Biological and Ultrastructural Properties of Acelagraft, a Freeze-Dried γ-Irradiated Human Amniotic Membrane

Laurence S. Lim, MRCSEd; Rebekah W. Y. Poh, BSc; Andri K. Riau, BSc; Roger W. Beuerman, PhD; Donald Tan, FRCOphth; Jod S. Mehta, MBBS, MD

Objectives: To compare the biological and ultrastructural properties of a commercially available decellularized and dehydrated human amniotic membrane (DDHAM) product with cryopreserved human amniotic membrane (CHAM) and to demonstrate the feasibility of DDHAM transplant in a case of chronic ocular surface disease.

Methods: Histologic examination, immunohistochemical examination for extracellular matrix molecules and growth factors, transmission and scanning electron microscopy, and atomic force microscopy were performed on both DDHAM and CHAM specimens. A DDHAM transplant was performed in a patient with chronic bulbar keratopathy and ocular surface instability.

Results: Histologic examination and transmission electron microscopy revealed the disruption of the trilaminar structure of the basement membrane compared with CHAM, and immunohistochemical examination confirmed the loss of collagen IV and VII, laminin, and fibronectin in DDHAM. Lower levels of several growth factors were also seen in DDHAM compared with CHAM. Clinical transplant of DDHAM was, however, successful, with rapid reepithelialization.

Conclusion: Significant differences in composition and ultrastructure exist between DDHAM and CHAM but do not appear to compromise cell survival in vivo.

Clinical Relevance: The ease of storage and handling of DDHAM make it potentially valuable in ocular surface surgery, but its biological properties have not been well characterized, and there are also few data on its clinical application.


Human Amniotic Membrane (HAM) has been used successfully in ocular surface reconstruction as a biological bandage and as a substrate for stem cell expansion. The combination of anti-inflammatory, antimicrobial and antiviral, antifibrotic, and antiangiogenic properties of HAM provides a favorable environment for cellular attachment and expansion in both in vivo and in vitro settings. Commercially available HAM products, such as AmnioGraft and ProKera (Bio-Tissue, Inc, Miami, Florida), are currently treated with cryopreservation at −80°C following harvesting to render amniotic epithelial cells nonviable and the tissue nonimmunogenic. This approach has 2 main drawbacks, namely incomplete sterilization with the possibility of disease transmission and the need for an expensive and cumbersome deep freezer. Acelagraft (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey) is a commercially available decellularized and dehydrated HAM (DDHAM) product. The tissue is sterilized by γ-irradiation and is acellular and freeze dried. It can thus be stored and shipped at room temperature, making handling much easier. Bhatia et al have previously reported that Acelagraft is a primarily collagenous extracellular matrix-like material and have demonstrated that it is able to support dermal fibroblast assembly and secretion of key wound healing cytokines.

The aim of this study was to compare the biological and ultrastructural properties of DDHAM with conventional cryopreserved HAM (CHAM) with respect to cellular components, biochemical composition, and membrane surface characteristics and to demonstrate its feasibility in a case of ocular surface disease requiring resurfacing with HAM.

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Methods

PROCUREMENT AND PREPARATION OF HAM

The CHAM was prepared from human placentas obtained from mothers who had undergone cesarean sections. The membranes were
washed with phosphate-buffered saline (PBS) to remove the blood clots. The HAM was peeled away from the chorion and flattened onto a sterilized nitrocellulose filter paper (Millipore, Bedford, Massachusetts). The CHAM was then stored in 50% Dulbecco modified Eagle medium and 50% glycerol (In-vitrogen Gibco, Carlsbad, California) at −80°C. In preparation for its use, the CHAM was thawed and rinsed with PBS.

The DDHAM (Acelagraft) was prepared using proprietary methods that have been described previously.12 Briefly, the amniotic membrane was excised from qualified term placentas and washed and scraped to remove extraneous tissue and cells. This was then followed by decellularization of the tissue using deoxycholic acid and drying of the tissue using a gel dryer. Sterilization of the product was achieved with electron-beam irradiation, and the recommended storage temperature for the product is between 15°C to 30°C.

IMMUNOHISTOCHEMICAL EXAMINATION FOR EXTRACELLULAR MATRIX MOLECULES AND GROWTH FACTORS

Tissues were embedded in optimal cutting temperature freez- ing compound (Leica, Nussloch, Germany). Six-micromole- thick sections were cut and positioned on polylysine-coated glass slides and then air dried for 20 minutes. They were then sub- jected to hematoxylin-eosin staining or indirect immunostain- ing analysis. The tissues were fixed in −20°C acetone for 15 min- utes, followed by washing 3 times for 5 minutes in PBS Triton, 0.15%. The samples were then immersed for 1 hour in blocking solution (bovine serum antibody, 4%, in 0.01M PBS) for 1 hour at room temperature in a humid chamber. After washing for 5 minutes in PBS Triton, 0.15%, they were incubated over- night, at 4°C, with primary antibody solutions (Table). Each sample (CHAM or DDHAM [Acelagraft]) was tested in quadruplicate. Epithelialized CHAM sections and normal mouse/goat/rabbit immunoglobulin were used as positive and negative controls, respectively. The tissues were then washed 3 times for 5 minutes in PBS (0.01M) and were subsequently incubated at room temperature with fluorescent (fluorescent isothio- cyanate conjugated) secondary antibody (Alexa fluor 488– labeled antimouse IgG, antirabbit IgG, and antigoat IgG; Invitrogen) at a dilution of 1:2000. After 1 hour, the tissues were washed 3 times for 5 minutes in PBS (0.01M), in the dark. Slides were then mounted with Floursave with 4,6-diamidino-2- phenylindole (Vector Laboratories, Burlingame, California). They were subsequently examined with a Zeiss Axioplan 2 fluo- rescence microscope (Zeiss, Oberkochen, Germany).

WESTERN BLOTTING

The CHAM was homogenized individually into 50mM TRIS- hydrochloride buffer containing protease inhibitors. In- soluble materials were removed by 15 minutes of centrifugation (10 000g at 4°C). An equal volume of 4× sodium dodecyl sulfate loading buffer was added to each sample, which was then subjected to boiling for 5 minutes at 100°C and centrifugation for 5 minutes (10 000g at 4°C). For DDHAM (Acelagraft), the membranes were divided into small pieces, then incubated in ristocetin-induced platelet agglutination lysis buffer at room temperature for 15 minutes. The samples were then sonicated.

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Abbreviations: EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; G, goat; IGF-I, insulin-like growth factor I; IHC, immunohistochemical examination; KGF, keratinocyte growth factor; M, mouse; Mo, monoclonal; PDGF, platelet-derived growth factor; Po, polyclonal; R, rabbit; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; WB, Western blotting.

a Used for IHC and WB.

b Santa Cruz Biotechnology Inc, Santa Cruz, California.

c Zymed Laboratories, San Francisco, California.
d Dako, Carpinteria, California.
e Acris Antibodies GmbH, Herford, Germany.
f Neomarkers Inc, Fremont, California.
g CHEMICON International Inc, Temecula, California.
h Upstate Chemicon, Temecula.

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for 15 minutes and heated at 100°C for 15 minutes. Following this, they were cooled to room temperature then centrifuged at 12,000g at 4°C for 10 minutes. The sample was then left on ice for 10 minutes and stored at −20°C until reduction on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (gradient, 6%–15%). The amounts of protein applied ranged from 30 to 60 µg. Resolved proteins were electrically transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California) and blocked in PBS containing 3% nonfat milk, followed by 2-hour incubation with the primary antibody (Table) at room temperature with agitation. Samples were then washed vigorously 3 times each for 5 minutes in PBS polysorbate 20, 0.05%. The appropriate secondary reagent was then applied, ie, goat-antimouse horseradish peroxidase, goat-antirabbit horseradish peroxidase, or rabbit-antigoat horseradish peroxidase (Santa Cruz Biotechnology Inc, Santa Cruz, California) at a dilution of 1:2,000. Immunoreactivity was visualized with a chemiluminescence reagent (SuperSignal West Pico; Pierce Biotechnology, Rockford, Illinois).

ELECTRON MICROSCOPY

Transmission Electron Microscopy

The CHAM and DDHAM tissues were fixed with cold paraformaldehyde, 2%, and glutaraldehyde, 2%, in 0.1M sodium cacodylate buffer (pH 7.4) (Electron Microscopy Sciences, Hatfield, Pennsylvania) at 4°C overnight. Tissues were washed in sodium cacodylate buffer, rinsed with distilled water, and trimmed into smaller pieces. Tissues were then postfixed in osmium tetroxide, 1%, and potassium ferrocyanide (Electron Microscopy Sciences) to enhance membrane contrast. After extensive rinsing with distilled water, tissues were dehydrated in a graded series of ethanol and embedded in Araldite (Electron Microscopy Sciences). Semithin sections of 0.5 to 1 µm thickness were cut with Reichert-Jung Ultracut E Ultramicrotome (C. Reichert Optische Werke AG, Vienna, Austria), counterstained with toluidine blue/basic fuchsin stain, and examined using a Zeiss Light Microscope (Zeiss). Ultrathin sections of 60 to 80 nm were collected on copper grids, double stained with uranyl acetate and lead citrate for 8 minutes each, and then viewed and photographed on a JEM 1220 electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Scanning Electron Microscopy

Specimens were immersed in a fixative containing glutaraldehyde, 2.0%; paraformaldehyde, 2.0%; and 0.1M sodium cacodylate (pH 7.4) overnight at 4°C. They were then transferred and stored in sodium cacodylate buffer (Electron Microscopy Sciences). Before processing, the samples were washed twice in distilled water for 10 minutes. They were then secondary fixed in osmium tetroxide, 1% (FMB, Singapore), for 2 hours at room temperature. Following this, they were dehydrated in a graded ethanol series of 25%, 50%, 75%, 95%, and 100% each for 10 minutes, with repetition in 100% ethanol 3 times. The samples were then dried in a critical point dryer (Bal-Tec, Liechtenstein, Germany) and mounted on a silicon wafer batch to alleviate variations in tip geometry. Atomic force microscopy imaging in air was conducted using a Nanoscope IV Multimode (Veeco Digital Instruments, Santa Barbara, California). Images were captured in tapping mode using TappingMode etched silicon probes (RTESP; Veeco Digital Instruments) with resonant frequency about 300 kHz, spring constant of around 40 N/m, and a tip radius of less than 10 nm. All images were acquired with a 256 × 256 point resolution at a scan rate of 5 Hz.

Ten reference areas were analyzed from each tissue. Each area of 625 µm² was scanned twice to ensure that the force exerted was not sufficient to damage the sample surface and cause artifacts. Areas scanned were selected based on a clock-face distribution. Height, amplitude, and phase images, as well as 3-dimensional images, were simultaneously acquired. Surface-roughness values were calculated from height-mode images using Nanoscope 6.11 software (Veeco Digital Instruments). The mean root-mean-square (RMS) values were calculated by collecting 10 RMS values over 10 areas of 625 µm² on each tissue and calculating the mean. The RMS values reflecting fluctuations of surface heights were calculated according to the following equation:

\[ R_{rms} = \sqrt{\frac{\sum_{j=1}^{N} (h_j - \bar{h})^2}{n}} \]

(where \( n \) is the number of data points) and taking the mean. All images used to gather RMS values were 25 × 23 µm in scan size to provide roughness measurements, and all tips were from the same wafer batch to alleviate variations in tip geometry. Skewness, representing the symmetry of surface data about a mean data profile, was calculated from the following equation with \( R_q \) as the RMS roughness:

\[ \text{Skewness} = \frac{1}{R_q} - \frac{3}{N} \sum_{j=1}^{N} Z_j^3 \]

RESULTS

HISTOLOGIC EXAMINATION

Histologic examination of a CHAM specimen stained with hematoxylin-eosin revealed a single layer of cuboidal epithelial cells on a densely eosinophilic basement membrane (BM) overlying a loosely arrayed basophilic stroma. In contrast, hematoxylin-eosin staining of a DDHAM specimen was notable for the lack of cellular structures or a prominent BM. The specimen showed a homogenous pale basophilia (Figure 1).

IMMUNOHISTOCHEMICAL EXAMINATION OF EXTRACELLULAR MATRIX MOLECULES

Normal sections incubated with mouse, goat, and rabbit immunoglobulins and without primary antibody demonstrated no immunoreactivity. Compared with CHAM, DDHAM expression of collagen I, II, and VI; elastin; tenascin; and thrombospondin were comparable. The DDHAM immunoreactivity to fibronectin was less intense than CHAM and negative for collagen IV and VII and laminin 5. Expression of all growth factors evaluated (epidermal growth factor receptor, fibroblast growth factor 2, insulinlike growth factor 1, platelet-derived growth factor A, platelet-derived
growth factor B, transforming growth factor (TGF) β1, TGF-α, TGF-β2 receptor, and vascular endothelial growth factor) was markedly lower in DDHAM compared with CHAM (Figure 2). Western blot analyses for fibronectin, collagens IV and VI, and elastin confirmed the differential expression of these extracellular matrix components in CHAM and DDHAM (Figure 3).

**Figure 2.** Immunohistochemical staining of various extracellular matrix and growth factor proteins in cryopreserved human amniotic membrane (CHAM) and decellularized and dehydrated human amniotic membrane (DDHAM) (original magnification ×200). EGFR indicates epidermal growth factor receptor; FGF-2, fibroblast growth factor 2; IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; +++, strong staining; ++, moderate staining; +, weak staining; −, negative staining.

**Figure 3.** Representative images of Western blot analysis of fibronectin; collagen types IV and VI; laminin 5; and elastin of cryopreserved human amniotic membrane (CHAM) and decellularized and dehydrated human amniotic membrane (DDHAM) (Acelagraft; Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey).

**TRANSMISSION ELECTRON MICROSCOPY AND SEM**

The BM of CHAM supports the overlaying epithelium and is composed of a 3-layered basal lamina and lamina fi-
broreticularis. Lamina rara externa (lucida) is an electron-lucent zone directly bordering the adjacent cell that makes up the upper portion of the basal lamina. Lamina densa is an electron-dense zone that appears somewhat amorphous and granular and constitutes the intermediate part of the basal lamina. Lamina rara interna comprises the basal portion of the basal lamina. The 3 layers of basal lamina sit on top of the lamina fibroreticularis, which is synthesized by cells from underlying connective tissue and contains fibrillar structures, namely anchoring fibrils, elastic fibrils, and microfibril bundles (Figure 4A).

In the transmission electron micrographs of DDHAM, the absence of epithelial cells was demonstrated and there was no lamina lucida. The lamina densa was present and approximately the same thickness as that of CHAM (Figure 4B).

The smoothness of DDHAM was evaluated by SEM and by atomic force microscopy (Figure 5). Surface structure morphology of DDHAM was cauterized using both SEM and atomic force microscopy. Although these 2 techniques provide complementary images, different information on the surface structure can be derived to give a more complete examination. The SEM micrographs at low magnification (Figure 5A) demonstrated a large area view of DDHAM showing the distinctive grids. At higher magnification (Figure 5B), it is clearly devoid of cellular structures and the surface appears smooth. On a smooth surface, SEM has difficulty resolving features because of the subtle variations in height. Moreover, tissues are subjected to shrinkage owing to chemical processing and the mandatory gold coating might conceal fine structures. To address these setbacks, atomic force microscopy was further used to image the samples at nanoscale resolution.

In the 3-dimensional (Figure 5F) reconstruction presented a topographical map on the imaged area.

**CLINICAL CASE REPORT**

A 40-year-old man with a history of irido-corneal endothelial syndrome, advanced chronic glaucoma, and an Ahmed glaucoma drainage device implantation was referred for bullous keratopathy. Penetrating keratoplasty was performed but the patient experienced endothelial rejection with an edematous graft, poor ocular surface, and severe pain (Figure 6A). The patient was keen to avoid enucleation, and conjunctival scarring from the Ahmed glaucoma drainage device implantation precluded a Gundersen flap. A DDHAM transplant was thus performed for ocular surface stabilization.

The DDHAM was punched with an 8.25-mm trephine and rehydrated for 10 minutes prior to use. The recipient epithelium was debrided and the prepared DDHAM was sutured with 11-0 nylon sutures and protected with a hydrophilic bandage contact lens. The postoperative regimen included topical preservative-free levofloxacin and dexamethasone eyedrops applied with a gradually tapering frequency.

On the first postoperative day, epithelialization had already begun with an epithelial defect over approximately 80% of the corneal surface (Figure 6B). Further recovery was uneventful, with only a small epithelial defect remaining at 17 days after surgery (Figure 6C) and restitution of a completely epithelialized ocular surface by 24 days.

![Figure 4. Cross-section ultrastructural comparison between the basement membrane of cryopreserved human amniotic membrane (CHAM) (A) and decellularized and dehydrated human amniotic membrane (DDHAM) (B) tissue. A, Transmission electron micrograph of CHAM tissue. E indicates epithelium; LD, lamina densa; S, stroma (original magnification ×30 000). B, High magnification of transmission electron micrograph of DDHAM tissue (original magnification ×30 000). Inset: Low magnification showing DDHAM tissue consists of thick basement membrane (arrow) and an avascular stroma (S) (original magnification ×12 000; bar = 1 µm).](https://www.archophthalmol.com/content/128/10/1307/F4)

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The treatment of ocular surface disease is challenging, and current treatment strategies are resource intensive and inaccessible to underdeveloped countries. In this context, the ease of handling of DDHAM presents notable advantages over the use of conventional CHAM because it can be transported at room temperature and simply rehydrated before use. Absolute microbial and viral sterility, which are of particular importance in a compromised ocular surface, are also assured. Extensive laboratory and clinical data on DDHAM are, however, limited. Bhatia et al\textsuperscript{12} have reported on the composition of DDHAM (Acelagraft) as well as the biology of fibroblasts grown on DDHAM, while Nakamura et al\textsuperscript{13} have described the physical characteristics and biocompat-

\textbf{Figure 5.} Smoothness of decellularized and dehydrated human amniotic membrane (DDHAM) evaluated by scanning electron microscopy (A and B) and by atomic force microscopy (C-F). A, Low magnification (original magnification ×18; bar=1 mm) of Acelagraft (Acelagraft Cellular Therapeutics, Cedar Knolls, New Jersey). B, High magnification (original magnification ×500; bar=50 µm) showing smoothness of DDHAM surface. C, Height data of DDHAM where dark areas represent depressions and brighter areas represent protrusions on the surface. D, Amplitude data. E, Phase data depicting hardness variations. Bright areas represent hard regions whereas dark areas represent soft regions on the surface. F, 3-Dimensional presentation of topographical map. Image scale: 50×50 µm.
ability of a noncommercial DDHAM in a rabbit model of ocular surface disease. To our knowledge, there are no reports of detailed analyses of Acelagraft or its use in human ocular surface disease.

In our study, a prominent feature of the transmission electron microscopy images of DDHAM was the disruption of the trilaminar structure of the BM compared with CHAM. Immunohistochemical staining for BM components likewise revealed the absence of several characteristic BM components, including collagens IV and VII and laminin, and reduced expression of fibronectin compared with CHAM. These findings were confirmed with Western blot. Solubilization of DDHAM and analysis of its components has shown that it is composed mainly of collagen types I and III (±4% dry weight) with only minimal amounts of collagen IV, laminin, and fibronectin. Nakamura et al were, however, able to demonstrate collagen IV, laminin, and fibronectin expression similar to CHAM in their preparation of DDHAM. These differences may be attributable to different preparation methods, because deoxycholic acid is used in the decellularization of Acelagraft prior to drying whereas EDTA was used by Nakamura et al. Laminin is localized primarily to the lamina lucida, which was disrupted in transmission electron microscopy images in the DDHAM in our study but well preserved in the freeze-dried HAM produced by Nakamura et al. The role of the BM in HAM as a substrate for cell growth, and particularly its collagen IV component, is presently unclear, but several authors have reported that HAM may serve as a more specific substrate for human conjunctival cells rather than corneal epithelial cells. Interestingly, the DDHAM used in our study, devoid of collagen IV and a normal BM, was able to support the growth of cells with conjunctival morphology in our case of human transplant, whereas Nakamura et al were able to cultivate rabbit corneal epithelial cells on their DDHAM preparation with an intact BM. It has been shown that limbal epithelial cells grown on HAM cause dissolution of the native BM prior to secreting a new BM. An intact BM hence may not be essential for cell attachment and expansion.

Several growth factors have been investigated for their roles in promoting epithelialization on HAM. High levels of epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor, TGF-β1, fibroblast growth factor, and several other growth factors have been identified in HAM, most of which—particularly epidermal growth factor, TGF-β1, and fibroblast growth factor—have well-documented roles in the epithelial-mesenchymal cell interactions involved in corneal wound healing. Significantly higher levels of growth factors are seen in HAM bearing epithelium compared with denuded HAM, suggesting that the amniotic epithelium actively secretes growth factors to support epithelial growth. Bhata et al, using guanidinium-hydrochloride extraction methods to solubilize DDHAM before enzyme-linked immunosorbent assay analysis, were unable to detect vascular endothelial growth factor, fibroblast growth factor, TGF-β1, platelet-derived growth factor A and B, or pigment epithelium-derived factor in DDHAM. Using immunohistochemical examination, we were able to detect the presence of fibroblast growth factor 2, platelet-derived growth factor A and B, TGF-β1, TGF-α, TGF-β2 receptor, and vascular endothelial growth factor in DDHAM, albeit in significantly lower quantities than in CHAM. The presence of these growth factors in detectable quantities may imply that the denudation and preservation process of DDHAM do not completely denature all growth factors from the membrane.

We were able to establish the feasibility of using DDHAM in human ocular surface reconstruction. The aim of surgery in this case was to achieve a stable, conjunctivialized ocular surface because the visual prognosis was poor. Trephination of DDHAM in the dehydrated state was much easier and more precise because of its stiffness, and the membrane was suitable for transplant after 10 minutes of rehydration. Complete corneal epithelialization was achieved within 24 days (3.4 weeks) after transplant. This is comparable with published reports on the use of CHAM for ocular surface reconstruction, in which the mean time to epithelialization has ranged from 3.5 to 16.6 weeks. Resurfacing with DDHAM also served to restore some of the barrier functions of the epithelium, with deturgescence of the corneal stroma. That a stable ocular surface was achieved even though DDHAM lacks an intact BM and expresses only low levels of growth factors—components conventionally thought to be important in supporting cell proliferation—implies alternative mechanisms for cell support and cell growth. Bhata et al have shown that dermal fibroblasts seeded onto DDHAM (Acelagraft) are able to recognize the small amounts of fibronectin in DDHAM and bind via integrin × fibronectin interactions. They can subsequently secrete fibronectin and assemble a new extracellular matrix on the DDHAM. These fibroblasts are also stimulated by DDHAM to secrete several key cyto-

![Figure 6. Slitlamp photography and fluorescein staining patterns of the clinical subject taken preoperatively (A), 1 day after surgery (B), and 17 days after surgery (C).](Image)
kines involved in chemotaxis and wound healing, including interferons, tumor necrosis factor α, and macrophage inflammatory protein. The interaction between fibronectin, which is relatively well conserved in DDHAM, and fibroblasts, which are abundant in scar tissue, may thus be a key mediator of wound healing augmentation by DDHAM.

In conclusion, our study has demonstrated the feasibility of a sterile, dehydrated HAM product for ocular surface reconstruction in a human subject. Decellularized and dehydrated HAM may be a more cost-effective solution for ocular surface reconstruction in smaller centers with lower patient numbers because the cost of purchasing and maintaining cryopreservation facilities are considerable. Larger tertiary centers for which the amortized costs of CHAM are lower may, however, still find DDHAM useful in situations of donor shortage. Significant differences in composition and ultrastructure exist between DDHAM and CHAM but do not appear to compromise cell survival in vivo. Further in vitro studies to demonstrate the viability of corneal, limbal, and conjunctival explants would help to establish the veracity of our findings, and more clinical trials evaluating the efficacy of DDHAM as an alternative to CHAM for a variety of indications may also expand the role of DDHAM in ocular surface reconstruction.

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