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Quantitative Analysis of Endothelial Cell Loss in Preloaded Descemet Membrane Endothelial Keratoplasty Grafts

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Abstract

Purpose—Availability of preloaded DMEK (pDMEK) tissue may increase acceptance of DMEK in surgical management of endothelial disease. The goal of this study is to determine the safety of pDMEK grafts for 24 hours prior to surgery by analyzing endothelial cell loss (ECL) using two image analysis software programs.

Methods—Eighteen cadaveric corneas were prepared for DMEK using a standardized technique and loaded in a modified jones tube injector. Nine of the corneas were injected into Calcein AM vital dye after 1 minute (controls) and the remaining nine corneas were left preloaded for 24 hours prior to injection into vital dye for staining. The stained corneas were imaged using an inverted confocal microscope. ECL was then analyzed and quantified by 2 different graders using two image analysis software programs.

Results—The control DMEK tissue resulted in 22.0 ± 4.0 % ECL compared to pDMEK tissue which resulted in 19.2 ± 7.2 % ECL ($p=0.31$). Inter-observer agreement was 0.93 for MetaMorph and 0.92 for Fiji. The average time required to process images with MetaMorph was 2 ± 1 minutes, and with Fiji was 20 ± 10 minutes. Intra-observer agreement was 0.97 for MetaMorph and 0.93 for Fiji.

Conclusions—Preloading DMEK tissue is safe and may provide an alternative technique for tissue distribution and surgery for DMEK. The use of MetaMorph software for quantifying ECL is a novel and accurate imaging software with increased efficiency and reproducibility compared to the previously validated Fiji.

Introduction

Endothelial Keratoplasty (EK) is a partial thickness corneal transplantation technique where only the posterior layers of the cornea are transplanted. The specific type of EK performed over the years has gone through many modifications with significant improvements in both surgical technique and visual outcomes.¹⁻³ The most recent form of EK being widely performed is Descemet Membrane Endothelial Keratoplasty (DMEK) which provides the most rapid visual recovery of current EK surgeries, with further improved final best-corrected visual acuity, and reduced rates of rejection.^{4, 5}

Outcomes of DMEK, specifically time to visual recovery and final visual outcome, have been consistently shown to be superior to DSAEK.⁶⁻⁹ Given these benefits, corneal surgeons are beginning to utilize DMEK over DSAEK. Over the past 3 years, there has been a sharp increase in the number of DMEK surgeries being performed (from 748 in 2012 to 6,459 in 2016). However, DMEK still only accounts for 23% of all EK procedures performed in the US.⁶⁻¹⁰ Surgeons have been slow to convert to DMEK because of the steep operative learning curve and the technical difficulty of donor corneal tissue preparation.^{11, 12} Any damage to the DMEK tissue can lead to endothelial cell loss (ECL) and may contribute to the higher rates of graft detachment and failure.^{4, 5, 13}

Eye bank preparation of pre-stripped tissue can aid in acceptance and increased conversion to DMEK, similar to DSAEK nearly a decade ago.^{10, 11, 14, 15} Eye banks began providing pre-stripped DMEK tissue around 2013; however, more tissue preparation is still needed by the surgeon in the operating room prior to surgery which can damage the fragile tissue. The optimal tissue delivery would be one where the tissue was completely stripped, trephined, and stored in the injector ready to go on the day of surgery. This can decrease tissue damage by the surgeon and thus decrease the likelihood of endothelial cell loss (ECL).

The purpose of this study is to develop an optimized DMEK corneal tissue storage and delivery method and to compare the accuracy and reproducibility of MetaMorph (Molecular Device Inc., Downingtown, PA, USA) ECL analysis as compared to analysis by Fiji (<http://Fiji.sc>). The novel DMEK tissue delivery system involves completely stripping, staining, trephining, and storing the DMEK tissue in a Straiko Modified Jones Tube (Gunther Weiss Scientific Glass, Portland, OR) injector 24 hours prior to surgery.

Methods

Corneal Tissue

Eighteen cadaver corneas stored in Optisol GS (Bausch and Lomb, Rochester, NY, USA) were obtained from Eversight (Ann Arbor, Michigan). All tissues in this study had normal endothelium but were deemed unsuitable for surgical use for other reasons including donor high-risk behaviors, positive serological results, or stromal infiltrate. Tissue from donors with a history of diabetes were excluded. Endothelial cells were quantified using specular microscopy (Konan Medical, Irvine, CA). The deceased donor corneal tissues were de-identified prior to receipt by the study investigators. The University of Michigan Institutional Review Board determined the study to not need regulation.

Corneal Tissue Preparation

The corneas were prepared for DMEK by an experienced eye bank technician using a standardized technique.¹¹ Briefly, the tissue was stabilized with the endothelium side up on the Barron Punch Cutting Block (Katena, Denville, NJ, USA). A partial thickness 9.5mm trephination was performed. Trypan 0.06% (DORC, Zuidland, Netherlands) staining was performed. The ring of EDM outside the 9.5mm trephination zone was peeled off. The peripheral EDM was then gently separated from the underlying stroma first circumferentially then limbus to limbus, stopping 1–2 mm short of complete separation and leaving a peripheral hinge. The EDM was gently folded back and a stromal punch was created. The tissue was positioned endothelium down and an S stamp was placed on Descemet's membrane through the stromal punch. The tissue was then placed endothelium side up in the Barron Punch Cutting Block (Katena, Denville, NJ, USA) and a 7.5mm partial thickness trephination was performed. 7.5 mm is the standard size donor graft used by the main surgeon in this study. The tissue was then placed in a viewing chamber.¹¹

An experienced DMEK surgeon (SM) prepared the DMEK tissue using a standardized technique previously described with some modifications.[16] Briefly, the tissue was gently removed from the viewing chamber and placed endothelium up on a Barron Punch Cutting Block. The DMEK graft was gently separated from the stromal base, which was filled with Trypan 0.06% (DORC, Zuidland, Netherlands), and the DMEK tissue placed in this pool for 30 seconds to allow for visualization of the tissue edges. The tissue was then gently floated in a petri dish of Optisol from which it was loaded in a Straiko Modified Jones Tube injector also filled with Optisol. The injector holds 200 ul of Optisol. Nine of the corneas were injected into Calcein AM (CAM; Catalog number C3100MP, Molecular Probes, Eugene, OR) vital dye after 1 minute, as controls. The remaining nine corneas were left in the injector for 24 hours prior to injection. Adjustments were made to the Straiko Modified Jones Tube injector. The single lumen #14 french nasogastric catheter plastic tubing (Covidien, Mansfield, MA) used as a coupler was cut long (14 cm) and looped around from the back end of the Straiko Modified Jones Tube and attached to the front end creating a closed loop system filled with Optisol GS; the system held approximately 1 ml of fluid. Care was taken to avoid any air bubbles in the system. Two plastic clips were used to seal off the catheter tubing at both ends to prevent the tissue from moving out of the injector and getting caught in the tubing (Figure 1). These pre-loaded injectors were each stored in an empty 50ml Eppendorf tube placed in a tube holder to prevent movement. The nine pre-loaded tissue were packaged in ice and driven around for an hour to give a semblance of transportation juggling. They were then returned to a 4 degree Celsius fridge for the remainder of the time.

Endothelial Cell Staining

Endothelial cell viability staining was performed using Calcein AM dye (CAM; Catalog number C3100MP, Molecular Probes, Eugene, OR) as previously described by Jardine et al.¹⁷ Briefly CAM was thawed, and 50 ul was added to 100 ul of dimethyl sulfoxide (DMSO; Molecular Probes, Eugene, OR). The solution was manually mixed, then pipetted into 20 ml of BSS (Alcon, Ft. Worth, TX, USA). Several drops of calcein were placed in the center of a glass bottom 35mm petri dish. The DMEK grafts were then injected into the pool

of calcein and unscrolled, endothelial side down, using Viscoat (Abbott Medical Optics, Santa Ana, CA).

Imaging

The unscrolled grafts were imaged on a Nikon A1 confocal microscope with a 10× objective (N.A. 0.45). The green fluorescence calcein signal was excited with a 488 nm argon laser and captured by a photomultiplier tube detector using a 500–530 nm filter. DMEK grafts were typically captured in an 8×8×20 XYZ-array at 256×256 resolution with an optical sectioning of 78.5 mm and z-step size of 45 mm. Alterations were sometimes made depending on the undulation of the graft (Figure 2). The images obtained were flattened using a maximum projection option in the Nikon Elements Software (Nikon Instruments Inc., America). The final images had areas of fluorescence, indicating viable cells, and areas of hypofluorescence, indicating cell drop out.

Standardized Image

A standardized image was created as described previously.¹⁷ Briefly, the image contained 500 × 500 pixels (250,000). Ten squares of multiple sizes and differing shades of grey were placed within the image; 150039 pixels, 60.02% area. The image was analyzed using Fiji and Metamorph to ensure accuracy in identification.

Image Analysis

Endothelial Cell Assessment—Two independent readers analyzed images for ECL on both Fiji and MetaMorph images per the protocols detailed below. They were blinded to the tissue preparation. One reader read the images twice, in random order and without access to prior grades, to allow for intraobserver agreement calculations. These numbers are averages of the percent ECL obtained by reader 1.

Fiji—Images were analyzed with Fiji enhanced image analysis software, part of ImageJ, an open source imaging program (<http://Fiji.sc>), as previously described by Jardine et al.¹⁷ Briefly, the images were converted to monochrome 16-bit images. The size of the images was decrease to 1000 pixels on their long axis. Contrast and brightness were enhanced to maximize differences in pixel intensities in areas of calcein uptake and calcein absence. The trainable segmentation (Weka) plug-in was opened. It was trained to recognize areas of hypofluorescence, hyperfluorescence, and the background. Once satisfied with training, a final segmentation image was created and saved. (Figure 3) The histogram feature was used to determine percent of ECL.

MetaMorph—Images were also analyzed using MetaMorph image analysis software (Molecular Device Inc., Downingtown, PA, USA), which has not been previously used for corneal endothelial cell imaging. The current standard in determining ECL in DMEK grafts is vital dye staining, microscopic imaging, and endothelial cell analysis with Fiji trainable segmentation software (<http://Fiji.sc>)¹⁸. Fiji has been previously validated for accuracy, however, it lacks in efficiency.[17] MetaMorph (Molecular Device Inc., Downingtown, PA, USA) is a commercially available image analysis software used by image analysis scientists

that provides an automated, and thus more efficient, and reliable approach to quantifying ECL.

Unlike Fiji, MetaMorph does not use local thresholds to determine signals from their immediate surroundings. MetaMorph uses an absolute threshold value that is applied across the whole image and any signal outside the upper or lower threshold limit is excluded from the analysis.

The MetaMorph protocol began by opening up the maximum projection images and a region was manually drawn around the tissue, with care taken to incorporate the edges of the graft which were always visible, separating it from the background. The region was saved and the threshold tool was opened and the defaults were set to a 12-bit dataset and inclusive threshold. The threshold intensity was manually adjusted by the user for each cornea until all of the dead cells were included within the range of the threshold while excluding all of the live cells. Then measurements were exported to a Microsoft Excel (Microsoft, Seattle, WA) spreadsheet. The post-analysis images were copied and pasted into Microsoft Excel as well. (Figure 3) The area of dead cell signal was used to calculate the ECL. The percentage ECL was measured as the percent threshold of drop signal related to the region drawn around the tissue.

Statistical Analysis

Differences between groups were compared. Baseline tissue characteristics were evaluated, and differences between the control and preloaded groups compared using 2 sample t tests. All eighteen corneal tissues were pooled to compare ECL grades by MetaMorph and Fiji image analysis software. Intra-observer and inter-observer differences within the same analysis software and between softwares was compared using intra-class correlation coefficients. MetaMorph software was used to assess the degree of ECL; the nine control and the nine preloaded tissues were compared using 2 sample t tests. All quantitative data analysis was performed using STATA version 14.0 (STATA-Corp LP, College Station, Texas, USA).

Results

Tissue Characteristics

A total of 18 tissues, from 14 donors, were obtained for study. Tissue characteristics included a mean donor age of 63 $\pm$ 10 years, mean death to preservation time of 12.2 $\pm$ 5.3 hours, and mean endothelial cell density at time of procurement of 2386 $\pm$ 339 cells/mm².

Nine DMEK lenticules were prepared for immediate injection (control group) and nine were pre-loaded for injection after 24 hours (study group). There were no differences in the tissue characteristics above between immediately injected tissue and pre-loaded tissue.

Standardized Image

The standardized image contained 10 square regions of varying levels of gray signal that covered 150,039 pixels of a total of 250,000 pixels (60.002%). Fiji detected 150,023 of the grey pixels (0.0% error). MetaMorph detected all of the 150,039 grey pixels (0.0% error).

Pre-loaded tissue versus immediately injected tissue

The control DMEK tissues resulted in 22.0 ± 4.1 % ECL (MetaMorph) and the study DMEK tissues stored for 24 hours prior to injection resulted in 19.2 ± 7.2 % ECL ($p=0.31$) listed in Table 1.

MetaMorph versus Fiji

The mean (SD) pooled ECL (pooled across all tissue) as analyzed by MetaMorph was 22.0 ± 6.6 % and by Fiji was 24.2 ± 6.4 % (Table 2, Figure 4). Interobserver agreement was 0.93 for MetaMorph and 0.92 for Fiji. Intraobserver agreement was 0.97 for MetaMorph and 0.93 for Fiji. The average time required for image analysis was 2 ± 0.5 min for MetaMorph and 15 ± 5 minutes for Fiji. Among the two readers, the mean time required to process images with MetaMorph was 2 ± 1 minutes, and with Fiji was 20 ± 10 minutes. The mean number of total Fiji tracings per image was 75 ± 51 , with a mean of 3 ± 2 segmentation trainings prior to image finalization amongst both readers.

Matching of Digital Stained Corneas to Their Processed Images

The main grader matched randomized images of the stained corneas to the appropriate MetaMorph and Fiji processed images of the same corneas. There was 100% success with both MetaMorph and Fiji.

Discussion

Pre-loaded DMEK Tissue

Preloading DMEK tissue in an injector and storing for 24 hours did not result in significantly increased endothelial cell loss showing the clinical feasibility of such an approach. This study adds to early literature on preloaded DMEK which can potentially make the surgery easier to perform, thus increasing the number of surgeons able to perform DMEK and benefit from improved outcomes.

A detailed report of eye bank preparation and preloading of DMEK donor tissue using was recently published by Tran et al.¹⁹ The DMEK tissue were stored in a Straiko modified Jones tubes similar to the DMEK tissue in this study. However the tube ends were not capped, and it was secured in the Krolman viewing chamber. ECL was evaluated after overnight storage. There was a statistically significant difference when comparing preloaded tissue to simply prestripped tissue, with the preloaded tissue incurring more ECL. The difference is likely due to comparison of preloaded tissue to non-preloaded tissue. However, both groups had clinically acceptable ECL.

Methods by which tissue can be analyzed post preloading were not addressed in our current study but they will need to be explored and studied prior to the clinical provision of

preloaded DMEK tissue to surgeons. In our study the preloaded scrolled tissue is visible in the Straiko modified jones tube as it is a clear glass tube. Techniques similar to those described by Tran et al. where images of the scroll are taken in glass tube may be considered with our methodology.

Parekh et. al described the first attempt at preloading DMEK tissue with good initial success.²⁰ However, ECL analysis and quantification techniques are not described in detail and are different to what has been recently described by studies from groups in the US and in this study.^{17, 21}

Surgeon's routinely stain the DMEK graft tissue with trypan blue for visualization during surgery. This is typically done immediately after stripping the tissue. Providing preloaded DMEK tissue requires trypan staining to be visible at the time of surgery. The tissue can be stained while it is in the injector/closed loop system; trypan can be introduced into the system and rinsed out with BSS. However, this creates turbulence of the stripped DMEK graft which may result in more ECL. Differing methods of introducing trypan blue staining will need to be evaluated.

ECL rates were high in our study, though these rates were after processing and injection with a method of quantification that is highly sensitive. The ECL rates are comparable to other similar studies.^{17, 21} Because we injected the tissue in order to analyze it, the technique likely resembles the rates of percent loss of ECD seen in clinical DMEK studies, where anywhere from 34% to 47% loss has been reported at 6 months.^{4, 9, 23, 24} In addition, unscrolling DMEK tissue on a slide prior to assessment may be more disruptive than clinically in the anterior chamber.

The main limitation of this study is a direct result of the staining technique utilized. CAM staining, as described by Jardine et al, is a relatively easy staining technique which highlights all viable cells with good results. However, it does not allow further differentiation of etiology of cell drop out, that is, cell death or cell separation/stunning where the latter may revive and repopulate thus leading to an overestimation of ECL. In addition, DMEK preloading technique in this study is not the preferred technique of many US surgeons.²² In our study DMEK tissue was preloaded using the Straiko Modified Jones Tube injector method, which loads DMEK tissue in a no-touch technique as a scroll with endothelium on the outside.¹⁶ The goal is to introduce a preloaded DMEK technique that will facilitate the preferred surgical technique for current DMEK surgeons and obviate the need to learn new technical skills.

MetaMorph Image Analysis Software

MetaMorph was used in a novel manner to analyze endothelial cells in DMEK tissue, and was found to be an accurate imaging software in this setting with increased efficiency and similar accuracy and reproducibility in quantifying ECL as compared to the previously validated Fiji software. It also allows both a quantitative and a qualitative analysis of the tissue. The use of MetaMorph may be limited by the high cost of acquiring the software as compared to Fiji, which is a free open source software.

Improving analysis techniques by improving sensitivity, accuracy, and reproducibility is of utmost importance in allowing timely analysis of potential safety and clinical success of new endothelial donor graft preparation and clinical surgical techniques, especially as the DMEK preparation technique evolves. Improved analysis techniques will have long lasting positive consequences on future experiments on endothelial graft preparation and surgical techniques.

Conclusion

This study shows the clinical feasibility of eye banks providing pDMEK to surgeons. This may be able to assist in reducing surgeon-induced tissue damage and decrease the operative time for DMEK surgeries. Most importantly, pDMEK may help lower the complexity of DMEK surgery by removing tissue preparation and tissue manipulation from the operative procedure. The study also shows a novel use for the commercially available Metamorph software in quantifying ECL and validates its accuracy as well as its increased efficiency compared to the previously validated Fiji.

Further investigation including a testing of both the final prototype for the pDMEK storage container and full eye bank preparation of pDMEK will be needed to draw final conclusions about clinical feasibility. Clinical trials with pDMEK will also need to follow to determine clinical efficacy. The long-term goal is that pDMEK will help surgeons convert to DMEK as the EK surgical procedure of choice and provide patients with the faster visual recovery and improved visual outcomes provided by DMEK.

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Figure 1.
Images of the catheter/injector set up used to load and store the DMEK grafts overnight
(without Optisol and DMEK grafts).*
* Pre-clinical experiment set up only.

Figure 2.

A. 3 dimension projection image of calcein AM vital dye stained cornea. B. Color map of A representing the variations in depth of the tissue (blue represents signal closest to the glass bottom of the dish and red represents the deepest signals) C. Cross sections through the adjacent full cornea image showing the undulations of the tissue on the z axis. The numbered lines in A represent the XZ cross sections in C. Scale bars = 1 mm.

Figure 3.
Final images after analysis showing ECL (hypofluorescent or black areas). A. MetaMorph (left) B. Fiji (right). Compared to C. Initial image obtained (bottom left).

Figure 4.

The mean distribution of ECL amongst the 18 grafts analyzed using the two quantification methods discussed. In all grafts except for tissue #4, #8, and #9, MetaMorph ECL was slightly less than Fiji ECL. This difference was not statistically significant.

Table 1

Endothelial Cell Loss Percentages of Immediately Injected Tissues vs. Preloaded Tissues

% ECL (MetaMorph [*])	Mean	SD	Min	Max
Immediately Injected Tissues (n=9)	22.1	4.1	17.2	29.3
Pre-loaded Tissues (n=9)	19.2	7.2	11.6	30.4

^{*} Reported ECL from MetaMorph Analysis only

Table 2

Endothelial Cell Loss Percentages measured by Metamorph versus Fiji

	%ECL as analyzed by Metamorph			%ECL as analyzed by Fiji		
	Reader 1 First	Reader 1 Second	Reader 2	Reader 1 First	Reader 1 Second	Reader 2
Average (SD)	20.3 (5.9)	20.9 (6.1)	23.4 (7.4)	24.0 (5.3)	23.7 (5.9)	24.6 (7.8)
Range (Min, Max)	11.7, 28.7	11.4, 32.5	12.4, 39.1	13.2, 30.0	11.1, 32.6	9.4, 39.8