**Abstract**

*Purpose:* To evaluate the biomechanical properties of DSAEK grafts when prepared by the surgeon for immediate transplantation compared with tissues prepared by eye bank technicians and shipped for transplant either as pre-cut or pre-loaded DSAEK tissues.

*Methods:* Corneal tissues were randomised to the following groups: a) surgeon cut DSAEK, b) pre-cut DSAEK and c) pre-loaded DSAEK. Endothelial cell density, immunostaining for tight junction protein ZO-1, elastic modulus and adhesion force were investigated.

*Results:* Endothelial cell loss was not different between groups; 7.8 (±6.5)% in the surgeon cut DSAEK group, 8.6 (±2.3)% in the pre-cut DSAEK group and 11.1 (±4.8)% in the pre-loaded DSAEK group (p=0.5910). ZO-1 was expressed equally across all groups. Overall, the surgeon cut grafts had a higher elastic modulus, which was significantly different when compared to pre-cut and pre-loaded DSAEK groups (p=0.0047 and p<0.0001 respectively). Similarly, adhesion force was significantly greater in the surgeon cut DSAEK group compared to pre-cut DSAEK (p<0.0001) or pre-loaded groups (p=0.0101).

*Conclusion:* Based on these biomechanical data, we could predict that surgeon cut DSAEK grafts may show a lower detachment rate when transplanted.

**Keywords**

DSAEK; pre-loaded; pre-stripped; adhesion; elastic modulus

**Introduction**

Currently, corneal endothelial failure is treated surgically by replacing the damaged endothelium with a healthy donor endothelium through a relatively small incision in an endothelial keratoplasty (EK) procedure.[1] Descemet stripping automated endothelial keratoplasty (DSAEK) and Descemet membrane endothelial keratoplasty (DMEK) have evolved in the last decade due to better visual recovery, fewer postoperative complications and faster recovery.[1,2] However, with these new techniques come new challenges such as a more complicated graft preparation procedure and higher graft detachment rates.[3-6] In order to overcome the issues associated with graft preparation such as damage or wastage of corneal tissue, there has been a rise in popularity of pre-cut and pre-loaded tissues offered by eye banks.[7-9] In addition to less corneal wastage, eye bank prepared tissues offer validation and quality control of the tissue to be grafted, for example, endothelial cell counts and optical coherence tomography (OCT) measurement of thickness, which cannot easily be obtained by surgeons. It has also been shown that endothelial graft preparation in the eye bank reduces the effort for the surgeon and cost of surgery due to the reduced theatre time required. These advantages are even more evident in the early stages of the learning curve. The graft detachment rate after EK varies and can affect the outcomes if not recognised and managed properly. It is important to determine if different graft preparation techniques contribute to the detachment rate as there is an additional storage phase involved in pre-loaded and pre-stripped tissues compared to surgeon cut. The purpose of this study was to examine and compare the difference observed in the biomechanical properties of DSAEK grafts either prepared by the surgeon for immediate transplantation or prepared by eye bank technicians and shipped for transplant either as pre-cut or pre-loaded DSAEK tissues.

**Materials and methods**

***Donor tissue***

The corneas used in this study (n=15), were obtained with written consent from the donor’s next-of-kin to be used for research purposes. The tissues were used and discarded as per the standards set by the respective government guidelines.

***Tissue evaluation before preparation***

All the tissues (n=15) were stained using trypan blue (0.25% wt/vol, VisionBlue, D.O.R.C., Zuidland, The Netherlands) to evaluate the percentage of dead cells. The endothelium was exposed to a hypotonic sucrose solution to aid in the measurement of the number of endothelial cells and to examine pleomorphism and polymegathism. Endothelial cell density (ECD) was expressed as a mean of five different counts using a 10x10 reticule mounted in the eye piece, each performed in a different region using the 10x objective of an inverted light microscope (Axiovision, Zeiss, Oberkochen, Germany).

***Preparation of tissues***

All tissues were prepared by one experienced eye bank technician for pre-cut and pre-loaded tissues and one experienced surgeon for the surgeon cut grafts.[10-12]

*Surgeon cut DSAEK (n=5):*

The corneal tissues were shipped (cross-country) in organ culture (OC) media supplemented with 6% dextran and on arrival they were mounted on an artificial anterior chamber (Moria, Antony, France) after a brief wash in phosphate buffered saline (PBS). The intra-chamber pressure was initially set at 50 mmHg (Schiøtz tonometer), but then increased before sectioning. The epithelium was carefully removed using sterile sponges. A microkeratome (Evolution-3; Moria) equipped with either a 350 µm depth blade was passed over the tissue to achieve a posterior lamellar thickness of around 100 µm. The blade depth was determined from the initial corneal thickness measured using an optical coherence tomography machine (OCT; Tomey Casia SS-1000, GmbH, Erlangen, Germany). Peripheral marginal dissection was performed. Finally, the tissues were punched using a trephine (8.5 mm; Moria) before further analyses. The tissues were not preserved in any additional medium to mimic the scenario in the surgical theatre.

*Pre-cut DSAEK (n=5):*

The DSAEK grafts were prepared as described above, however, at the end of the procedure, the anterior lamellae of the stroma were repositioned back in their original place and the pre-cut tissues were clipped to a cornea claw and shipped for further analyses.

*Pre-loaded DSAEK (n=5):*

Following the procedure described above to obtain a DSAEK graft, the anterior stromal lamellae were used as a base support to reduce any potential damage to the posterior lenticule during punching and loading phases. Pre-cut tissues were then transferred to a standard punching block (Moria, Antony, France) with the endothelial side facing up. The tissues were trephined with an 8.5 mm punch. The posterior lenticule was gently picked up and placed in an iGlide device (Eurobio, Les Ulis, France). The device was filled with transportation medium using a 1 mL syringe to remove any air inside the glide. The cap was closed, and the glide was gently fixed into the preservation container (Eurobio, Les, Ulis, France) and shipped.[7,9]

***Endothelial cell loss***

After the shipping/preparation of each group of tissues, the cells were re-stained with trypan blue for 20 seconds and placed in sucrose solution to visualize the cell mortality and count the number of cells present as described in the tissue evaluation paragraph. The endothelial cell loss (ECL) was determined as the difference between the endothelial cell count before and after the preparation or transportation phases for surgeon cut or eye bank prepared tissues respectively, plus the number of trypan blue positive cells.

***Immunostaining for tight junction protein Zonula Occludens-1***

The tissues (n=2 per group) were washed with phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 20 minutes. The cells were permeabilized with 0.5% Triton X-100 in PBS for 30 minutes. After blocking with 5% goat serum for 1 hour at RT, the tissues were incubated overnight at 4oC with ZO-1 monoclonal antibody conjugated with FITC (2.5μg/ml; ZO1-1A12, Thermo Fisher Scientific, Rochester, NY, USA). 3 μL of Hoescht 33342 was mixed in 1mL PBS and 100 μL of the solution was added on the tissues to stain the nucleus. After each step, the tissues were washed 3 times with 1x PBS. The tissues were covered with mounting medium (Vector Laboratories, Peterborough, UK), and cover slips and examined with a Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) using NIS Elements software (Nikon).

***Elasticity and adhesion force***

The DSAEK tissues (n=3 per group) were washed with PBS and fixed on circular glass coverslips (12 mm diameter), which were glued on to metal disks for mounting into the atomic force microscope (AFM). Elastic modulus and adhesion force of the anterior surface of the DSAEK tissues were measured utilizing a Bruker MultiMode 8 AFM (Bruker Nano Inc., Nano Surfaces Division, CA, USA). A silicon probe with a rectangular tip, type RTESPA-300 (Bruker Nano Inc., CA, USA) was used. The PeakForce quantitative nanomechanical mapping (PF-QNM) mode in air with the Derjaguin-Muller-Toporov (DMT) model were used and calibrated using relative method before every test as previously described.[13,14] A Vishay Photostress PS1 Polymer reference sample (Vishay; Wendell, NC, USA) of a known elastic modulus (2.7±0.1 GPa) and a sapphire sample (Sapphire-12M; Bruker Nano Inc., Nano Surfaces Division, CA, USA) were used in the calibration process. Adhesion force was maintained at less than 1 nN on the sapphire sample during the calibration. The tip radius of the probes was approximately 20 nm in all experiments.

AFM images were collected from six different positions on each DSAEK tissue. The optical microscopy integrated with the AFM machine helped identify the centre of the samples that were scanned in three places approximately 500 µm from each other. Another three places were scanned at the mid-periphery of the samples, 3.5 mm from the first central scans. Image scanning size of 1 µm was chosen, and resolution was set to 256 pixel/line. AFM images were scanned at a scan rate of 0.666 Hz. The peak force frequency and amplitude at 2 kHz and 150 nm, respectively, were chosen. Elastic modulus and adhesion force were measured from the AFM images of the DSAEK tissues after processing the images using NanoScope Analysis 1.8 software (Bruker Nano Inc., Nano Surfaces Division, CA, USA).

***Statistical analysis***

A Kruskal-Wallis test with Dunn’s multiple comparisons was used to compare data from more than two groups, with significance level of alpha = 0.05 (95% confidence intervals). A Mann-Whitney test was used to compare elasticity and adhesion in the mid-periphery with the centre using Prism 8 software (Graphpad, San Diego, CA USA).

**Results**

***Donor characteristics***

All the tissues were randomly assigned to groups for the laboratory investigation. The mean age of the donors was found to be 72.9 (±8.7) years with 7 males and 4 females. Average time from death to enucleation was 13.6 (±9.8) hours. The tissues were stored in tissue culture medium for 29 (±6.8) days in the eye bank followed by <72 hours of storage in transportation medium before use.

***Endothelial cell loss was not different between groups***

The endothelial cells appeared normal with typical cobblestone morphology and minimal trypan blue positive cells before processing in the surgeon cut group (Figure 1A), pre-cut (Figure 1B) and pre-loaded groups (Figure 1C). After processing, the cells from the surgeon cut DSAEK (Figure 1D), pre-cut DSAEK (Figure 1E) and pre-loaded DSAEK (Figure 1F) were counted. ECL of 7.8 (±6.5)% was observed from the surgeon cut DSAEK group compared to 8.6 (±2.3)% in the pre-cut DSAEK group and 11.1 (±4.8)% in the pre-loaded DSAEK group, which was not found to be a statistically significant difference (p=0.5910).

***Expression of tight junction protein ZO-1 was not affected by preparation and transport***

The expression of tight junction protein ZO-1 was maintained in all tissues even after preparation and shipping (Figure 1G-I). Some small areas of cell loss were observed in all three conditions, but the majority of cells had typical cobblestone morphology with staining seen at the junctional borders.

***Elastic modulus was higher in surgeon cut DSAEK grafts***

Average elastic modulus from the surgeon cut DSAEK group was found to be 2134 (±246)MPa in the centre compared to 2056 (±217)MPa in the mid-periphery (p=0.8571; Figure 2A), which was not a significant difference. Conversely, average elastic modulus from the pre-cut DSAEK group was found to be higher (1642 ±48MPa) in the centre compared to (1451±108MPa) the mid-periphery, which was found to be statistically significant (p=0.0007; Figure 2A). In addition, average elastic modulus from the pre-loaded DSAEK group was found to be 1583 (±122)MPa compared to 1343 (±80)MPa in the mid-periphery, which was also found to be statistically significantly different (p<0001; Figure 2A). Combining the centre and mid-periphery data to compare between the graft groups, the surgeon cut grafts had a higher elastic modulus, which was a significant difference when comparing the surgeon cut and pre-cut, and surgeon cut and pre-loaded DSAEK groups (Figure 2B; p=0.0047 and p<0.0001 respectively). The difference between pre-cut and pre-loaded was not significant (p=0.7646).

***Adhesion force was also higher in surgeon cut DSAEK grafts***

Average adhesion force from the surgeon cut DSAEK group was not found to be significantly different in the centre (55.8 ±1.4nN) compared to the mid-periphery (72.2 ±9.9nN; p=0.0571, Figure 2C). However, average adhesion from the pre-cut DSAEK group was found to be 6.1 (±0.6)nN in the centre compared to 9.0 (±0.5)nN in the mid-periphery, which was found to be statistically significant (p=0.0007; Figure 2C). Average adhesion from the pre-loaded DSAEK group was found to be 19.4 (±3.8)nN in the centre compared to 31.5 (±2.7)nN in the mid-periphery, which was also found to be statistically significantly different (p<0.0001; Figure 2C). When combining the data from mid-periphery and centre to compare the three graft groups (Figure 2D) adhesion force in the surgeon cut DSAEK was significantly higher than in the two other groups. There was a significant difference between the surgeon cut DSAEK and pre-cut DSAEK groups (p<0.0001), surgeon cut and the pre-loaded groups (p=0.0101) and the pre-cut and pre-loaded DSAEK groups p<0.0001).

**Discussion**

In this present study, the surgeon cut DSAEK grafts generally showed a higher elastic modulus (stiffness) compared with pre-cut and pre-loaded DSAEK grafts. As detachments tend to initiate in the periphery, we investigated if there was any difference in the biomechanical properties in the centre of the graft compared to the periphery. We measured the elastic modulus and adhesion force in the centre and at the edge of the 8.5mm grafts (equivalent to the mid-periphery of the whole endothelial layer). There were no significant differences seen in the surgeon cut tissues but there were small but significant differences between the centre and the mid-periphery in the pre-cut and pre-loaded groups for both elastic modulus and adhesion force. The elastic modulus was higher in the centre compared to the mid-periphery in both groups. Looking at the surgeon cut data, a higher elastic modulus appears to be correlated with a higher adhesion force but in the eye bank prepared tissues the opposite is true; a higher elastic modulus in the centre correlated with a lower adhesion force in that region. It may be that the slightly contradictory results are due to the small sample size. The number of samples in this study was limited by the fact that we do not have unlimited access to human tissue. However, randomisation to groups and sampling at multiple sites for mechanical testing allowed us to minimise any bias related to donor characteristics.

The higher elastic modulus in the surgeon cut DSAEK compared to eye bank prepared could be related to the hydration of the tissue. A previous study showed that the thickness of a DSAEK lenticle was 143.90μm right after cutting but this increased to 170μm after pre-loading.[7] Others have shown that when corneas are placed in dextran, leading to subsequent dehydration and thinning, there is an increase in elastic modulus.[15] These studies support our data that show the surgeon cut tissue has a higher elastic modulus than the eye bank prepared tissues. The increased thickness seen in the eye bank prepared tissues is likely to be due to the absence of the epithelial layer, which is still present in the surgeon cut tissues and usually aids in the maintenance of fluid homeostasis in the stroma.

The surgeon cut tissue displayed a higher elastic modulus and related stiffness as well as a higher adhesion force. If we look to nature we see that many insects possess specialised attachment organs to enable them to adhere to surfaces and climb. These features have been studied extensively by engineers trying to create artificial reusable adhesion devices. The mechanical properties of these adhesive organs have been analysed and in one particular study looking at *carausius morosus* (stick insects), they determined that the outer contacting surface of the organ in fact had a high elastic modulus.[16] This was surprising to the authors as it goes against the Dahlquist criterion that states that “adhesive organs must be very soft exhibiting an effective Young's modulus of below 100 kPa to adhere well to substrates”.[17-19] This is based on the principle that adhesion would be maximised if the surface could flow round all the nanopeaks and troughs in a surface to make perfect contact. When the contacting surface of the adhesive organ of the *carausius morosus* was analysed without the influence of its subjacent layer it was noted that the outer contacting layer had a high elastic modulus but the underlying layer had a much lower modulus. This outer layer is likely to be rather stiff, as this would make it less susceptible to the inevitable abrasion that would occur when contacting substrates. This suggests that stiff outer surfaces are able to adhere as long as there is a complaint underlayer present. This may explain why DMEK grafts, Descemet’s membrane and endothelial layer alone, appear to be less adhesive, with a much higher detachment rate, compared to DSAEK grafts.[2,5,20] Others have shown that DM is more stiff than corneal stroma: measured using AFM the stiffness of the hydrated anterior stroma is reported as 33.1±6.1 kPa,[20] whereas the DM has been reported as 1.8±0.8 MPa hydrated and 4.8GPa dehydrated.[21] This agrees with our hypothesis that stiff surfaces require a compliant underlayer for good adherence, which is not present in a DMEK graft and may explain poor detachment rates. In our study, we found that the tested layer, which is a cut stromal interface, had relatively high elastic modulus but taking into consideration previous published data, the underlying stromal portion is likely to be complaint. The discrepancy between our figures for elastic modulus and published figures for stroma are likely due to the fact that samples were tested in air having been out of medium for approximately 20 minutes before testing, additionally, the prolonged storage period in tissue culture medium before use, which is common practice in European eye banks, could have affected the samples relative to fresh tissue.

A surgeon cut DSAEK graft is prepared and transplanted without undergoing any further preservation phases, this allows the tissue to remain in its natural form for a longer period of time before transplantation. In contrast both, pre-cut DSAEK and pre-loaded DSAEK tissues are preserved in a dextran based medium with the pre-cut stromal interface directly exposed to the medium. Dextran is a complex branched glucan (a polysaccharide derived from the condensation of glucose) and is used as a hypertonic solution for restoring the stromal thickness by removing excess water from the tissue.[22] One explanation for the differences in adhesion force between surgeon cut and eye bank prepared could be that the additional exposure to the dextran solution may result in the deposition of a thin film that disrupts the exposed stromal surface leading to a decrease in adhesion force. It would be interesting to see if this effect is observed clinically by making a direct comparison of detachment rates in surgeon cut compared to pre-cut and pre-loaded grafts. If the decreased adhesion force in eye bank cut grafts was a predictor for increased detachment rate it might be prudent to limit the time in dextran containing medium to the minimum time required for deswelling and also perform wash steps before transplantation to remove any dextran that may be interfering with the attachment surface.

**References**

1. Veldman PB, Terry MA, Straiko MD. Evolving indications for descemet's stripping automated endothelial keratoplasty. Curr Opin Ophthalmol. 2014;25:306-311.

2. Stuart AJ, Romano V, Virgili G, Shortt AJ. Descemet's membrane endothelial keratoplasty (dmek) versus descemet's stripping automated endothelial keratoplasty (dsaek) for corneal endothelial failure. Cochrane Database Syst Rev. 2018;6:CD012097.

3. Price FW Jr, Price MO. Descemet's stripping with endothelial keratoplasty in 200 eyes: Early challenges and techniques to enhance donor adherence. J Cataract Refract Surg. 2006;32: 411-418.

4. Lee WB, Jacobs DS, Musch DC, Kaufman SC, Reinhart WJ, Shtein RM. Descemet's stripping endothelial keratoplasty: Safety and outcomes: A report by the american academy of ophthalmology. Ophthalmology. 2009:116: 1818-1830.

5. Parekh M, Leon P, Ruzza A, Borroni D, Ferrari S, Ponzin D, Romano V. Graft detachment and rebubbling rate in descemet membrane endothelial keratoplasty. Surv Ophthalmol. 2018:63: 245-250.

6. Parekh M, Borroni D, Ruzza A, Levis HJ, Ferrari S, Ponzin D, Romano V. A comparative study on different descemet membrane endothelial keratoplasty graft preparation techniques. Acta Ophthalmol. 2018;96:e718-e726.

7. Ruzza A, Parekh M, Ferrari S, Salvalaio G, Nahum Y, Bovone C, Ponzin D, Busin M. Preloaded donor corneal lenticules in a new validated 3d printed smart storage glide for descemet stripping automated endothelial keratoplasty. Br J Ophthalmol. 2015;99: 1388-1395.

8. Price MO, Baig KM, Brubaker JW, Price FW Jr. Randomized, prospective comparison of precut vs surgeon-dissected grafts for descemet stripping automated endothelial keratoplasty. Am J Ophthalmol. 2008;146: 36-41.

9. Parekh M, Ruzza A, Steger B, Willoughby CE, Rehman S, Ferrari S, Ponzin D, Kaye SB, Romano V. Cross-country transportation efficacy and clinical outcomes of preloaded large-diameter ultra-thin descemet stripping automated endothelial keratoplasty grafts. Cornea. 2019;38: 30-34.

10. Romano V, Steger B, Myneni J, Batterbury M, Willoughby CE, Kaye SB. Preparation of ultrathin grafts for descemet-stripping endothelial keratoplasty with a single microkeratome pass. J Cataract Refract Surg. 2017;43: 12-15.

11. Romano V, Tey A, Hill NM, Ahmad S, Britten C, Batterbury M, Willoughby C, Kaye SB. Influence of graft size on graft survival following descemet stripping automated endothelial keratoplasty. Br J Ophthalmol. 2015:99: 784-788.

12. Romano V, Steger B, Chen JY, Hassaan S, Batterbury M, Willoughby CE, Ahmad S, Elsheikh A, Kaye SB. Reliability of the effect of artificial anterior chamber pressure and corneal drying on corneal graft thickness. Cornea. 2015;34: 866-869.

13. Pittenger B EN, Su C. Quantitative mechanical property mapping at the nanoscale with peakforce qnm. In. Quantitative mechanical property mapping at the nanoscale with peakforce qnm. Application Note 128; Veeco Instruments Inc.; 2010.

14. Dokukin ME, Sokolov I. Quantitative mapping of the elastic modulus of soft materials with harmonix and peakforce qnm afm modes. Langmuir. 2012;28: 16060-16071.

15. Singh M, Han Z, Li J, Vantipalli S, Aglyamov SR, Twa MD, Larin KV. Quantifying the effects of hydration on corneal stiffness with noncontact optical coherence elastography. J Cataract Refract Surg. 2018;44: 1023-1031.

16. Bennemann M, Backhaus S, Scholz I, Park D, Mayer J, Baumgartner W. Determination of the young's modulus of the epicuticle of the smooth adhesive organs of carausius morosus using tensile testing. J Exp Biol. 2014;217: 3677-3687.

17. Last JA, Liliensiek SJ, Nealey PF, Murphy CJ. Determining the mechanical properties of human corneal basement membranes with atomic force microscopy. J Struct Biol. 2009;167: 19-24.

18. Kishore Cholkar SRD, Dhananjay Pal, Ashim K. Mitra. Eye: Anatomy, physiology and barriers to drug delivery. In: Mitra AK editor. Eye: Anatomy, physiology and barriers to drug delivery. Ocular transporters and receptors: Woodhead Publishing; 2013.

19. A. DC. Pressure-sensitive adhesives. In: R.L.Patrick editor. Pressure-sensitive adhesives. Treatise on adhesion and adhesives: New York: Dekker; 1969.

20. Ali M, Raghunathan V, Li JY, Murphy CJ, Thomasy SM. Biomechanical relationships between the corneal endothelium and descemet's membrane. Exp Eye Res. 2016;152:57-70.

21. Xia D, Zhang S, Nielsen E, Ivarsen AR, Liang C, Li Q, Thomsen K, Hjortdal JO, Dong M. The ultrastructures and mechanical properties of the descement's membrane in fuchs endothelial corneal dystrophy. Sci Rep. 2016;6:23096.

22. Parekh M, Ruzza A, Di Mundo R, Ferrari S, Recchia G, Elbadawy H, Carbone G, Ponzin D. Role of dextran in maintaining adhesive and stiffness properties of prestripped dmek lenticules. Eur J Ophthalmol. 2017;27: 270-277.

**Figure captions**

**Figure 1:** Corneal endothelial cell density and morphology using trypan blue staining compared before processing the tissues for A) surgeon cut DSAEK, B) pre-cut DSAEK and C) pre-loaded DSAEK grafts and after processing the tissues for D) surgeon cut DSAEK, E) pre-cut DSAEK and F) pre-loaded DSAEK grafts. Representative images of immunoflurorescence staining of phenotypical marker ZO-1 (green) and nuclear DAPI staining (blue) of G) surgeon cut H) pre-cut and I) pre-loaded tissues after processing. Scale bars A-F 100μm, G-I 50μm.

**Figure 2:** Elastic modulus in A) the centre and mid-periphery of DSAEK grafts. B) Comparison of elastic modulus in the entire tissue between all the groups. C) Adhesion force in the centre and mid-periphery of DMEK grafts. D) Comparison of adhesion force in the entire tissue between all the groups. The data are represented in violin plots showing median (dashed line) and quartiles (dotted lines) A,C= Mann Whitney and B,D = Kruskal-Wallis test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.