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Elucidation of the migratory behaviour of the corneal endothelium

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CHAPTER 3

Endothelial cell viability after DMEK graft preparation

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ABSTRACT

Aim: To evaluate the effect of graft preparation and organ-culture storage on endothelial cell density (ECD) and viability of Descemet membrane endothelial keratoplasty (DMEK) grafts.

Materials and methods: DMEK grafts (n = 27) were prepared at Amnitrans EyeBank Rotterdam from 27 corneas (15 donors) that were eligible for transplantation but could not be allocated due to the Covid-19-related cancellation of elective surgeries. Cell viability (by Calcein-AM staining) and ECD of five grafts originally scheduled for transplantation were evaluated on the originally planned surgery day, whereas 22 grafts from paired donor corneas were evaluated either directly post-preparation or after 3–7 days of storage. ECD was analyzed by light microscopy (LM ECD) and Calcein-AM staining (Calcein-ECD).

Results: Light microscopy (LM) evaluation of all grafts showed an unremarkable endothelial cell monolayer directly after preparation. However, median Calcein-ECD for the five grafts initially allocated for transplantation was 18% (range 9–73%) lower than median LM ECD. For the paired DMEK grafts, Calcein-ECD determined by Calcein-AM staining on the day of graft preparation and after 3–7 days of graft storage showed a median decrease of 1% and 2%, respectively. Median percentage of central graft area populated by viable cells after preparation and after 3–7 days of graft storage was 88% and 92%, respectively.

Conclusion: Cell viability of most of the grafts will not be affected by preparation and storage. Endothelial cell damage may be observed for some grafts within hours after preparation, with insignificant additional ECD changes during 3–7 days of graft storage. Implementing an additional post-preparation step in the eye bank to evaluate cell density before graft release for transplantation may help to reduce postoperative DMEK complications

INTRODUCTION

Descemet membrane endothelial keratoplasty (DMEK) is an increasingly popular treatment option for patients with corneal endothelial dysfunction, optimizing visual out- comes, recovery time, and rejection risk relative to previous generation of corneal transplantation, such as penetrating keratoplasty (PK) and Descemet stripping (automated) endothelial keratoplasty (DSAEK). Typical endothelial cell loss rates within the first 6 months after DMEK are reported to be 25–40%, followed by a slower decrease thereafter.[1–5] The majority of the observed cell loss after DMEK, however, actually occurs within the first week after DMEK.[6] At the same time, it was shown that endothelial cell loss after DMEK showed a high degree of variability between patients. Several studies tried to identify donor, recipient and surgery-related risk factors for endothelial cell loss after DMEK, with no consistent results so far.[5,7–10]

Next to focusing on the postoperative DMEK outcomes regarding endothelial cell loss, other studies aimed at validating graft preparation techniques. Some studies compared different graft preparation techniques[11,12] in terms of efficiency and observed endothelial cell loss. It was also evaluated whether, e.g., the speed of stripping,[13] type of graft storage,[14,15] other eye bank[16,17] or surgeon-related graft manipulations,[18,19] or the use of pre-loaded systems[20–23] would be associated with a reduction in endothelial cell density.

In a recent study,[6] we speculated that overestimation of endothelial cell viability on the DMEK graft may play a role and may result in an unrealistically high ECD drop in the early postoperative phase after DMEK. However, there is currently no possibility for eye banks to evaluate the endothelial cell viability of grafts allocated for transplantation. Typical graft evaluation in the eye bank is performed by trypan blue staining that fails to recognize apoptotic cells[24–26] and stained nuclei in a non-continuous area will not be discerned macroscopically.[27] Although in vitro analysis of endothelial integrity, including the analysis of sample subpopulations (apoptotic and necrotic), by using Calcein-AM staining was reported to be safe for pre-stained, pre-stripped, or pre-loaded DMEK grafts,[16,20–23] this protocol has not yet been approved for eye bank use.

While in vitro studies indicated that the endothelial cell counts provided by eye banks seem to overestimate the actual number of viable endothelial cells on a graft,[12,28] the use of research-grade tissue, i.e., corneas not eligible for transplantation, may not be directly comparable to surgery-grade tissue as the endothelial quality may often be lower to start with.

As a consequence of the COVID-19-related cancellation of elective surgeries in the Netherlands, we were able to analyze the endothelial cell density and viability of DMEK grafts on the day of the planned surgeries and additionally evaluated the effect of organ-culture storage on the endothelial cell density and viability of surgery-grade DMEK grafts.

MATERIALS AND METHODS

Corneas

Twenty-seven human corneas were obtained from 15 donors (mean age 70 (±8) years; range 59–85 years), for a total of 27 DMEK grafts. Prior to graft preparation, the average storage time was 21 (±6) days (range 9–29 days) and average ECD was 2588 (±139) cells/mm^2 (range 2300–2800 cells/mm^2) (**Table 1**). All corneas would have been eligible for transplantation but could either not be sent out after allocation (corneas #1–5) or could not be allocated (corneas #6–27) due to the COVID-19 related cancellation of elective surgeries. In all cases, the donors had stated to have no objection against transplant-related research and the study adhered to the tenets of the Declaration of Helsinki and the Barcelona Principles.[29] No institutional review board approval was obtained as under national regulation no approval is required for this research if no extra procedure was

performed to obtain the samples and donors had consented to having the samples used for research purposes (https://www.ccmo.nl/onderzoekers/aanvullende-informatie-over-bepaalde-soorten-onderzoek/niet-wmo-onderzoek/onderzoek-met-lichaamsmateriaal).

DMEK graft preparation techniques

All DMEK-grafts were prepared at Amnitrans EyeBank Rotterdam by means of hydrodissection[30] and/or no-touch DMEK graft preparation.[31,32] Briefly, to prepare the DMEK grafts, corneo-scleral rims were placed endothelial-side-up on a custom-made holder with a suction cup. The endothelium was then stained with 0.04% hypotonic trypan blue solution (Hippocratech, Rotterdam, The Netherlands) for 10 seconds for visualization. A 30-gauge needle mounted on a 2.5 mL syringe filled with 0.9% NaCl was inserted superficially through the tissue just peripheral to the pigmented trabecular meshwork and advanced until the entire bevel was in the cornea just past Schwalbe's line. NaCl was injected slowly until a peripheral separation "bubble" between the stroma and the Descemet membrane (DM) appeared, after which the needle was inserted in the bubble to enlarge the bubble until complete separation of the DM. In case of unsuccessful bubble formation, the same process was attempted at another site. After complete bubble formation, the peripheral DM was pierced with a 30-gauge needle and liquid was drawn from the bubble before an 8.5 mm trephination was performed directly on the cornea to complete the DMEK graft preparation.

In cases that no bubble could be formed, the "no-touch" DMEK graft preparation method was applied.[31,32] First, trabecular meshwork was loosened over 360° by pushing a hockey stick blade from the trabecular meshwork towards the corneal center. Then, by holding the trabecular meshwork with McPherson forceps and making gentle centripetal movements, the DM was carefully peeled from the posterior stroma before trephination with an 8.5 mm trephine.

Endothelial cell density of the corneas used for graft preparation was assessed in the eye bank with an inverted light microscope (Zeiss Axiovert 40C, Carl Zeiss International, Zaventem, Belgium) using the fixed frame method.[32] Endothelial cell density (ECD) of the DMEK rolls directly after preparation was determined on light microscopy (LM) images by averaging the counts performed manually on three fixed frames of 0.01 mm^2 per graft (reported as LM ECD). Grafts were considered eligible for transplantation if outcome of visual inspection after graft preparation was unremarkable (i.e., absence of bare DM areas or other irregularities), an ECD ≥ 2000 cells/mm^2 and negative microbiological testing to exclude possible contamination. For further analysis, grafts were transferred into a glass vial for storage in dextran-free organ-culture medium (CorneaMax, Eurobio, Cortaboeuf, France) at 31°C until the time of evaluation.

Light microscopy imaging and cell viability assay

For further analysis, grafts were carefully unfolded endothelial-side-up on a silane-precoated glass (Sigma–Aldrich Chemistry BV, Zwijndrecht, The Netherlands) by transferring the graft with a glass pipette from the storage vial to the glass support, and gently dropping organ-culture medium onto the graft until complete graft unfolding (**Figure 1a**). During the procedure, cellulose sponges were used to guide the movement of the liquid in such way that it would force the graft edges to flatten over the glass support. Next, DMEK grafts were stained with hypotonic trypan blue 0.04% solution to better visualize the tissue on the glass slide and then photographed with an AxioVert.A1 microscope with AxioCam 305 color camera (Zeiss, Oberkochen, Germany) (**Figure 1b,c**).

After light microscopy imaging, grafts were subjected to cell viability analysis. Each sample was covered with 100–150 µl of Dulbecco's phosphate-buffered saline (PBS) (Sigma–Aldrich Chemistry BV, Zwijndrecht, The Netherlands) containing Calcein-AM (4 µM) (Sigma–Aldrich Chemistry BV, Zwijndrecht, The Netherlands) and

incubated at room temperature (RT) for 45 min. After incubation, samples were washed with PBS and then imaged using an inverted fluorescence microscope. Multiple image tiles taken with a 50x magnification were combined and stitched together to create the graft panorama using Microsoft PowerPoint program.

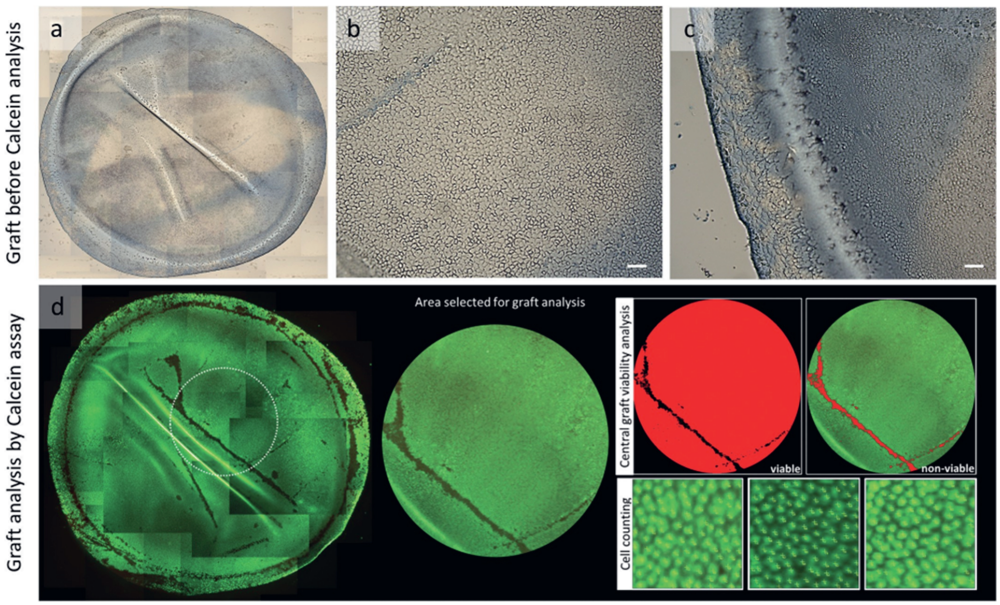

Figure 1| Representative images for the graft analysis before and after Calcein-AM staining. (a) Collage of light microscopy images (×50) to create the graft overview before cell viability investigation. (b) and (c) represent higher magnification areas (×100) of the graft displayed in (a), showing the endothelium at the central (b) and peripheral (c) regions. (d) Graft viability analysis process includes composite photos (×50) stitched together to create an image panorama of the graft stained with Calcein-AM (left). The central graft area (white dashed area in graft overview image) is then selected for cell viability analysis and endothelial cell density counting. The central graft viability analysis panel shows image segmentation and thresholding, with the viable and non-viable cell areas displayed in red. The cell counting panel displays three different frames (200 × 200 µm), selected in the white dashed area, wherein cell counting was performed using the multi-point function in ImageJ. Images are from graft #1 (**Table 1**). Scale bars = 100 µm.

Image analysis

Composite images were imported into FIJI open software for processing and analysis (https://imagej.net/Welcome). For each Calcein-AM-labeled DMEK graft, the graft area was manually defined, the background noise was removed, and the image underwent thresholding at different levels of image intensity and saturation. The presence of hyperfluorescent folds on some grafts caused some areas populated by viable cells to appear hypofluorescent rendering a low viability signal and, thus, underestimating graft viability. For this reason, next to analyzing the entire graft surface, also a circular area was selected centrally on the graft and with a diameter equal to one third of the entire graft diameter (**Figure 1d**, white-dashed line area). For both the entire graft and the selected central graft area, we were able to estimate the percentage of the area of the graft covered by viable cells (=viable cell area/total graft area) and the percentage of the non-viable graft area (=apoptotic and/or denuded graft area/total graft area). ECD on Calcein-stained grafts, reported as Calcein-ECD, was determined centrally on the graft by using the fixed-frame method like for the LM images taken directly after graft preparation. For Calcein-ECD, counting was performed in fixed frames of 0.04 mm^2 using the multi-

point tool to mark all cell centers and counts from three frames per graft were averaged. A half-cell was marked only if it could be paired with another unlabeled half- cell (**Figure 1d**, Cell counting panel). ECD differences were reported between the Calcein-AM-labeled DMEK graft (Calcein-ECD) and LM imaged DMEK rolls (LM ECD).

Statistical Analysis

Paired t-tests were performed to identify significant differences in outcomes between data (ECD and cell viability) collected at graft preparation time (day 0) and post-storage time (3 and 7 days). $P < .05$ was considered significant.

RESULTS

Cell density and viability analysis of DMEK grafts allocated for surgery

Due to the Covid-19 related cancellation of elective surgeries in the Netherlands in March 2020, 5 DMEK grafts from 4 donors that had been allocated and prepared for transplantation became available for research (**Table 1**, corneas #1-5). Endothelial cell density and viability of these grafts were evaluated on the days of the scheduled surgeries. Graft preparation by hydrodissection had been uneventful, except for one graft for which bubble formation was not successful and the graft had to be harvested using the "no-touch" peeling technique. Directly after preparation, standard visual inspection of the DMEK rolls by light microscopy (LM) had shown an unremarkable endothelial cell monolayer with a median LM ECD of 2600 cells/mm^2 and 2617 cells/mm^2 before and after preparation, respectively (mean LM ECD 2600 (±122) cells/mm^2 before and 2575 (±112) cells/mm^2 after preparation; P = .687). After 6 days of organ-culture storage, i.e., at the time of the scheduled surgery, median Calcein-ECD based on analysis of the Calcein-AM images had decreased to 2025 cells/mm^2 (mean ECD 1678 (±687) cells/mm^2; P=.058). Analysis of Calcein-AM images revealed that the percentage of central surface area covered by viable cells ranged from 57% to 97% (median 85%, mean 84 (±16)%) and for the entire graft surface area this ranged from 59% to 92% (median 80%, mean 78 (±13)%) (**Table 1** and **Figure 2**).

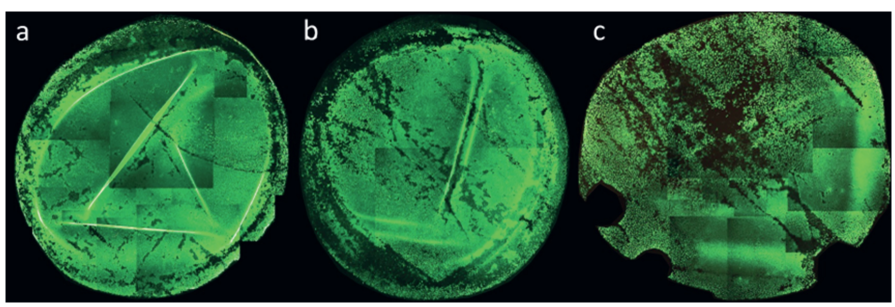

Figure 2| Example images representing Calcein-AM-stained DMEK grafts that had been allocated for transplantation. (a) Graft with a well maintained endothelial cell viability after 6 days in organ culture medium; (b) graft displaying mainly peripheral edge damage, probably induced during the trephination step in the graft preparation procedure; Calcein-AM-labeled cells showing a low level of fluorescence; (c) marked graft showing cell loss of about half of the surface area. Grafts were imaged on the planned day of the cancelled surgeries. Displayed grafts are (a) graft #2, (b) graft #4, and (c) graft #5.

Table 1. Basic donor demographics of corneas used for DMEK graft preparation.

# Cornea (Donor)	Donor age (y)	Gender	Cause of death	Diabetic status	Sepsis status	IOL scars	Preserv. time before graft prep. (days)	Preserv. time between graft prep. and cell viability analysis (days)	Graft prep. technique	ECD (cells/mm²) At cornea preserv. based on LM images	ECD (cells/mm²) Directly after DM stripping based on LM images	ECD (cells/mm²) Based on Ca-AM images (viable cells)	ECD difference between Ca-AM and LM images after DM stripping (%)	Central surface area covered by viable cells (%)	Graft area covered by viable cells (%)
Grafts initially allocated for transplantation, but surgery was cancelled due Covid-19-related cancellation of elective surgeries															
1 (1 R)	74	M	Circulatory syst.	No	Yes	No	9	6	Hydrod.	2600	2558	2317	9	97	92
2 (2 R)	78	M	Respiratory syst.	No	No	No	16	6	Hydrod.	2500	2392	2025	15	96	87
3 (2 L)	78	M	Respiratory syst.	No	No	No	16	6	Hydrod.	2500	2625	1175	55	85	80
4 (3 L)	71	F	Respiratory syst.	No	No	No	15	6	Hydrod.	2800	2617	2142	18	83	70
5 (4 L)	73	M	Circulatory syst.	No	No	No	12	6	Hydrod./NT	2600	2683	733	73	57	59
Median	74						15			2600	2617	2025	18	85	80
Mean (±SD)	75 (±5)						14 (±3)			2600 (±122)	2575 (±112)	1678 (±687)	34 (±28)	84 (±16)	78 (±13)
Paired grafts considered eligible for transplantation (but no allocation due to Covid-19 related cancellation of elective surgeries)															
6 (5 R)	70	M	Respiratory syst	No	Yes	No	29	0	Hydrod.	n.p.	2617	2583	1	83	71
7 (5 L)	70	M	Respiratory syst	No	Yes	No	29	3	Hydrod.	n.p.	2483	2592	-4	92	92
8 (6 R)	85	M	Other	Yes	Yes	No	28	0	Hydrod./NT	2400	2192	2283	-4	94	83
9 (6 L)	85	M	Other	Yes	Yes	No	28	3	Hydrod./NT	2300	2233	2367	-6	92	87
10 (7 R)	73	M	Circulatory syst.	No	No	No	21	0	Hydrod./NT	2800	2950	2750	7	85	81
11 (7 L)	73	M	Circulatory syst.	No	No	No	21	7	Hydrod./NT	2800	2850	2000	30	83	66
12 (8 R)	67	M	Circulatory syst.	No	Yes	Yes	19	0	Hydrod.	2700	2308	1625	30	38	44
13 (8 L)	67	M	Circulatory syst.	No	Yes	No	19	7	Hydrod.	2500	2533	333	87	60	27
14 (9 R)	73	M	Digestive syst	No	Yes	No	24	0	Hydrod.	2800	2650	2642	0	90	75
15 (9 L)	73	M	Digestive syst	No	Yes	No	24	7	Hydrod.	2700	2300	2317	-1	97	81
16 (10 R)	63	M	Circulatory syst.	No	No	No	23	0	Hydrod.	2500	2383	2350	1	95	83
17 (10 L)	63	M	Circulatory syst.	No	No	No	23	7	Hydrod.	2800	2449	2408	2	96	79
18 (11 R)	61	M	Other	No	No	No	22	0	Hydrod.	2500	2433	2417	1	88	91
19 (11 L)	61	M	Other	No	No	No	22	7	Hydrod./NT	2500	2500	2392	4	99	93
20 (12 R)	59	M	Respiratory syst	Yes	Yes	No	14	0	Hydrod.	2500	2300	2333	-1	89	82
21 (12 L)	59	M	Respiratory syst	Yes	Yes	No	14	0	Hydrod.	2500	2342	2317	1	91	77
22 (13 R)	77	M	Malign. neoplasms	No	Yes	No	29	0	Hydrod./NT	2500	2300	2017	12	47	53
23 (13 L)	77	M	Malign. neoplasms	No	Yes	No	29	0	NT	2600	2250	2175	3	88	75
24 (14 R)	60	M	Circulatory syst.	Yes	Yes	No	18	3	Hydrod.	2500	2475	2258	9	68	55
25 (14 L)	60	M	Circulatory syst.	Yes	Yes	No	18	3	Hydrod.	2500	2313	2125	8	81	68
26 (15 R)	75	F	Malign. neoplasms	No	Yes	No	18	3	Hydrod.	2600	2642	1558	41	73	62
27 (15 L)	75	F	Malign. neoplasms	No	Yes	No	18	3	Hydrod.	2700	2967	2683	10	90	79
Median	70						22			2500	2441	2325	3	89	78
Mean (±SD)	69 (±8)						22 (±5)			2585 (±146)	2476 (±224)	2206 (±515)	10 (±21)	83 (±16)	73 (±16)
All Grafts (n=27)															
Median	73						21			2500	2475	2317	7	88	79
Mean (±SD)	70 (±8)						21 (±6)			2588 (±139)	2494 (±210)	2108 (±575)	15 (±24)	83 (±16)	74 (±16)

Ca-AM: Calcein-AM staining; DM: Descemet membrane; ECD: endothelial cell density; F: female; Hydrod.: hydrodissection technique; IOL: intraocular lens; L: left donor cornea; LM: Light microscopy; M: male; Malign.: malignant; n.p.: not possible; NT: No-touch DMEK-graft preparation technique; Prep.: preparation; Preserv.: preservation; R: right donor cornea; SD: standard deviation; syst.: system

Table 2. Endothelial cell density and viability for grafts from paired donor corneas (corneas #6-27)

Statistical Parameters	ECD (cells/mm²) At cornea preservation based on LM images	ECD (cells/mm²) Directly after DM Stripping based on LM images	ECD (cells/mm²) Based on Ca-AM images	ECD difference between Ca-AM and LM images after DM stripping (%) [range]	Central surface area covered by viable cells (%) [range]	Graft surface area covered by viable cells (%) [range]
Evaluating effect of organ culture storage time (n=14)						
Evaluated directly after preparation (n=7)						
Median [range]	2600 [2400-2800]	2433 [2192-2950]	2417 [1625-2750]	1 [-4-30]	88 [38-95]	81 [44- 91]
Mean (±SD)	2617(±172)	2505 (±254)	2379 (±372)	5 (±11)	82 (±20)	75 (±15)
Evaluated after 3-7 days storage (n=7)						
Median [range]	2600 [2300-2800]	2483 [2233-2850]	2367 [333-2592]	2 [-6-87]	92 [60-99]	81 [27- 93]
Mean (±SD)	2600 (±200)	2478 (±198)	2058 (±781)	16 (±33)	88 (±14)	75 (±23)
All grafts (n=14)						
Median [range]	2600 [2300-2800]	2466 [2192-2950]	2380 [333-2750]	1 [-6-87]	91 [38-99]	81 [27-93]
Mean (±SD)	2608 (±178)	2492 (±219)	2219 (±611)	11 (±25)	85 (±17)	75 (±19)
Evaluating intra-donor variability (n=8)						
Grafts from right donor cornea (n=4)						
Median [range]	2500 [2500-2600]	2388 [2300-2642]	2138 [1558-2333]	11 [0-41]	71 [47-89]	59 [53-82]
Mean (±SD)	2525 (±50)	2429 (164)	2042 (±349)	15 (±18)	69 (±17)	63 (±13)
Grafts from left donor cornea (n=4)						
Median [range]	2550 [2500-2700]	2328 [2250-2967]	2246 [2125-2683]	6 [1-10]	89 [81-91]	76 [68-79]
Mean (±SD)	2575 (±96)	2468 (±335)	2325 (±252)	6 (±4)	88 (±5)	75 (±5)
All grafts (n=8)						
Median [range]	2500 [2500-2700]	2328 [2250-2967]	2217 [1558-2683]	8 [0-41]	85 [47-91]	72 [53-82]
Mean (±SD)	2550 (±76)	2449 (±245)	2183 (±320)	10 (±13)	78 (±15)	69 (±11)

Ca-AM: Calcein-AM staining; DM: Descemet membrane; ECD: endothelial cell density; LM: Light microscopy; Preserv.: preservation; SD: standard deviation

Effect of organ-culture storage on cell density and viability analysis of paired DMEK grafts

Based on the wide range of cell viability observed for the five grafts initially allocated for transplantation, additional tests with 14 DMEK grafts from paired donor corneas (**Table 1**, corneas #6–19) were performed to assess whether freshly prepared grafts would show less variability than grafts after organ-culture storage.

Median ECD assessed by light microscopy directly after DMEK graft preparation was 2466 cells/mm^2 (mean LM ECD 2492 (±219) cells/mm^2) (n = 14) as compared to 2600 cells/mm^2 (mean LM ECD 2608 (±178) cells/mm^2; P = .138) (n = 12) (Table 2) before preparation, with pre-preparation LM ECD calculated centrally on the donor cornea at the time of cornea preservation.

For grafts subjected to Calcein-AM staining on the day of the graft preparation (n = 7), median Calcein-ECD was 2417 cells/mm^2 (mean Calcein-ECD 2379 (±372) cells/mm^2) as compared to median LM-ECD of 2433 cells/mm^2 (mean LM ECD 2505 (±254) cells/mm^2; P = .247) determined directly after preparation. This corresponds to an average ECD difference between LM ECD and Calcein-ECD of 5 (±11)% (median decrease of 1%). Cell viability analysis performed on the day of graft preparation showed that, on average, 82 (±20)% (median 88%) (n = 7) of the central graft surface area and 75 (±15)% (median 81%) of the entire graft surface area was covered by viable cells (**Table 2**).

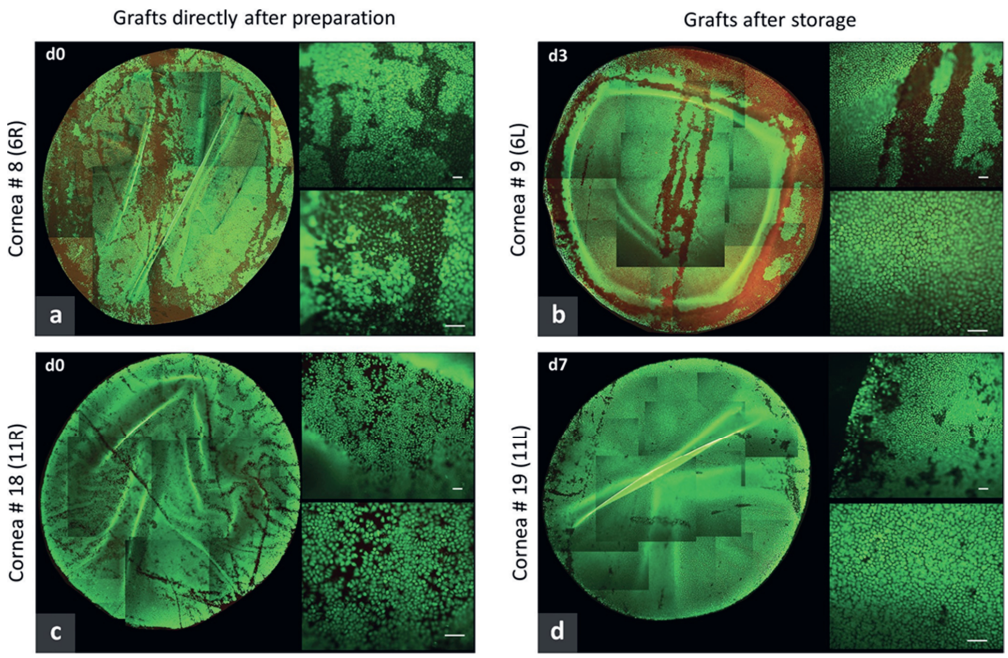

Figure 3| Representative images of paired grafts immediately after preparation and organ culture storage. Top row: Paired grafts #8 and 9 were imaged at d0 (day of preparation, (a) and after 3 days of organ culture storage (b). Bottom row: Paired grafts #18 and 19 were imaged at d0 (c) and after 7 days of organ culture storage (d). Every graft overview image is a collage of 50× image tiles stitched together, and two higher magnification images were included for a better visualization of endothelium integrity. Dark areas on the graft indicate apoptotic cells (a) or bare areas of DM (c). Scale bars = 100 µm.

Median Calcein-ECD of DMEK grafts analyzed after 3 to 7 days of graft storage in organ-culture medium (n = 7) was 2367 cells/mm^2 (mean Calcein-ECD 2058 (±781) cells/mm^2) as compared to 2417 cells/mm^2 (P = .155)

measured directly after preparation of grafts prepared from the contralateral corneas (n = 7) (**Table 2**). Assessment of cell viability following graft storage for 3–7 days showed that the median % central surface area populated by viable cells on the grafts was 92% (mean 88 (±14)%) (n = 7) compared to 88% (P = .0.089) measured directly after preparation of grafts prepared from the contralateral corneas and for the entire graft surface area the median remained at 81% (mean 75 (±23)%) (**Table 2** and **Figure 3**).

Noteworthy, the graft pair with the lowest area covered by viable cells (**Table 1**, corneas # 12 and 13), showed unremarkable endothelial cells upon LM inspection in the eye bank directly after preparation (**Figure 4a,f**) and both grafts were considered eligible for transplantation. However, about 3 hours later, LM inspection in the lab before Calcein-AM staining already showed cells with large nuclei (**Figure 4b**) and Calcein-AM staining revealed large areas of the graft devoid of viable cells (**Figure 4c–e**). Light microscopy and fluorescence imaging of the contralateral graft after 7 days of organ-culture storage showed sparsely distributed elongated endothelial cells (**Figure 4g–i**) that were not contact-inhibited.

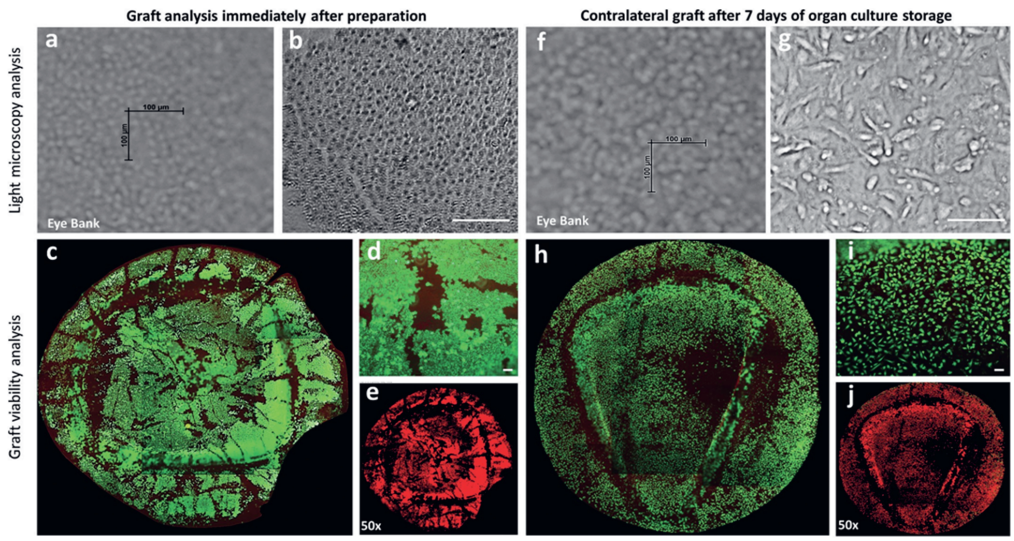

Figure 4| Paired donor graft analysis before and after Calcein-AM staining. (a–e): Analysis of graft #12 at day 0 after preparation in the eye bank; (f–j): Analysis of the contralateral graft (#13) after 7 days of organ culture storage. Displayed are representative brightfield images of the graft taken directly after preparation in the eye bank (a, f), taken about 3 hours after the preparation in the lab mounted on a glass cover slide (b, g), and fluorescence composite images after staining with Calcein-AM (c, h). (d) and (i) represent higher magnification images of the graft endothelium shown in (c) and (h), respectively. Especially in (d), the difference in fluorescence intensity between viable cells (higher fluorescence signal) and dying cells (low fluorescence signal) is evident. (e) and (j) represent the FIJI segmented images of the viable areas on the graft in (c) and (h), respectively. Scale bars = 100 µm.

Intra-donor variability of endothelial cell density and viability

Of four graft pairs (Table 1, corneas #20-27), grafts of two pairs were assessed directly after preparation and grafts of the other two pairs after 3 days of organ-culture storage. Overall, there was no significant difference between contralateral grafts in terms of average Calcein-ECD based on fluorescence images (mean Calcein-ECD 2042 (±349) cells/mm^2 vs. 2325 (±252) cells/mm^2; median Calcein-ECD 2138 cells/mm^2 vs. 2246 cells/mm^2) and central surface area covered by viable cells (mean 69 (±17)% vs. 88 (±5)%) (P = .396 and P =

.113, respectively) (**Table 2**). However, one pair (**Table 1**; corneas #26 and 27) had comparable LM ECD before and directly after preparation, but lower Calcein-ECD and central graft viability for one of the grafts after 3 days of graft storage (1558 cells/mm^2 vs. 2683 cells/mm^2 and 73% vs. 90%, respectively).

DISCUSSION

In this study, we have shown that vital dye staining of surgery-grade DMEK grafts revealed a high degree of variability in endothelial cell loss and in surface area covered by viable cells.

Overall, LM ECD determined centrally on the donor cornea at the time of cornea preservation and directly after DMEK graft preparation by counting in fixed-frames of 0.01 mm^2 with trypan blue staining in the eye bank, was almost similar in our study. All grafts were considered eligible for transplantation based on post-preparation LM ECD and light microscopy inspection of the endothelial cell layer. Mean Calcein-ECD determined centrally on the graft based on Calcein-AM analysis performed on the day of graft preparation was comparable to LM-ECD within the error margin of the fixed-frame method[33] for the majority of the grafts (8/11 grafts (73%)), while 2/11 grafts (18%) showed a slightly lower Calcein-ECD and one graft (9%) had a 30% lower Calcein-ECD. Overall, there was no significant difference in median Calcein-ECD depending on storage time, but the highest losses in Calcein-ECD were observed for grafts stored for 6 or 7 days, respectively.

Since the difference in LM-ECD and Calcein-ECD was within the error margin of the counting technique for most of the grafts, it is unlikely that the observed difference in eye bank determined LM ECD and Calcein-ECD based on Calcein-AM staining is due to cell damage induced by the graft handling for the Calcein-AM staining. Most likely the observed difference is due to the presence of apoptotic cells on the graft after preparation, which cannot be detected by trypan blue staining[34] and will be counted as viable endothelial cells in the eye bank. The presence of apoptotic cells on the grafts may not necessarily only be related to mechanical strain injury during graft preparation but also to stress-induced damage associated with donor death or even cornea procurement.[35] An already vulnerable endothelium before graft harvesting may not be able to maintain a proper cell repair mechanism after DM stripping, with some cells turning apoptotic and dropping off the DM. Another important parameter that could potentially affect the endothelial viability would be the ultrastructure of the DM. A DM may show resistance during cornea swelling in organ culture, forming folds that will deepen as swelling progresses, thereby compromising the surrounding cells.[36,37] Thus, after graft preparation, cells that had surrounded the folds could be shed off allowing the endothelium to heal but clearly resulting in a lower ECD compared to pre-preparation ECD.

Our data showed a median difference of about 2% between Calcein-ECD evaluated on the day of graft preparation (2417 cells/mm^2) and after 3–7 days of storage (2367 cells/mm^2) and suggest that in general the apoptotic cell population observed after graft preparation may not increase during graft storage as this would in time translate to a loss of Calcein-ECD. This finding is in line with a recent study showing an 11% ECD loss caused by the preparation with no further statistically significant Calcein-ECD loss occurring during 5 days of culture storage.[38] A series of recent studies, focusing on the effect of short-term storage time on cell viability showed that organ-cultured DMEK grafts[16,39] or grafts preloaded and then stored for up to 3 days in cold-storage[20,21,23,40] did not show a consistent or significant difference in ECD or cell viability when compared to uncultured grafts. However, in some cases of grafts with pronounced endothelial cell damage developed during the preparation, a further reduction in ECD during storage seems to occur as shown in our study, which can probably be caused by the redistribution of viable endothelial cells by migration to the areas where the cells were dead and detached. This may then result in a reported reduction of graft surface area covered by viable cells of on average 40%.[40] Larger studies with high-quality grafts, however, may be needed to shed more light on the overall effect of storage time on endothelial cells as this effect may not only be directly reflected by an

ECD loss but could also be more subtle and resulting in an elevated cellular stress level. The latter could lead to cells being more susceptible to damage caused by surgical manipulation.[41]

The large variability in the observed loss of Calcein-ECD after graft preparation may explain the clinical observation of a large variability in early endothelial cell loss after DMEK with an ECD decrease of up to 50% in some eyes as early as 1 day postoperatively.[6] This variability in postoperative ECD has been shown to persist also at longer follow-up times[5,42] and DMEK grafts with a low ECD at 6-month postoperatively have a significantly lower graft survival probability at 5-year postoperatively.[43]

This underlines the clinical importance of developing a better method for post-preparation graft screening in the eye bank to avoid transplanting grafts with a low density of viable endothelial cells. Therefore, the use of a florescent vital dye, such as Calcein-AM, to clearly visualize live and ideally also apoptotic cells could possibly assist eye bankers in the early detection of DMEK tissue of poor quality. Though Bhogal et al.[18,44] demonstrated the efficiency and safety of using Calcein-AM for cell evaluation across the entire surface of a corneal transplant in vitro, regulatory and safety concerns as well as economic considerations may prevent eye banks from implementing such a step in their current protocol. Another less invasive and less time-consuming alternative may be an additional graft evaluation by light microscopy within 1 day after graft preparation. Any loss in cell density and integrity of the endothelial cell layer could already be recognizable at this time and may allow to detect low-quality grafts before sending them out for transplantation.

One limitation of our analysis is the small number of grafts tested in every group from graft preparation through storage in organ-culture, limiting the accuracy of the cell viability analysis. On the other hand, these grafts were prepared from surgical-grade corneas that arrived in our eye bank before the cancellation of elective surgeries due to the COVID-19 pandemic and would have never been made available for research outside the nationwide lockdown context. In addition, our single dye method cannot ascertain whether non-stained areas contain dead cells or no cells. However, Calcein-AM is known to recognize cells in both early and late stages of cells death[45] and these areas will be accurately identified by the image segmentation software and rendered “non-viable” in the analysis.

CONCLUSION

In conclusion, our findings show that endothelial cell damage may be observed within hours after DMEK graft preparation with insignificant additional changes in ECD during 3–7 days of organ-culture storage before use in DMEK surgery. Implementing an additional step for checking tissue quality in the eye bank after preparation may improve the quality of DMEK grafts released for transplantation and thereby contribute to further reducing post-operative DMEK complications.

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