The Matlab 2022 program was used for the image analysis. The corneal epithelial growth rate and total re-epithelialization time were recorded. Anterior segment photographs of fluorescein-stained corneas were taken under a cobalt blue light filter. The day the corneal wound model was created was labeled "day 0." The fluorescein-stained area was taken as the reference at day 0 and was considered 100% for each rabbit. Then, for each rabbit, the fluorescein-stained area was calculated daily and compared with the reference image to analyze the daily rate of corneal epithelial growth in percent. Given that the semi-opaque amniotic membranes obscured the corneal details, epithelial defect measurements in Group 2 (AMT) could only be performed after the amniotic membranes were removed (day 7).

Histopathological assessment: On day 7, the rabbits in all groups were euthanized using a high-dose anesthetic. The corneas were dissected, fixed in 10% formaldehyde, and embedded in paraffin. Five-micron-thick sections were cut and stained with hematoxylin & eosin (H&E). The corneas were examined semiquantitatively for wound healing under light microscopy.

The number of newly formed epithelial layers, epithelial cell polarity, epithelial cell arrangement, degree of keratocyte loss, and organization of stromal collagen were evaluated as wound healing markers in the cornea. The degree of stromal mononuclear lymphocyte (MNL) and polymorphonuclear leukocyte (PMNL) infiltration and the presence of vascular proliferation were recorded as inflammatory markers. Digital photographs of corneal sections at ×100, ×200, and ×400 magnifications were taken and recorded.

Statistical analysis: The number of animals per group and the total number of subjects were determined according to the E resource equation (Equation 1):

$$n = DF/k+1 \quad (1)$$

(DF = degrees of freedom; k = number of groups; n = number of individuals per group)

Data are expressed as mean ± standard deviation. All experiments were conducted at least in triplicate. Statistical analysis was performed using IBM® SPSS® Statistics 26.

A one-way ANOVA was used for multiple comparisons, and the least significant difference (LSD) post hoc test was used for pairwise comparisons after confirming that the samples exhibited a normal distribution (Kolmogorov–Smirnov test) and had equal variances (Levene's test for homogeneity of variances). When either or both assumptions were violated, non-parametric analysis was conducted using the Kruskal–Wallis test for multiple comparisons and the Mann–Whitney test for pairwise comparisons. Differences between selective experimental groups were considered statistically significant at *p*-value <0.05.

RESULTS

Total protein amount and growth factor levels: The total protein amount in the AME, as measured by the Bradford method, was 0.149 mg/ml ± 0.01 (mg total proteins/ml AME). The growth factor (EGF, FGF, HGF, KGF, NGF, and TGF- β) concentrations are given in Table 1. In the present study, high total protein amounts and growth factor levels were found in the lyophilized and sterilized AME samples.

Corneal epithelial wound healing: Following corneal epithelial wound modeling, the mean wound closure times were 2.75 ± 0.50 (2–3) days in Group 1, 3.5 ± 1 (3–5) days in Group 3, and 3.33 ± 1.52 (2–5) days in Group 4 (p > 0.05). Although there was no statistically significant difference between the groups, the mean wound closure time in Group 1 was shorter than in Groups 3 and 4 (Figure 4 and Figure 5).

The amniotic membrane was dislocated in one rabbit in Group 2 on day 3 of modeling, and the epithelial wound closure of this rabbit was delayed to day 6. Due to the semi-opaque nature of the amniotic membrane, the corneal wound healing of the other rabbits in Group 2 could not be monitored and recorded daily. At the end of the experiment (day 7), after the amniotic membranes were removed, the corneal wounds of the other three rabbits in Group 2 were detected to be completely healed (Figure 6).

The epithelial defect area on the day of modeling (day 0) was defined as 100%, and the corneal epithelial growth rate was analyzed. The daily changes in the epithelial defect area for Group 1, Group 3, and Group 4 are given in Figure 7 as percentages.

Histopathological documentation of wound healing: There were five layers of newly formed epithelial cells in all corneas in Group 1, and this result was significantly higher than in Group 4 ($p < 0.010$). The mean number of newly formed epithelial cell layers was 3.25 ± 0.9 (2–4) in Group 2 and 3 ± 0.8 (2–4) in Group 3; there was no significant difference when compared with Group 4 (mean 3.33 ± 0.5, range 3–4; p < 0.879; p < 0.547, respectively).

In Group 1, epithelial cell polarity and arrangement were close to normal compared to Group 4 (p < 0.019). The presence of well-organized lamellar collagen with the absence of keratocyte loss indicated enhanced stromal remodeling (p < 0.019). In Groups 2 and 3, newly formed epithelial cells were disordered with inadequate polarity, and there was no significant difference when compared with Group 4 (p < 0.452; p < 0.704, respectively). Hypocellular areas in the stromal collagen were present due to marked keratocyte loss in Groups 2 and 3, similar to Group 4 (p < 0.595; p < 0.760, respectively).

In summary, proliferating epithelial layers were more regular in structure, newly formed subepithelial collagen was more organized, and stromal keratocyte loss was milder in Group 1 than in the other three groups. There was no significant difference among Groups 2, 3, and 4 in terms of proliferating epithelial layers, collagen remodeling, and keratocyte loss (Figure 8).

Table 1. Total protein amount (mg total proteins/ml AME†) and growth factor levels (pg growth factor/ml AME) in AME samples.

Total protein	Mean (mg total proteins/ml AME)	Std. Dev.	Min.	Max.
	0.149	0.01	0.137	0.164
Growth factors	Mean (pg growth factor/ml AME)	Std. Dev.	Min.	Max.
EGF	41.19	14.86	31.48	58.29
FGF	43.11	15.19	30.75	60.07
HGF	203.67	31.09	178.38	238.37
KGF	328.03	51.38	280.88	382.78
NGF	207.92	64.32	133.66	246.02
TGF-β	506.93	69.97	436.73	576.67

† Amniotic membrane extract

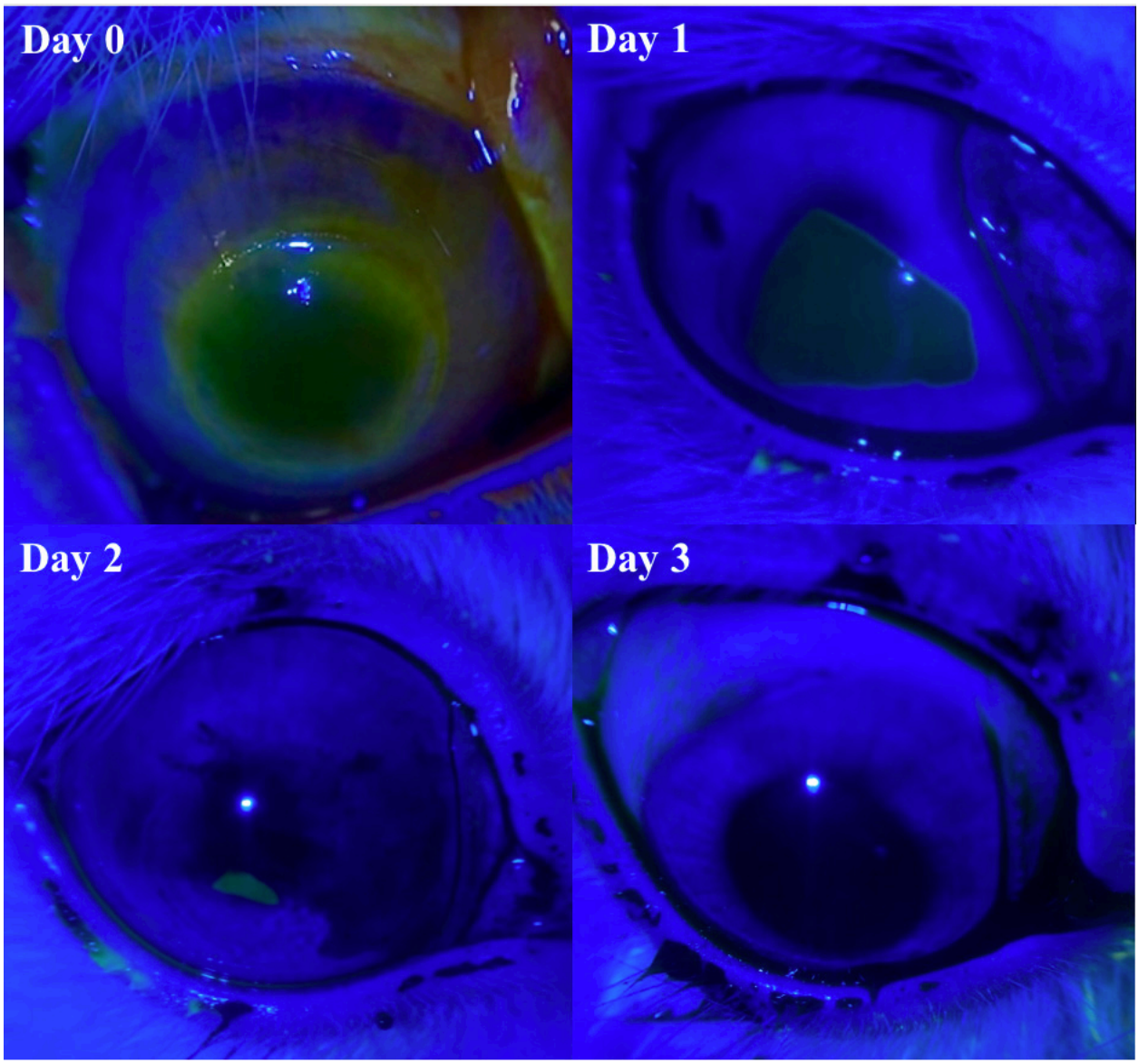

Figure 4. Anterior segment photographs of one of the rabbits in Group 1. The epithelial defect was completely closed on the 3rd day.

Inflammatory markers such as PMNL/MNL infiltration and the presence of vascular proliferation increased in Group 2 compared to Group 4 ($p < 0.047$; $p < 0.033$, respectively). There was no significant difference in inflammatory cell infiltration or vascular proliferation in Group 1 and Group 3 compared to Group 4 ($p < 0.286$; $p < 0.248$, respectively, for Group 1 and $p < 0.586$; $p < 0.767$, respectively, for Group 3).

DISCUSSION

AME and AMEED are amniotic membrane-derived products that were recently introduced in ophthalmology. AMEED has great potential to overcome the limitations of the AMT technique while preserving the clinical activity of HAM [17]. The major advantages of AMEED over AMT are non-invasive application without the need for a surgical procedure and the absence of complications such as residual membrane in the visual axis, premature degradation, or dislocation of the membrane [18]. Topical application of AMEED allows for accurate assessment of clinical response to treatment, visualization of anterior segment details, and adjustment of therapeutic dose and duration. Furthermore, unlike AMT, AMEED maintains biologic activity and efficacy throughout the treatment period by providing growth factor supplementation with each drop [19,20].

Despite these advantages, some fundamental questions and limitations remain regarding the preparation of AME

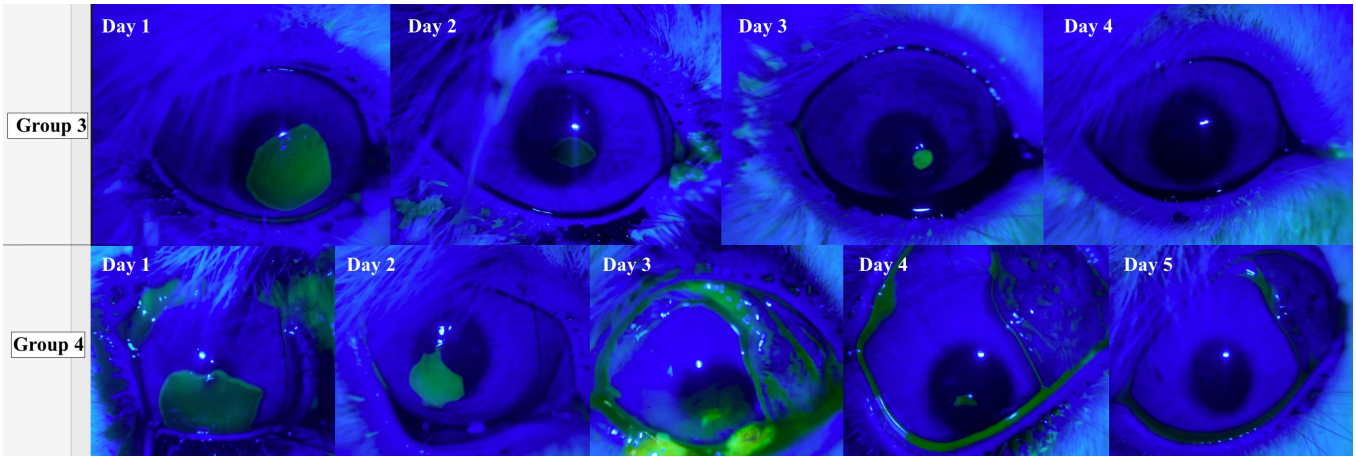

Figure 5. Anterior segment photographs of a rabbit in Group 3 (top row) and Group 4 (bottom row) after corneal wound modeling. While the epithelial defect closed on day 4 in the subject in Group 3, it closed on day 5 in the subject in Group 4.

and AMEED, one of which is avoiding donor-induced variability in the biochemical composition of HAM. Since it is a human-derived product, donor-related factors, such as age, gestational age parity, and gravidity, affect the characteristics of HAM [21,22]. Lopez-Valladares et al. [21] reported lower amounts of total protein and growth factor levels in HAMs from donors with higher donor and gestational ages. This variability in biochemical content, which plays a major role in the therapeutic efficacy of HAM, results in non-standardized AME with varying clinical activities [21-23]. Preparation of AME by the collection of amniotic membranes from different donors to be gathered in a pool minimizes inter-donor-induced

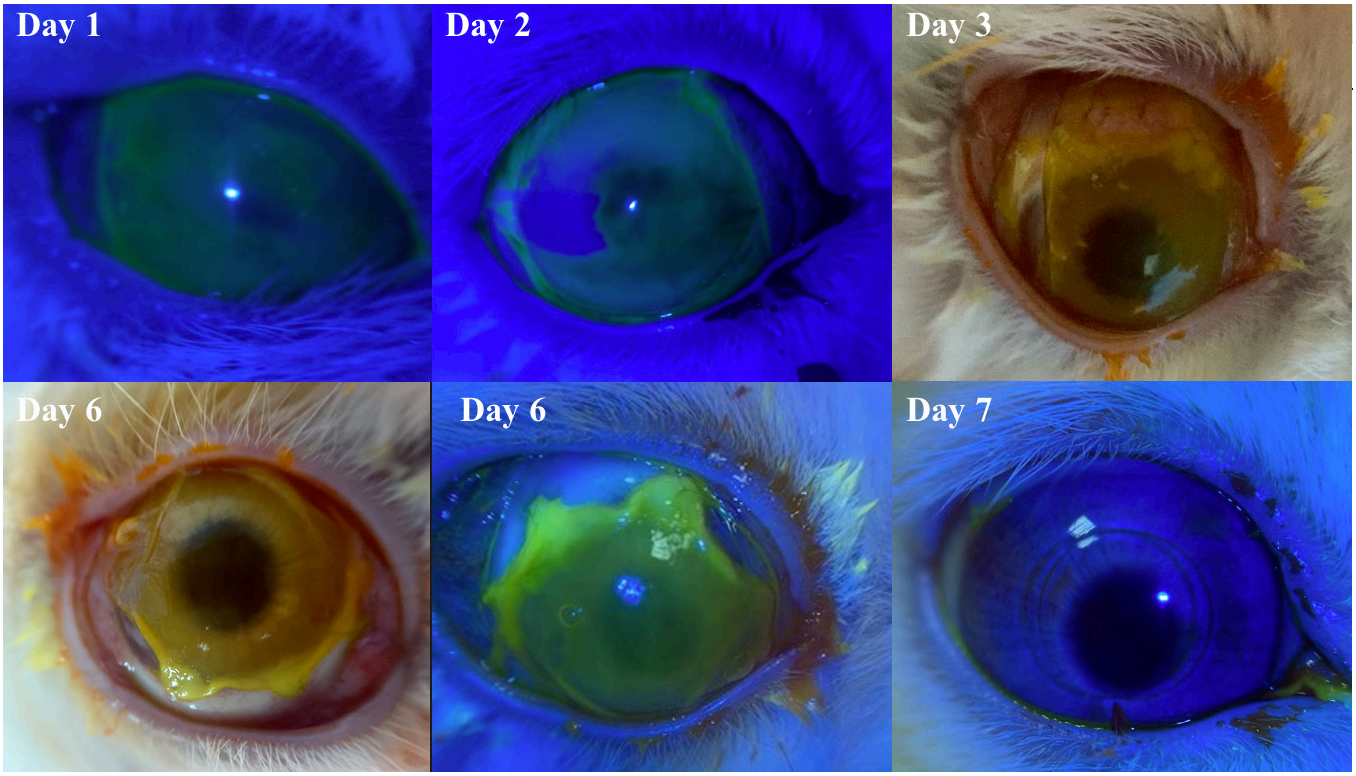

Figure 6. Anterior segment photographs of one rabbit that underwent amniotic membrane transplantation. Due to the semi-opaque nature of the amniotic membrane, corneal wound healing could not be monitored daily.

variability. In the present study, five amniotic membranes from five different donors were collected and pooled, and AME and AMEED were prepared from homogeneous distribution of these different HAMs.

Preservation and storage are other controversial issues for AME preparation. Following the processing of HAM, various preservation methods, such as cryopreservation, lyophilization, and dehydration, are used to preserve the biochemical composition of AME [11]. Cryopreservation is the most commonly preferred preservation method for HAM-derived products. It limits enzymatic reactions in tissue under low temperature conditions (−70 to −80 °C) containing special storage medium. However, the requirement for deep-freezing facilities, difficulty in transportation, and limited shelf life (1–2 years) are important limitations [24-26]. However, lyophilization, a relatively new preservation method that removes water from tissue by sublimation, can prevent the deterioration of tissues by limiting enzymatic reaction, thereby preserving the biochemical activity of the products for a long time. Lyophilization is an advantageous method that is suitable for storage between 4 °C and 25 °C, eliminates the need for cold chain transportation, and overcomes the technical difficulties of cryopreservation [14,27]. However, the inability to determine specific and standard lyophilization conditions for AME is a significant drawback of this method. In the present study, lyophilization conditions specific to AME were also investigated, and standard lyophilization conditions were determined by repeated laboratory experiments. The lyophilized AME was stored at 4 °C and dissolved with PBS before use.

Since it is a human-derived product, another important consideration with AME is the risk of infection. In most previous studies, after all processing steps (including homogenization and centrifugation) were completed, the AME was filtered through a 0.2 µm pore size membrane to be sterilized [11]. However, recent research highlights that gamma radiation is an effective sterilization method for AME [14]. Gamma sterilization does not cause any damage to the structural integrity or biochemical composition of the tissue and is often combined with lyophilization. Nakamura et al. [28] reported that gamma-sterilized and lyophilized amniotic membranes preserved their biologic and morphological structures. In the current study, AME was sterilized by gamma radiation after the lyophilization process. The lack of a standard and precise

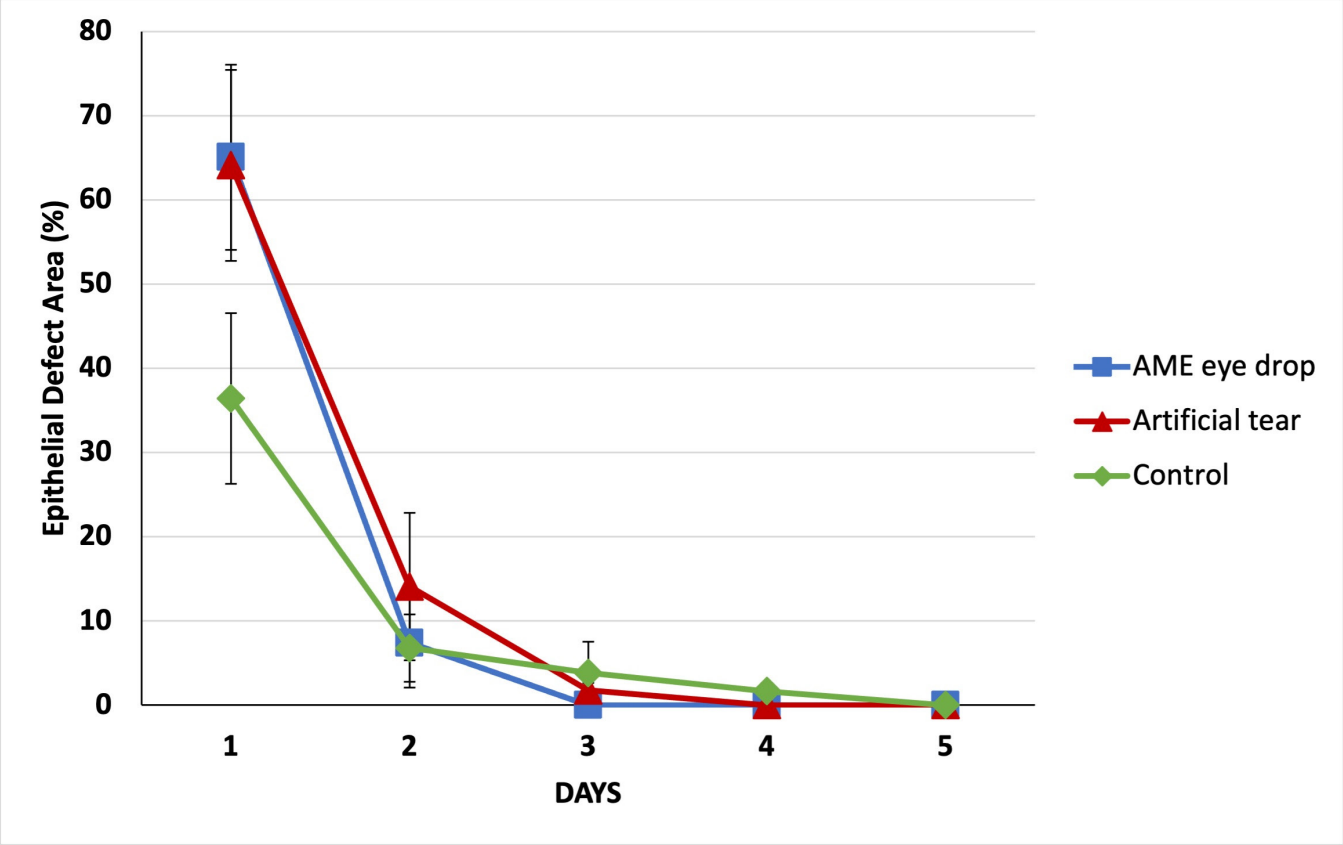

Figure 7. Daily corneal epithelial growth rates in Group 1, Group 3, and Group 4.

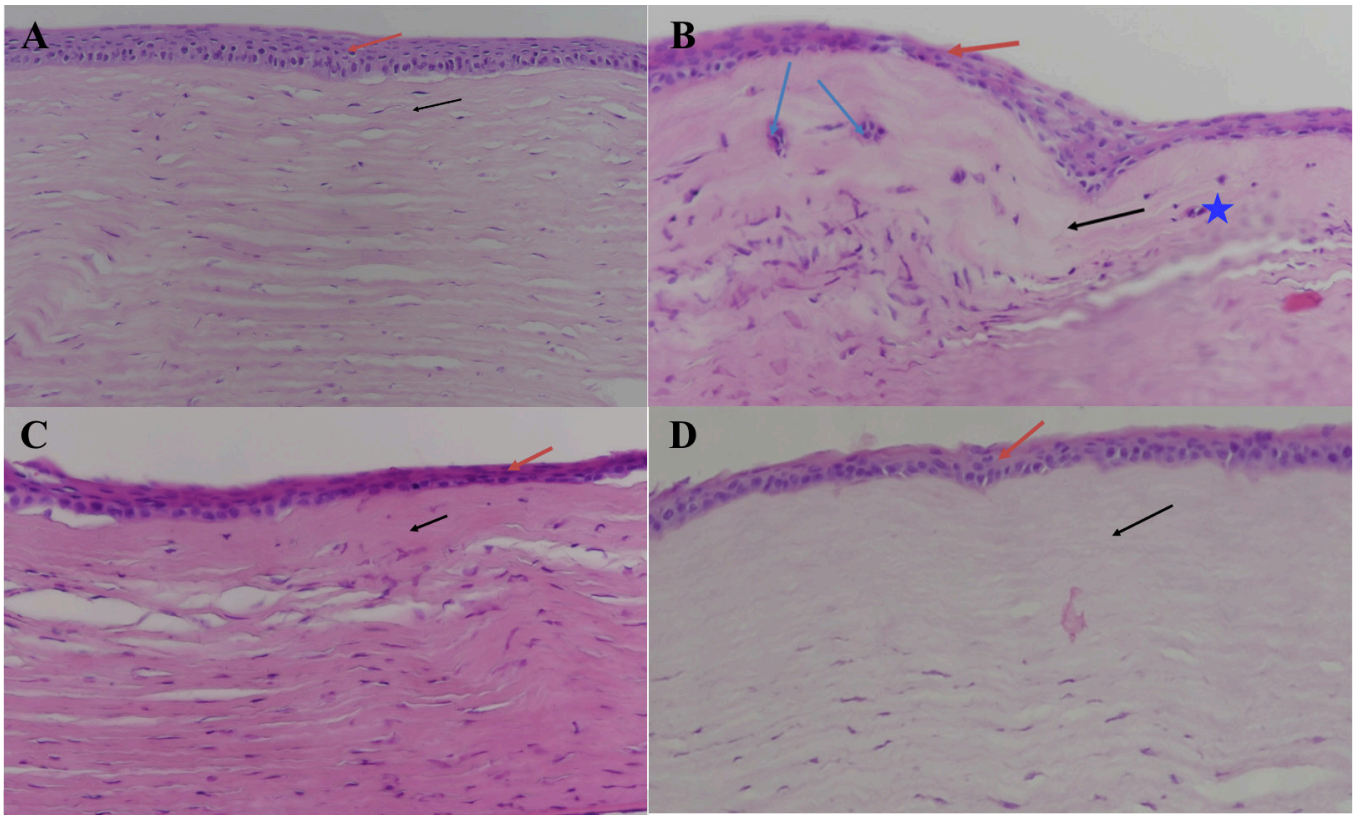

Figure 8. Histopathological examination examples of Group 1 (A), Group 2 (B), Group 3 (C), and Group 4 (D). Red arrows indicate the newly formed epithelial layers. Black arrows indicate the degree of stromal collogen remodeling and keratocyte loss. Blue arrows indicate the newly formed vessels, and blue stars indicate PMNL infiltration (HE; x200). Abbreviations: PMNL, polymorphonuclear leukocyte.

method for the preparation and preservation of AME leads to variations in its biochemical composition [21,29,30].

The biochemical composition of HAM is responsible for its therapeutic effect on ocular surface diseases [21]. HAM is rich in growth factors, including EGF, FGF, HGF, KGF, NGF and TGF-β. Each growth factor plays a vital role in corneal epithelial healing [31-33]. Consistent with the literature, AME prepared in this study was shown to contain high levels of EGF, FGF, HGF, KGF, NGF and TGF-β. Although ELISA is the preferred method for the quantitative analysis of growth factors in AME, its high sensitivity may cause different growth factor concentration measurements in the analyzes of the same AME samples at different times. This variability becomes more prominent, especially for growth factors found in lower concentrations in the samples. Therefore, to interpret growth factor concentrations more accurately, repeated measurements are made on the same AME samples, and the average value is usually stated. We observed that the AME growth factor concentrations found in our study were comparable to those in the literature. Similarly, high standard deviation values were found for EGF and FGF in the literature, which were detected at lower concentrations compared to other growth factors [29,30].

Persistent epithelial defect is one of the most common indications for the use of amniotic membrane in ophthalmology [5]. The use of AMT to accelerate corneal wound healing is an effective method that is often preferred in routine ophthalmology practice. The present study aimed to demonstrate the clinical efficacy of AMEED in a corneal wound model. A 6.5 mm diameter trephine was used to create an epithelial defect of equal size in all groups, and thus to accurately evaluate corneal wound healing. Animal experimental studies in the literature support the idea that AMEED may be a treatment option that accelerates corneal re-epithelialization [34]. Choi et al. [35,36] demonstrated that topical amniotic membrane suspension promotes corneal epithelial proliferation both in-vivo in rat alkaline burn models and in-vitro in human corneal epithelial cell cultures. However, only one study in the literature has investigated the effectiveness of AME, which was developed for use as an alternative to AMT, in corneal wound healing with AMT. Guo et al. [16] reported that amniotic membrane homogenate and AMT had

similar clinical efficacy in promoting corneal wound healing in rabbits. The pool method recommended for AME was not used in the aforementioned study, and detailed information on the clinical application dose was not presented. In the present study, the dose of AME was correlated with clinical use for the first time, and for the first time, both sterilized and lyophilized AME were used in a corneal wound model. We demonstrated that the use of AME obtained by the pool method, which is not affected by inter-donor variation, is clinically and histopathologically superior to AMT in corneal wound healing.

Histopathological confirmation of the clinical outcomes of AMEED treatment is critical in corneal wound healing. Following corneal epithelial injury, epithelial cells adjacent to the wound flatten and migrate to re-establish corneal epithelial integrity. Thus, the wound area is closed with a single layer of epithelial cells. The wound area is then repopulated by the proliferation of single-layered epithelial cells, eventually reaching the normal thickness of the corneal epithelium [37,38]. In the early phases of healing, when the wound is covered with a single layer of epithelial cells, the cornea is no longer stained with fluorescent dye. At this stage, epithelial healing seems to be complete. However, although the wound appears to be clinically healed, it takes time for the newly formed epithelium to reach its normal multilayered thickness [32]. Therefore, the absence of fluorescein staining of the cornea alone is not sufficient to indicate the degree or quality of epithelial healing. In this regard, it is essential to support the clinical findings of corneal wound healing with histopathological analysis. In the present study, the number of newly formed epithelial layers was significantly higher in rabbits treated with AMEED than in the other groups. This histopathological finding was consistent with the clinical outcomes of the current study.

The asymmetry in the shape and structure among cells is defined as “cell polarity.” An example of cell polarity in the corneal epithelium is that the cells in the basal layer are columnar, and the cells on the surface are squamous. Cell polarity is essential for the functioning of epithelial cells and is also an indicator of proper corneal wound healing [32,39]. In this study, epithelial cell polarity and arrangement were close to normal in rabbits treated with AMEED. By contrast, the newly formed epithelial cells were disordered in the other three groups.

Simultaneous changes occur in the stroma due to increased proinflammatory cytokines (especially IL-1) released by the injured epithelium. IL-1 penetrates the anterior stroma and binds to the IL-1 receptors expressed by stromal keratocytes. IL-1 stimulates loss by keratosit modulating the Fas-Fas ligand system. As a result, anterior stromal keratocytes undergo loss in the early phase of epithelial damage [37,40]. Matrix metalloproteinases and collagenases are released by deeply located stromal keratocytes that do not undergo apoptosis. Degradation of the disordered extracellular matrix occurs through increased matrix metalloproteinases and collagenases in the stroma [41]. Simultaneously, TGF-β secreted by epithelial cells stimulates stromal collagen and extracellular matrix remodeling [42]. Therefore, stromal collagen is hypocellular and homogeneous in the early stages of healing due to keratocyte apoptosis, while in the advanced stages, stromal collagen has a well-organized lamellar arrangement [32]. In this study, well-organized, cellular stromal collagen was present in rabbits treated with AMEED. On the contrary, hypocellular stromal collagen due to marked keratocyte loss was present in the other three groups. In addition, inflammatory markers were not present in any of the rabbits treated with AMEED. By contrast, there were few inflammatory cells in the preservative-free artificial tears and control groups. Inflammatory markers were more pronounced in the AMT group than in the other groups. These findings suggest that since AMT is a surgical procedure, it may additionally trigger ocular surface inflammation.

The main limitations of this study are the undetermined stabilization conditions of the AME and the limited number of subjects in the groups. In addition, the growth factor concentrations of lyophilized and sterilized AME have not been compared to fresh (unprocessed) and cryopreserved (for surgical use) amniotic membranes. To overcome this limitation, the AME dose in this study was determined in correlation with the standard-sized amniotic membrane used in AMT, whose clinical efficacy has been well established.

In conclusion, AME is an alternative that overcomes the limitations of AMT while possessing the clinical activity of HAM. However, there are controversial issues regarding the preparation of AME, such as inter-donor variability, long-term preservation of its biochemical composition, risk of transmission of infection, and administration dose. Inter-donor variability in AME can be minimized by collecting amniotic membranes from different donors in a pool. Lyophilization combined with gamma sterilization can preserve the biochemical composition of AME and eliminate the risk of infection transmission. Again, we think that the dose to be determined in correlation with clinical practice, instead of proceeding it on a protein dose that is not based on evidence, is an important point that can be taken as a basis for efficacy evaluation. More work is needed to determine a standard and precise method for the preparation and preservation of AME. The concentration of growth factors in AME can be affected

by several variables, and further studies are still needed to confirm the analyzes with multiple measurements to obtain a standard product. Although AME has been clinically shown to promote corneal epithelial healing and suppress inflammation, clinical findings alone may be insufficient to assess wound healing. AMEED appears to not only promote epithelial proliferation during wound healing but also inhibit stromal keratocyte loss and accelerate collagen remodeling. However, larger studies evaluating the effect of AMEED on the cornea and stroma in detail are needed to confirm the results of this study.

ACKNOWLEDGMENTS

The authors would like to thank to "Ege University, Faculty of Pharmacy, Pharmaceutical Sciences Research Center (FABAL)" and "Ege University, Faculty of Medicine, Ocular Surface Research Laboratory" for providing facilities for extraction studies and total protein analysis. This work was supported publicly by the Ege University Project Coordination Center, Izmir, Turkey (Project No: TOA-2020–21402 and TGA-2021–23222). The authors report there are no competing interests to declare. This study was presented at Turkish Ophthalmological Association 56th National Congress, 2022, Antalya, Turkey and was chosen as the best project.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 28 March 2024. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.