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Transport and Preservation Comparison of Preloaded and Prestripped-Only DMEK Grafts

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Purpose: This study compares the effect of the transport of conventionally prestripped Descemet membrane endothelial keratoplasty (DMEK) tissue with the DMEK revolutionary advanced Preloadable Injection Device (RAPID) preloaded transport system from Geuder AG (Heidelberg, Germany). Endothelial cell loss, tissue integrity, endothelial cell phenotype, and viability were assessed and compared.

Methods: Twelve DMEK grafts were prestripped by the cornea bank and transported using the following 2 conditions: conventional flask (n = 6) or a preloaded transport cartridge (DMEK RAPID, n = 6). After transport, tissues were analyzed for cell density; denuded areas; immunolocalization of corneal endothelial markers, such as ZO-1, CD166, and Na^+/K^+ ATPase; histology analysis; and cell viability staining with Hoechst, calcein AM, and ethidium homodimer.

Results: Endothelial cell loss (10.35% vs. 9.15%) did not differ between transport conditions. Histological analysis confirmed the integrity of the Descemet membrane and endothelial cell layer with both transport conditions. Similarly, the corneal endothelial cell mosaic was conserved in both conditions. The ZO-1 tight junctions confirmed the integrity of the confluent corneal endothelial cell monolayer. CD166 and Na^+/K^+ ATPase detection with immunofluorescence was also comparable. A similar percentage of dead cells was reported in both conditions (18.1% vs. 16.73%). Moreover, the surface covered with calcein-positive cells (59.02% vs. 61.95%) did not differ between transport conditions.

Conclusions: Our results suggest that DMEK grafts can be prestripped or preloaded into a novel transport cartridge and shipped to the clinic with comparable endothelial cell loss, phenotypical marker expression, and viability to the conventional prestripped donor tissue.

Key Words: DMEK, preloaded tissue, eye banking

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Descemet membrane endothelial keratoplasty (DMEK) has emerged as the treatment of choice for corneal endothelial dysfunction owing to excellent visual recovery and low risk of rejection.[1–5] Eye bank–prepared tissue has helped to reduce intraoperative complications related to tissue preparation. However, the final steps of tissue preparation, namely separating the donor tissue from the corneal button, staining with trypan blue, rinsing with balanced salt solution, and loading into an injector, are left to the surgeon. The recent development of preloaded DMEK transport systems has the potential to reduce costly operation theater time and the risk of iatrogenic tissue damage. Moreover, they will allow eye banks to send only the tissue necessary for DMEK surgery, optimizing donor availability for other purposes.[6,7] To date, 2 preloaded DMEK transport protocols have been developed and studied.[8–10] These allow the transport of prestripped DMEK tissue in either a modified Jones tube[8] or a modified lens insertion carrier.[9] More recently, Geuder AG (Heidelberg, Germany) has developed a method for transporting the prestripped DMEK tissue in a preloaded glass cannula similar to the one used for its injection in the operation theater. The aim of this study is to compare the DMEK RAPID preloaded transport method, developed by Geuder AG, with the conventional prestripped method used to date. Determining whether the DMEK tissue arrives equally viable for transplant in both conditions is necessary before the implementation of this preloaded transport system in the clinical setting.

MATERIALS AND METHODS

Ethical Statement

This research was performed in compliance with the tenets of the Declaration of Helsinki. Twelve human cadaveric corneoscleral tissues (Table 1), which were unsuitable for

TABLE 1. Donor Information

Cornea Number	Age	Sex	Hours Between Death and Excision	Days in Preservation	Cause of Death	ECD Before Stripping	ECD After Stripping	ECD After Transport	Experiment
Preloaded 01	34	M	15	22	Stroke	3000	3000	2500	Brightfield, histology, immunofluorescence
Preloaded 02	58	M	16	28	Non-specified	2700	2700	2500	Brightfield, histology, immunofluorescence
Preloaded 03	76	F	20	16	Stroke	2600	2600	2400	Brightfield, immunofluorescence
Preloaded 04	62	M	26	26	Respiratory failure	2800	2800	2800	Viability assay
Preloaded 05	68	F	37	8	Aortic aneurysm	2800	2800	2600	Viability assay
Preloaded 06	69	M	32	25	Septicemia	2500	2500	2100	Viability assay
Conventional 01	34	M	15	22	Stroke	3100	3100	2700	Brightfield, histology, immunofluorescence
Conventional 02	57	F	27	27	Hemorrhage	2300	2300	2000	Brightfield, histology, immunofluorescence
Conventional 03	76	F	20	16	Stroke	2600	2600	2400	Brightfield, immunofluorescence
Conventional 04	66	F	29	21	Chronic obstructive pulmonary disease	2800	2800	2600	Viability assay
Conventional 05	76	F	26	22	Acute myocardial infraction	2900	2900	2400	Viability assay
Conventional 06	69	M	32	25	Septicemia	2700	2700	2500	Viability assay

ECD, endothelial cell density (cells/mm^2).

transplantation because of the medical history reasons, such as high-risk behavior, were obtained from the Cornea Department of the ETB-BISLIFE Multi-Tissue Center (Beverwijk, the Netherlands) after obtaining consent from the next of kin of all deceased donors.

Shipping Conditions

All tissues were preserved postmortem in organ culture media at 31°C before use. Organ culture media comprised the following: minimum essential medium (Biowest, Nuaillé, France) supplemented with 20 mM HEPES (Sigma, St. Louis, MO), 26 mM sodium bicarbonate (Sigma), 2% (vol/vol) newborn calf serum (Thermo Fisher Scientific, Waltham, MA), 10 IU/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin (Sigma). The tissues were processed and transported in 2 different conditions; the first condition used was the conventional method used for shipping DMEK tissue grafts, in which the prestripped endothelial tissue was laid-flat on the corneal stromal bed. The second condition was the preloaded DMEK RAPID from Geuder (Geuder AG) (Fig. 1), in which corneal endothelium was prestripped with the endothelial cells facing outward and was loaded in a Geuder preloadable glass cannula for DMEK (Geuder AG). During transportation, the tissue was conserved in transport media [organ culture media with 6% (wt/vol) dextran (Sigma)] at ambient temperature in-line with clinical practice. Transport of the DMEK tissues was performed within the Netherlands, from the ETB-BISLIFE Multi-Tissue Center in Beverwijk (North Holland) to the Maastricht University Medical Center+ (Limburg). All tissues were received and analyzed 36 to 48 hours after the DMEK prestripping only or preloading.

Preparation of Tissue for Transport

The corneas (n = 12) were vacuum fixed in a punch base. The corneal endothelium was first trephined using a 10-mm diameter trephine (e.janach, Como, Italy), and Descemet membrane was gently stripped, leaving it attached to the cornea by a hinge. After the first stripping, the DMEK

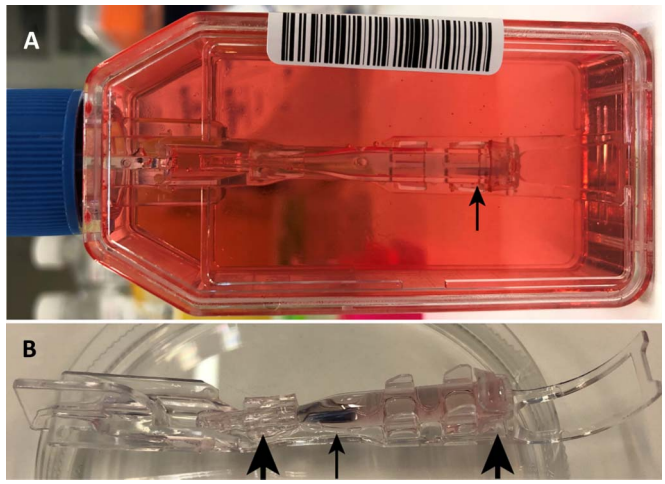

FIGURE 1. Representative images of DMEK tissue in the preloaded transport condition by using the DMEK RAPID Geuder system. A is the cartridge with preloaded DMEK tissue in the transport flask containing transport media. B is the cartridge with transport support for the preloaded DMEK tissue. Full arrows indicate the stained DMEK tissue roll in the Geuder cartridge. Arrow heads indicate 2 liquid permeable plugs that allow gentle washing steps and staining of the graft within the transport cartridge.

graft was left to lay on top of the corneal stromal bed and further trephined using an 8.5-mm diameter trephine (Moria, Doylestown, PA). For the prestripped-only transport method (n = 6), the 8.5-mm diameter DMEK graft was left on top of the corneal stroma attached by a hinge. The prestripped cornea was then transported in a glass bottle containing approximately 50 mL of transport media. The average total time required for preparation of prestripped-only DMEK tissue was 20 minutes. For the preloaded transport method (n = 6), the 8.5-mm DMEK graft was separated from the cornea and rolled with the endothelial side facing outward. The endothelial roll was then stained by dragging it through a drop of trypan blue solution (0.4%), washed with BSS, transferred to a 35-mm culture dish with transport media and gently suctioned by using a syringe into a preloadable glass cannula for DMEK containing transport media (Geuder AG). The loaded glass cannula was sealed with plastic plugs at both the funnel and rear sides and transferred to a T25 tissue culture flask (Corning, Corning, NY) completely filled with approximately 70 mL of transport media for transport. The total time required for the preparation of preloaded transport DMEK tissue was approximately 30 minutes. Tissue marking for graft orientation was not used in this study because tissue marking may be more traumatic to preloaded tissue.[11]

Study Design

This study included 12 donor corneas, 6 in the prestripped-only DMEK group and 6 in the preloaded DMEK group. From each group, 3 whole-DMEK rolls (n = 6) were used for viability staining. The remaining DMEK rolls (n = 6) were used as following; 2 DMEK rolls from each group (n = 4) were divided, one half was used for immunofluorescence and the other half used for hematoxylin and eosin staining. The remaining rolls (n = 2) were used for immunofluorescence (Fig. 2).

Tissue Analysis With Brightfield Microscopy

All tissues (n = 12) were analyzed before and after Descemet membrane stripping at the Cornea Department of ETB-BISLIFE to determine the cell density and identify possible denuded areas by using an upright brightfield microscope. After transport, the preloaded DMEK rolls were released into a 35-mm culture dish filled with BSS ophthalmic irrigation solution (Alcon, MO), and prestripped-only DMEK tissues were fully stripped. DMEK tissues for immunofluorescence and histology were stained with 0.4% trypan blue solution for 30 seconds and washed with BSS ophthalmic irrigation solution (Alcon), then unfolded on a glass slide and analyzed with an Eclipse TS100 brightfield microscope (Nikon, Tokyo, Japan) to determine the cell density and denuded areas (n = 6). The tissues for viability assay (n = 6) were immediately incubated with the viability assay.

Histological Analysis

Four corneal endothelial rolls (2 of each transport condition, n = 4) were fixed in 4% paraformaldehyde for 20 minutes at ambient temperature. After fixation, the tissues were halved, with one half used for histological analysis and the other for immunofluorescence analysis. The half used for histological analysis was placed overnight in a Citadel 2000 automated tissue processor (Thermo Fisher Scientific) and embedded in paraffin. The paraffin-embedded endothelial rolls were sectioned by using a Microm HM 355S microtome (Thermo Fisher Scientific) and mounted on glass slides. The mounted sections were then stained for hematoxylin and eosin to identify any morphological changes in the Descemet membrane and endothelium integrity. In brief, samples were first dewaxed with xylene and rehydrated with a decreasing series of ethanol (100%, 96%, 70%, and 50%) followed by water. After rehydration, the tissue samples were stained with hematoxylin Gill III (Sigma) for 5 minutes after a wash with running water for 5 minutes. Then, the samples were stained

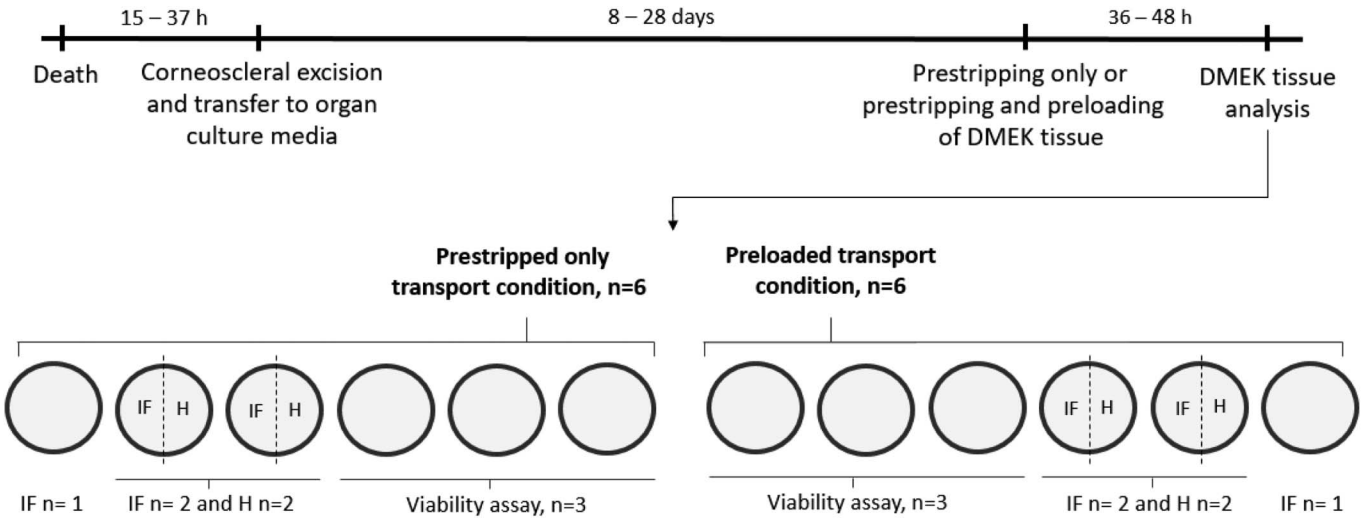

FIGURE 2. Schematic representation of timings and tissue use in this study. IF, immunofluorescence assay; H, histology.

with eosin (Sigma) for 1 minute, dehydrated with increasing series of ethanol (70%, 96%, 100%), and then cleared with xylene. Finally, the samples were mounted using DPX mounting media (Sigma). Descemet membrane thickness was analyzed using the ImageJ software distributor Fiji.[12] Briefly, a line was drawn across the Descemet membrane with the line-drawing tool and measured to determine the thickness of the membrane.

Immunofluorescence Analysis

Two full DMEK tissues (one of each transport condition, n = 2) and the 4 remaining halves of fixed DMEK tissues (2 of each transport condition, n = 4) were used for immunofluorescence analysis. The tissues were stained for ZO-1, Na^+/K^+ ATPase, and CD166 (n = 3 for each transport condition). Briefly, the DMEK tissues were fixed in 4% paraformaldehyde for 20 minutes at ambient temperature, and the cells were permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. After permeabilization, nonspecific antibody interactions were blocked with 2% BSA solution in PBS for 1 hour at ambient temperature. Tissues were incubated overnight at 4°C with primary antibodies diluted in 2% bovine serum albumin (BSA) solution. After primary antibody incubation, tissues were washed 3 times in PBS and then incubated with secondary antibodies and Hoechst 33342 for 50 minutes at ambient temperature in the dark. The DMEK tissues were then washed 3 times in PBS and mounted with coverslips with ProLong Gold antifade reagent (Thermo Fisher Scientific). Cells were examined on an Eclipse Ti-E inverted microscope equipped with a spinning disk (Nikon). All the information of the primary and secondary antibodies used can be found in Table 2.

Endothelial Cell Triple Staining for Viability

Six DMEK tissues (3 of each transport condition) were triple-stained with Hoechst 33342, ethidium homodimer-1, and calcein AM to determine the viability of the corneal endothelial cells after transport, as previously described.[13] Briefly, the tissues were first washed in BSS solution to remove serum esterase from the media. The whole endothelial rolls were then incubated in a BSS solution containing 4 μM ethidium homodimer-1, 2 μM calcein AM, and 3 μM Hoechst 33342 (Thermo Fisher Scientific) for 45 minutes at ambient temperature in the dark. After the incubation, the corneal endothelial rolls were unfolded on a glass slide and flat mounted using relaxation incisions without mounting media. A tile scan of the whole corneal endothelium was performed on an Eclipse Ti-E inverted widefield microscope (Nikon). The percentage of dead cells (nuclei stained with ethidium homodimer) compared with the total number of cells (nuclei stained with Hoechst) found in the unfolded corneal endothelial rolls was calculated using ImageJ software distributor Fiji.[12] To perform the particle analysis, the images were converted to binary format, and the stained cell nuclei were selected by circularity (circularity range 0.3–1) and size (size range: 25–670 μm^2) and were counted. The surface percentage covered with calcein AM and the denuded areas were also calculated using Fiji. For calcein AM quantification, the images were converted to binary, and the percentage of area covered was calculated. For the denuded areas, the denuded regions were manually selected and their surface area was measured.

TABLE 2. Primary and Secondary Antibody Details

Antibody/Stain	Manufacturer	Reference Number	Concentration, μg/mL
Mouse anti-CD166 primary	BD Biosciences (Allschwill, Switzerland)	559260	2.5
Rabbit anti-ZO-1 primary	Thermo Fisher Scientific (Waltham, MA)	402200	2.5
Mouse anti-Na^+/K^+ ATPase primary	Abcam (Cambridge, UK)	Ab7671	10
Goat antimouse Alexa Fluor 488 secondary	Thermo Fisher Scientific (Waltham, MA)	A11001	5
Donkey antirabbit Alexa Fluor 568 secondary	Thermo Fisher Scientific (Waltham, MA)	A10042	5
Hoechst 33342	Thermo Fisher Scientific (Waltham, MA)	H1399	1

Statistical Analysis

R Statistical Software[14] (v. 3.2.4) was used for statistical analysis. Descriptive values are shown as mean ± SD. Data were analyzed to check for statistical significance using a Student *t* test, considering samples with equal variances and a 0.05 significance level.

RESULTS

Donor Characteristics

Baseline donor characteristics did not differ between both transport conditions. The average donor ages for the prestripped-only and preloaded transport conditions were 63 (±15.87) and 61.2 (±14.68) years, respectively. The interval between death and tissue retrieval was 24.83 (±6.24) hours for the prestripped-only and 24.33 (±8.91) hours for the preloaded conditions. The average days in culture media of the donor tissue for prestripped-only and preloaded conditions were 22.17 (±3.76) and 20.83 (±7.55) days, respectively.

Tissue Preparation

Eye bank prestripping (n = 12) and preloading (n = 6) were performed by one experienced technician and was successful in all cases.

Tissue Analysis After Shipping

All tissues (n = 12) were received without evident damage. One of the preloaded DMEK tissues lost the trypan blue coloring and was not visible inside the cannula; the remaining 5 maintained the trypan blue coloring on arrival.

All tissues analyzed with light microscopy (n = 3 per transport condition) preserved the integrity of the corneal endothelial cell mosaic, regardless of the transport condition (Figs. 3A, C). The DMEK tissues had minimally denuded areas in the periphery, which were comparable in both transport conditions (Figs. 3B, D). Baseline endothelial cell count and cell loss did not differ between transport conditions. There was also no significant difference in central endothelial cell count (cells/mm^2) before and after transport in both conditions (prestripped only: 2733 ±273 vs. 2450 ±243; preloaded: 2733 ±175 vs. 2483 ±232).

Histological Analysis of DMEK Tissues

The hematoxylin and eosin staining of the DMEK tissue transported in both conditions showed the presence and integrity of a monolayer of endothelial cells on the Descemet membrane (Fig. 4). Descemet membrane thickness was statistically similar in both conditions, namely 3.67 μm (±0.21) for preloaded and 3.86 μm (±0.18) for prestripped only. These data indicated no macroscopic alterations in the Descemet membrane or the endothelial cells for either transport condition.

Immunofluorescence Analysis of Corneal Endothelial Phenotypical Markers on DMEK Tissue

Samples transported in both conditions were analyzed to detect the presence of CD166, Na^+/K^+ ATPase, and ZO-1 proteins (Fig. 5). The phenotype markers for the corneal endothelium CD166 and Na^+/K^+ ATPase showed a similar expression in both conditions (Figs. 5A, B, D, E). Moreover, the presence and expression of ZO-1 indicates the integrity of the cell monolayer and the maintenance of these tight junctions after the DMEK processing in both conditions (Figs. 5C, F). The data obtained indicated that the phenotypical markers of corneal endothelial cells were maintained in both conditions after transport.

Viability Staining for DMEK Tissues

The viability triple staining with Hoechst, calcein AM, and ethidium homodimer was used to assess the viability of the DMEK tissues transported in both conditions (Fig. 6). The whole-DMEK tissue staining of calcein AM (Figs. 6C, G) and ethidium homodimer (Figs. 6D, H) was similar in tissues transported in both conditions. The percentage of dead cells, calcein-covered area, and denuded areas was calculated with Fiji analysis software and was statistically similar in DMEK tissue transported in either condition. The percentage of dead cells was 16.73% (±7.45) for the preloaded transport and 18.1% (±5.01) for the prestripped-only transport condition. The calcein-covered surface in the preloaded condition was 61.95% (±7.63) and in the prestripped-only condition was 59.02% (±8.58). The denuded areas were 8.11% (±3.2) in the preloaded condition and 5.83% (±1.61) in the prestripped-only condition. These data reveal that the transport condition had no effect on the viability of the DMEK tissue for transplantation.

DISCUSSION

In this laboratory study, we compared the DMEK RAPID transport system from Geuder AG with conventional eye bank prestripped DMEK. Our results show no significant difference in endothelial cell density, tissue histology, surface marker expression, and endothelial cell viability, suggesting that this system is suitable for clinical use.

Previous studies describe different methods to preload DMEK grafts at the eye bank.[8–10] These allow the transport of prestripped DMEK tissue in either a modified Jones tube[8] or a modified lens insertion carrier.[9] Unlike the other preloaded transport systems, the DMEK RAPID uses a smooth-surface

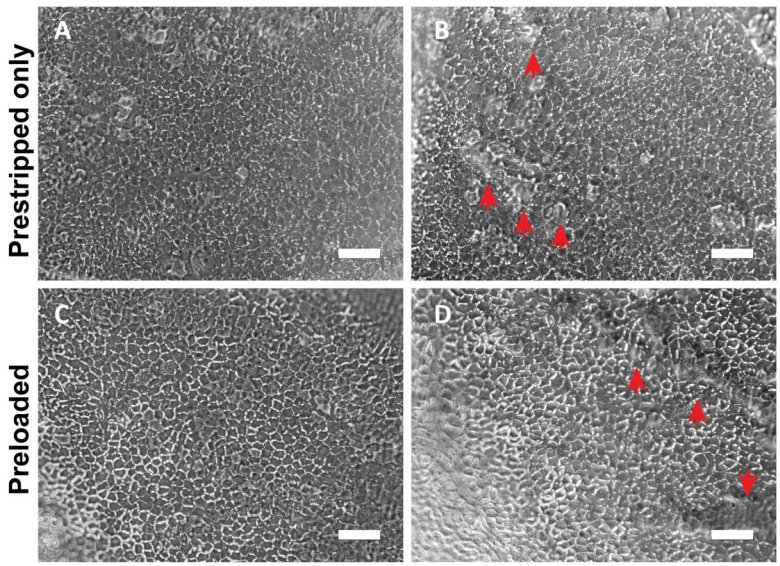

FIGURE 3. Representative brightfield images after the transport of prestripped-only (upper row) and preloaded (lower row) DMEK rolls. A and C are images of the central part of the corneal endothelial rolls, showing that the cell morphology and confluency were unaffected by either transport condition. B and C show minimally denuded areas (arrow heads) in the periphery of tissues transported in both conditions. Scale bar: 100 μm.

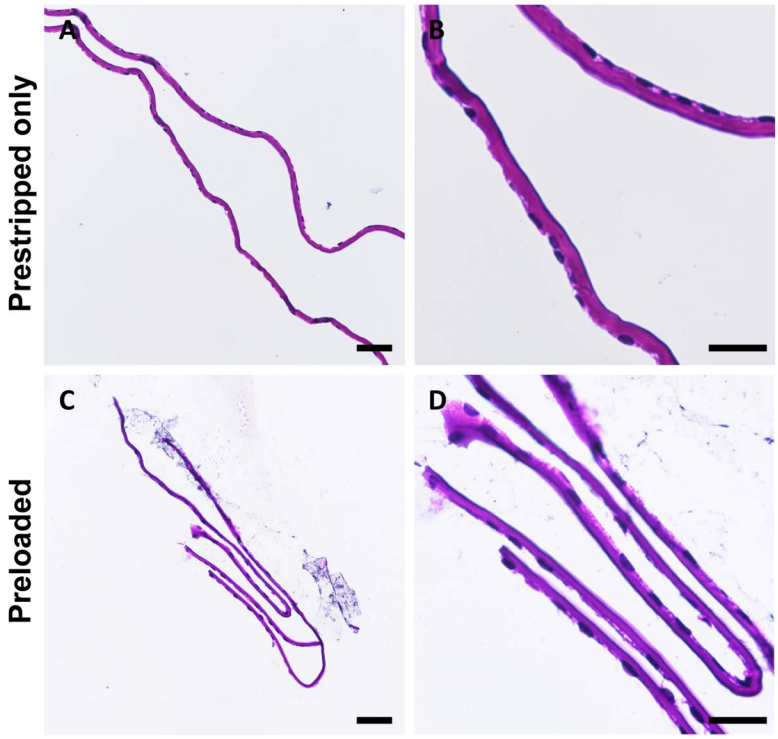

FIGURE 4. Representative images of hematoxylin and eosin stained sections of corneal endothelial DMEK tissues transported as either prestripped-only or preloaded endothelial roll. The thickness of the Descemet membrane in the endothelial rolls was comparable in both conditions, namely 3.67 μm (±0.21) for preloaded and 3.86 μm (±0.18) for conventional, $P = 0.48$. Moreover, a monolayer of cell nuclei stained with hematoxylin was present on the Descemet membrane in both conditions. B and D are a zoomed region of interest from images A and C, respectively. Scale bar: A and C: 50 μm; B and D: 25 μm.

and smooth-edged borosilicate glass cartridge that allows the surgeon to inject the sensitive donor tissue directly into the patient's anterior chamber without manipulation of the endothelium through an astigmatism neutral incision.[15–17] Moreover, the high transparency of the glass cannula enables correct orientation of the lamella to be controlled during surgery.

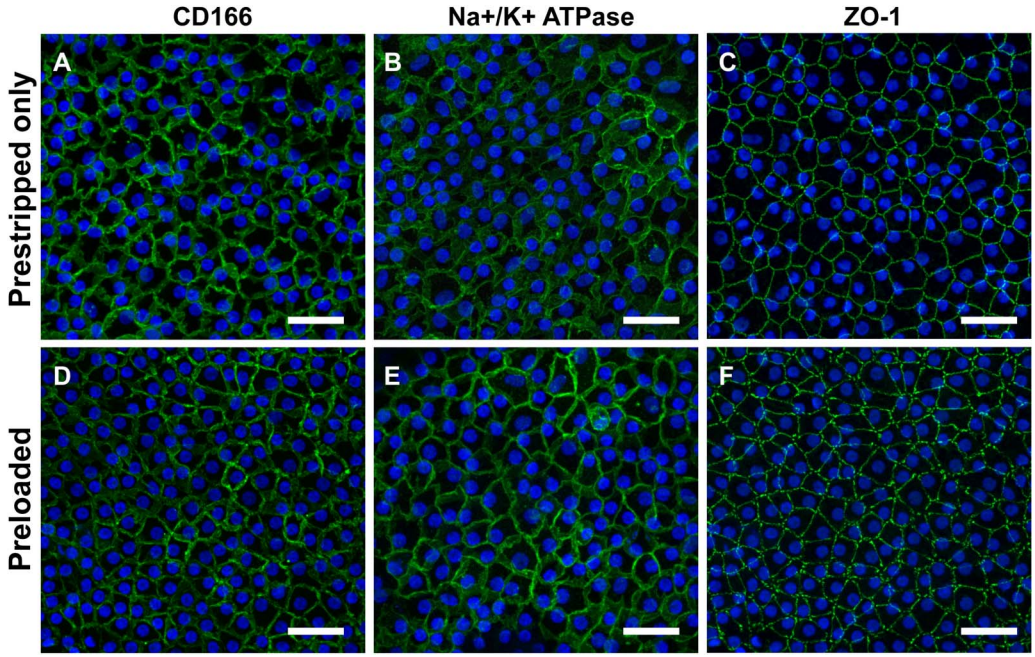

FIGURE 5. Representative images of immunofluorescence stainings of phenotypical and functional protein markers (green) of the corneal endothelial DMEK tissues transported in both prestripped-only and preloaded conditions. Cell nuclei (blue) were stained with Hoechst. A and D are immunofluorescence analysis for CD166, B and E for Na^+/K^+ ATPase, and C and F for ZO-1. All the protein markers expression was comparable in both transport conditions and showed integrity of the tight junctions in the corneal endothelial monolayer in both conditions. Scale bars: 50 μm.

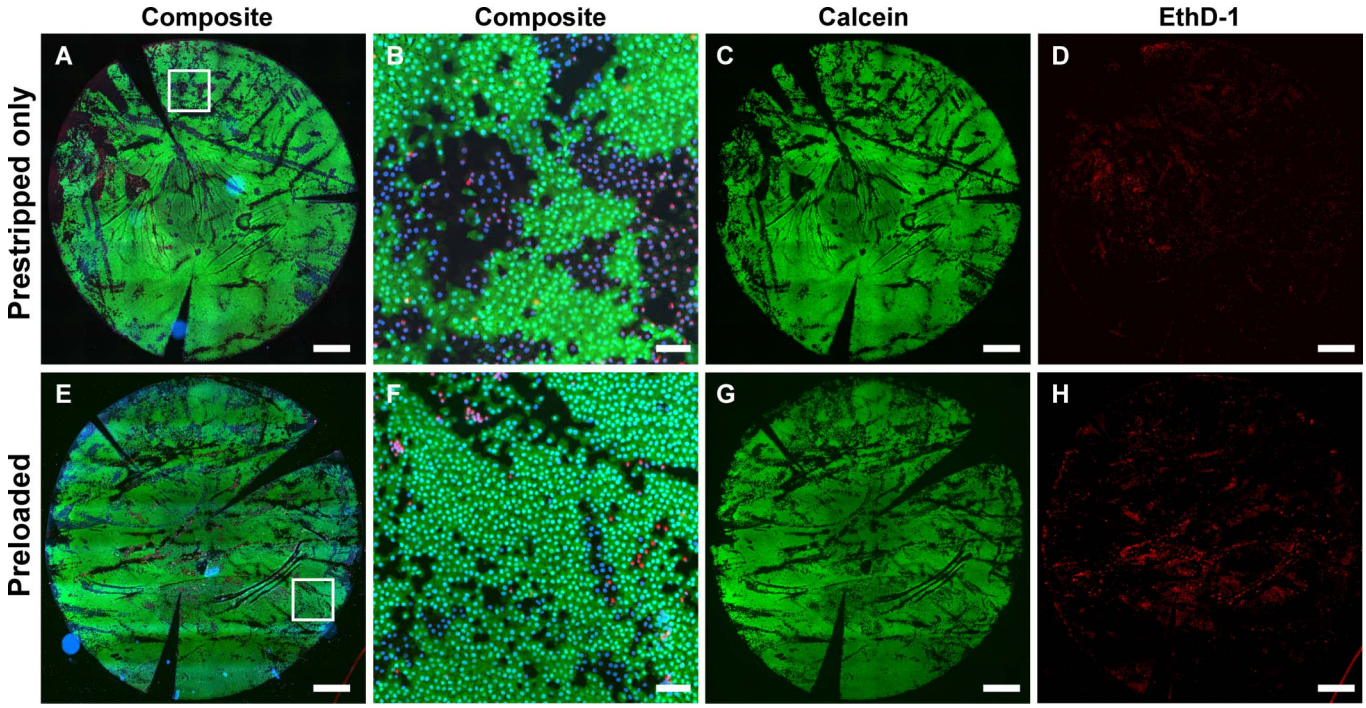

FIGURE 6. Representative images of live/dead triple staining of full corneal endothelial DMEK tissues transported in both prestripped-only (A–C) and preloaded (E–H) transport conditions. A and E are merged images of Hoechst (blue), calcein (green), and ethidium homodimer (red). B and F are zoomed in of the regions indicated in A and E with a square. C and G are calcein fluorescent regions. D and H are ethidium homodimer fluorescent regions. The percentage of dead cells is statistically equal in DMEK tissue transported in either condition, being 16.73% (±7.45) for the preloaded transport condition and 18.1% (±5.01) for the prestripped-only transport condition, $P = 0.81$. Furthermore, the calcein-covered surface (green surface) and the denuded areas (acellular areas) seem to be equal in DMEK tissue transported in either condition. The calcein-covered surface in the preloaded condition was 61.95% (±7.63), whereas in the prestripped-only condition was 59.02% (±8.58), $P = 0.68$. The denuded areas were 8.11% (±3.2) in the preloaded condition and 5.83% (±1.61) in the prestripped-only condition, $P = 0.33$. n = 3 preloaded, n = 3 prestripped only. Scale bar: A, C, D, E, G and H: 1 mm; B and F: 100 μm.

Storage and transport were not associated with significant central endothelial cell loss in both conditions, despite the use of Dextran because of the eye bank regulations.[18,19] The denuded areas in the periphery of the donor grafts (Fig. 3), irrespective of transport conditions, likely represent stress lines caused by prestripping and/or tissue handling during experiments. Histological analysis (Fig. 4) confirmed the integrity of Descemet membrane and endothelial cell layer and revealed no difference in Descemet membrane thickness (3.67 vs. 3.86 μm) between transport conditions.

Regarding the endothelial cell phenotype, in both conditions, the corneal endothelial cell mosaic was conserved. The ZO-1 tight junctions detected with immunofluorescence (Figs. 5C, F) confirmed the integrity of the confluent corneal endothelial cell monolayer. CD166 and Na^+/K^+ ATPase detection with immunofluorescence (Figs. 5A, B, D, F) was also comparable in both conditions, confirming the maintenance of corneal endothelial–specific proteins (CD166) and metabolic functional endothelial transporters (Na^+/K^+ ATPase) in both conditions.

The viability assay showed a similar percentage of dead cells in both transport methods (18.1% vs. 16.73%). Moreover, the calcein-covered surface (59.02% vs. 61.95%) did not differ between transport conditions. A comparable percentage of cells in both transport conditions were still alive, but not metabolically active, reflected by the lack of calcein-associated fluorescence and the lack of ethidium homodimer nuclear staining. This cell population was only recognized; thanks to the triple staining (Hoechst, calcein AM, and ethidium homodimer) used in these experiments. Viability studies performed only with calcein AM would not recognize this population of cells. The findings reported in this study are in-line with previous publications on preloaded tissue for DMEK using a modified Jones tube[8] or a modified lens insertion carrier.[9] The cell viability determined by the calcein-covered surface ranged from 37.8% to 70.1% with these transport techniques.[10] Compared with the previously studied preloaded techniques, the Geuder transport system shows favorable cell viability (61.95%) compared with the modified Jones tube (61.95% vs. 37.8%) and a comparable cell viability to the modified lens insertion carrier (61.95% vs. 70.1%).[10] Given the limited number of suitable tissues, the current study focused on the effects of the new transport system from Geuder in comparison to conventional prestripped-only method. A future side-by-side comparison of existing preloaded DMEK transport methods would give further clinically relevant data.

With this study, it can be concluded that the DMEK RAPID preloaded transport system delivers DMEK donor

tissue with comparable endothelial cell density, phenotypical marker expression, and viability to conventional prestripped donor tissue. Implementation of this system could facilitate the transition to DMEK for novice surgeons, shorten the duration of surgery, and take advantage of the tissue preparation skills of eye bank professionals to minimize iatrogenic endothelial cell loss.

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