Endothelial Approach Ultrathin Corneal Grafts Prepared by Femtosecond Laser for Descemet Stripping Endothelial Keratoplasty

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PURPOSE. To investigate the quality of the ultrathin corneal grafts prepared by femtosecond laser from the endothelial side for Descemet stripping endothelial keratoplasty.

METHODS. Thirty human corneoscleral buttons were cut from the endothelial side by laser Doppler velocimetry (LDV) with or without viscoelastic materials coating. Two cutting depths were selected: 70 and 90 µm. The postcut endothelial count was determined by specular microscopy, and the graft thickness was evaluated by anterior segment optical coherence tomography. The endothelial viability was determined using Trypan blue/Alizarin red staining, calcein-AM/EthD-1 live/dead cell assay, and scanning electron microscope (SEM). The graft interface smoothness was evaluated by SEM. Another 18 corneoscleral buttons were used as controls for the comparisons.

RESULTS. The overall targeted cutting depth and achieved cutting depth were significantly highly correlated (r = 0.84). The central to peripheral corneal thickness ratio was 0.976 and 0.998 for the 70- and 90-µm grafts. The percentage of the damaged endothelial cells assessed by vital staining and SEM showed the 70-µm grafts had noticeably more endothelial damage compared with the 90-µm grafts. But the damage was significantly reduced, to the control corneas level, after coating the endothelium with Viscoat. The 90-µm grafts had a slightly rougher graft interface than the 70-µm grafts, but all the grafts dissected by a Chansue dissector exhibited a generally smooth interface.

CONCLUSIONS. The corneal endothelial grafts prepared by LDV femtosecond laser with endothelial approach produced consistently ultrathin grafts in uniform shape with high accuracy and good endothelial and stromal interface quality.

Keywords: endothelial keratoplasty, femtosecond laser, ultrathin graft

During the past decade, endothelial keratoplasty (EK) has rapidly increased in popularity as an alternative to conventional penetrating keratoplasty (PK) for the treatment of endothelial diseases.1 In our own service at the Singapore National Eye Centre, there has been an increase in BK numbers compared with PK over the last 8 years, with EK becoming the dominant procedure in the past 4 years. Corneal grafts for Descemet’s stripping automated endothelial keratoplasty (DSAEK) are traditionally cut by a mechanical microkeratome from the epithelial side. This approach often results in grafts of variable thickness and a meniscus shape, that are typically thinner centrally than peripherally, leading to an average of +1.25 diopters (D) postoperative hyperopia.2 Several attempts have been made to address these problems, including the use of femtosecond lasers. Currently, there is no precise definition of ultrathin corneal tissue, but most surgeons would consider such tissue to be 100 µm or thinner.3 Although there is no clear consensus to support the notion that thinner grafts yield better visual outcomes in DSAEK,4,5 ultrathin DSAEK grafts have been reported to be associated with faster visual recovery and higher percentage of patients with 20/20 final visual acuity.6 However, the perforation rates were higher when using a microkeratome to prepare ultrathin tissues compared with nonultrathin tissue.7,8 Hence, newer techniques have been proposed for better preparation of ultrathin grafts, including the use of a femtosecond laser,3,9 or two sequential cuts by femtosecond laser and microkeratome.8 Femtosecond lasers have been used to produce highly reproducible LASIK flaps.10,11 However, deeper stromal cutting poses more challenges in terms of accuracy and smoothness of interface quality because of greater possibility of scattering of laser energy.12 Soong et al.2 reported that the femtosecond laser excised posterior lamellae were 55 ± 61 µm thicker than the preoperative programmed values,13 due to problems in accuracy and focusing. Moreover, the stromal surface of deep lamellar cuts appeared to be rougher when made with femtosecond lasers compared with a microkeratome.3,12,14 Cheng et al.15,16 conducted a randomized clinical trial...
comparing the visual outcomes of deep-cut (400 μm) femtosecond laser-assisted EK versus conventional PK, and reported that the mean best spectacle-corrected visual acuity was significantly better after PK compared with femtosecond laser-assisted EK at 3, 6, and 12 months’ follow-up. The authors thought the possible explanation was laser-assisted EK had a suboptimal smoothness of the stromal bed compared with a microkeratome-prepared bed. In order to eliminate these limitations, some investigators have proposed the idea of lasering from the endothelial side (i.e., endothelial approach) to obtain reproducible, smooth, thin grafts of even thickness.\textsuperscript{17,18}

The inverse cutting technique, or so-called “endothelial approach,” has been described for several femtosecond laser systems.\textsuperscript{17,18} Hjortdal et al.\textsuperscript{17} used a femtosecond laser cut from an endothelial side with viscoelastic coating protection from the application of a curved applicator. Uniform thickness posterior lamellar grafts were obtained with acceptable endothelial cell counts, but the stromal surfaces were not smooth. Singh et al.\textsuperscript{2} described a novel device that utilized a low-energy femtosecond laser system as both a light source for optical coherence tomography and for guiding the laser to cut consistent and uniform grafts from the endothelial side with no application or contact onto the cornea. Therefore, the endothelial approach with femtosecond laser lamellars may be a viable and promising technique in the preparation of EK grafts. Furthermore, the newer generation of femtosecond lasers (e.g., Femto LDV laser; Ziemer Ophthalmic System, Port, Switzerland) use lower pulse energies and tighter spot size, which are two features reported to be associated with smoother stromal interface quality.\textsuperscript{19}

Hence, current research on femtosecond laser-prepared endothelial grafts focuses on maintaining good endothelial viability as well as stromal interface quality, and the inverse cutting with viscoelastic materials coating on endothelium may be potentially a promising way. The purpose of this present study was to investigate the quality of the ultrathin corneal endothelial grafts prepared by femtosecond laser from the endothelial side for Descemet stripping endothelial keratoplasty (DSEK), with regard to its reproducibility, accuracy, endothelial viability, and graft interface quality.

**METHODS**

**Human Corneoscleral Buttons**

Fifty-one human donor corneoscleral buttons unsuitable for clinical transplantation were used (Lions Eye Institute, Tampa, FL, USA), and were randomly divided to the femtosecond laser (FL; \( n = 30 \)) and control groups (\( n = 21 \)). The mean death-to-experimentation time was 10.9 ± 2.3 days (range, 8–15 days) and 10.4 ± 2.8 days (range, 6–15 days) for the FL and control groups, respectively (\( P = 0.386 \), Mann-Whitney U test).

**Femtosecond Laser Inverse Cutting Procedure**

The corneas were mounted onto the Ziemer artificial anterior chamber (AAC) with the endothelial side upwards onto the center of the AAC column. The fixation ring assembly was carefully placed over the donor button, and this fixed the scleral rim of the donor downward onto the column. The normal saline infusion bottle was set at the height of 80 cm above the base level of the AAC, and the donor was gently inverted into a convex position by slowly opening a 3-way tap attached to the AAC. Viscoelastic materials (\( n = 9 \); Viscoat; Alcon, Ft. Worth, TX), or a few drops of Optisol-GS (\( n = 39 \)), were instilled over the endothelial surface, and the laser application head with a ring adapter was gently placed onto the AAC column. The cornea was then gently touched the flat Intershield (Akzo Nobel, Amsterdam, The Netherlands) of the laser application head by turning the adjusting ring until the apposition area reached 9.5 mm. The laser process was performed using the Femto LDV Z6 laser machine and programmed to cut a double pocket pattern: firing frequency of >5 MHz, pulse energy of ~100 nJ, spot size of 2 μm with overlapping spots, two pocket side cut lengths of 2 and 4 mm with a diameter of 9.2 mm (Fig. 1A), and the angle of the vertical side cuts of 90°. The lamellar dissection depth was set at 70 or 90 μm from the endothelial surface, and the entire laser firing duration was 55 seconds. Following completion of the laser procedure, the AAC column was gently withdrawn by reducing the amount of application and the laser head was removed. The posterior lamellar was gently dissected, using either a Chansue dissector (\( n = 6 \); Bilcon, India) or Tan Femto lamellar dissector (\( n = 3 \); ASICO, USA), through the double pocket incisions (Fig. 1B). The AAC pressure was gently reduced by lowering the bottle height to a level beneath that of the AAC, and this maneuver reverted the cornea into its physiological shape. The inverse cutting procedure was performed by a single surgeon (JSM). The cut cornea was then placed in the original storage medium-filled container (Optisol GS; Chiron Ophthalmics, Irvine, CA, USA) for trephination in the operating room.

A total of 21 control corneas were used as controls. Six were in the positive control group to study the effect of the laser head application, and another six corneas were used to study the effect of inversion of the corneas on the endothelium only (inverted control group). All the control corneas were mounted onto the AAC and endothelial side upward. For the positive control group, the laser head was applied to the cornea for the same 55 seconds to simulate the laser procedure duration with storage medium (Chiron Ophthalmics) only. For the inverted control group, the corneas were just inverted and mounted onto the AAC for 55 seconds without application of the laser head. The remaining nine corneas without any procedure performed served as the naïve controls to evaluate the baseline endothelial damage of the donor corneas. The flow diagram for the detailed experimental design is described in Figure 2.

**Evaluation of the Accuracy of Cutting Depth**

After the laser photodisruption procedure, the graft was placed in the storage medium (Chiron Ophthalmics)-filled container and the cutting depth was immediately evaluated by anterior segment optical coherence tomography (ASOCT; RTVue; Optovue, Inc., Fremont, CA, USA). For each cornea, a total of eight high-resolution corneal cross-sectional scans (8 mm scan length, single scan mode, approximately 45° axis apart) were obtained by the same ophthalmologist (YCL). The central graft thickness at the vertex (C), and peripheral graft thickness (P), which was the average of the thickness 2 mm away from the central cornea at each side, were measured by two independent observers (YCL, ETP-W), and then the average was taken. The C:P ratio was subsequently calculated to evaluate the graft shape.

**Evaluation of Graft Interface Smoothness**

The graft interface smoothness was assessed by scanning electron microscopy (SEM). Nine corneoscleral buttons that have undergone the laser cutting procedure were trephined centrally to 8 mm diameter for graft interface evaluation, and the remnant corneoscleral rims were used for further histopathology examination. The sample preparation for SEM
assessment was performed as we described previously. For each ultrathin graft, five images, one at the center and one at each quadrant, were taken for each graft (×50 magnification), and the graft interface smoothness was graded based on a previously described scoring system: 1 = very smooth, 2 = smooth, 3 = rough, and 4 = very rough. The smoothness was graded by two independent observers (one unmasked and one masked: YCL, ETP-W).

Evaluation of Endothelial Damage

Before and following laser photodisruption, the central endothelial cell density (ECD) was measured using a fixed-frame method of cell counting with specular microscopy (EB-10; Konan Medical, Inc., Irvine, CA, USA) by an experienced eye bank technician, marking at least 50 cells per image. The mean ECD before laser cutting was 2746 ± 326 cells/mm² for the FL group and 2722 ± 232 cells/mm² for the control group (P = 0.811, Mann–Whitney U test). Corneal endothelial damage was subsequently assessed by Trypan blue/Alizarin red staining and SEM. Trypan blue stained damaged and dead corneal endothelial cells, whereas Alizarin red stained cell borders and exposed areas of Descemet membrane. The corneas were stained with 0.25% Trypan blue (Sigma-Aldrich Corp., Singapore) for 3 minutes and rinsed with PBS (0.01 M, Life Technologies, Carlsbad, CA, USA). The corneas were subsequently stained with 0.5% Alizarin red S (Sigma-Aldrich Corp.) for 2 minutes, and rinsed again with PBS. Thirteen micrographs were taken per cornea, one at the center and one at each clock hour. The percentage of the damaged endothelial cells was determined by counting the number of Trypan blue positively stained cells.
stained cells and the total number of cells. For the SEM sample preparation, the corneas were processed as we described previously. Four images with magnification of ×50 were taken per cornea, and the percentage of the area of disrupted cells was semi-quantified by ImageJ software (http://imagej.nih.gov/ij/) provided in the public domain by the National Institutes of Health (NIH, Bethesda, MD, USA). Another 13 micrographs with ×500 magnification were also taken per cornea, one in the center and one in each clock hour. The percentage of disrupted cells was determined by counting the number of cells with disrupted cell membranes and the total number of cells.

As the Intershield of the laser head was in contact with the endothelial surface, the in vitro endothelial viability experiments were also designed to evaluate the possible endothelial toxicity of the Intershield by performing calcein-AM/ EthD-1 live/dead cell assay. Human corneal endothelial cells (HCECs) were seeded onto the Intershield at a seeding density of 3000 cells/mm² (n = 4). Comparatively, donor-matched HCECs seeded on FNC-coated glass coverslips at the same seeding density were used as control (n = 4). Live cell samples were then assessed for viability using an assay kit (LIVE/DEAD Viability/Cytoxicity; Life Technologies) according to manufacturer's instructions. Briefly, the dye components Calcein AM (3 μM) and Ethidium homodimer-1 (6 μM) were prepared in ×1 PBS, and subsequently mixed together. The Endo medium was first removed and the combined LIVE/DEAD assay reagents were added to the HCECs grown on the Intershield or glass coverslips, and incubated for 30 minutes at room temperature, in the dark. Samples were then mounted in medium containing DAPI (Vectorshield; Vector Laboratories, Burlingame, CA, USA) onto glass slides and observed under a fluorescence microscope (Nikon Ti-Eclipse; Nikon Corp., Tokyo, Japan). The percentage of the dead cells were then counted and calculated.

Histology

The samples were fixed in neutral 4% buffered paraformaldehyde (Sigma-Aldrich Corp.), dehydrated, cleared, and embedded in paraffin, and then cut in 7-μm sections. The sections were then stained with hematoxylin for 2.5 minutes and eosin for 2 minutes. The images of sections were examined using a light microscope (Axioplan, Zeiss; Carl Zeiss Microlimaging, Thornwood, NY, USA) under bright field mode.

Statistical Analysis

All data was expressed as mean ± standard deviation. Statistical comparisons among the different groups were performed using Kruskal–Wallis test with Dunn post hoc tests. Spearman’s correlation test was used to assess the correlation between the targeted cutting depth and achieved cutting depth. Statistical analyses were performed using statistical software (STATA version 13; StataCorp LP, College Station, TX, USA). Values of P < 0.05 were considered statistically significant.

RESULTS

Accuracy of Inverse Cutting in Depth

The overall targeted cutting depth and achieved cutting depth were significantly highly correlated (r = 0.84; P = 0.032). For the 70-μm group, the mean central thickness (mean deviation) and peripheral thickness were 72.2 ± 4.7 μm (3.2%) and 74.0 ± 5.6 μm (5.7%) for the corneas without Viscoat coating. For Viscoat-coated corneas, the mean thickness were 74.1 ± 5.8 μm (5.9%) centrally and 76.2 ± 6.7 μm (8.9%) peripherally (P = 0.092 and P = 0.186, respectively, compared with the Viscoat and non-Viscoat–coated corneas). For the 90-μm group, the mean central thickness and peripheral thickness were 91.7 ± 4.9 μm (1.9%) and 91.9 ± 2.2 μm (2.1%) for the corneas without Viscoat coating, and were 92.0 ± 4.8 μm (2.2%) and 92.2 ± 3.6 μm (2.4%) for the corneas with Viscoat coating (P = 0.216 and P = 0.247, respectively, compared with the Viscoat and non-Viscoat–coated corneas). The ratios of C:P were 0.976 and 0.972 for the Viscoat and non-Viscoat–coated groups in the 70-μm grafts, and were 0.998 and 0.998 for the Viscoat and non-Viscoat–coated groups in the 90 μm grafts. The C:P ratios were all close to 1, indicating the laser cut the grafts close to a planar shape. Figure 3 shows representative ASOCT images of both 70- and 90-μm grafts with the hyper-reflective demarcation line visible at the graft margin.

Graft Interface Smoothness

Representative SEM images for analysis of the graft interface smoothness are shown in Figure 4. When cutting with a 70 μm depth, the grafts dissected by a Tan Femto lamellar dissector had rougher interface compared to that dissected by a Chansue dissector (mean score 3.5 ± 0.6 versus 2.3 ± 0.6 graded by observer #1; mean score 3.3 ± 0.6 versus 2.0 ± 0.0 graded by observer #2; P = 0.102 and P = 0.023, respectively), and therefore a Chansue dissector was used for the subsequent experiments of graft interface smoothness. When using a Chansue dissector, the 90-μm grafts appeared slightly less smooth than the 70-μm grafts. The average scores of the smoothness graded by observer #1 were 2.3 ± 0.6 and 2.7 ± 0.6 for the 70- and 90-μm grafts (P = 0.525). The average scores of the smoothness graded by observer #2 were 2.0 ± 0.0 and 2.3 ± 0.6 for the 70- and 90-μm grafts (P = 0.378).

Endothelial Damage

The mean percentage of endothelial damage evaluated by Trypan blue and Alizarin red vital staining was 11.4% ± 2.1%, 14.7% ± 4.5%, 25.5% ± 4.9%, 22.0% ± 3.7%, 29.4% ± 4.0%, and 15.9% ± 2.4%, for the naïve, inverted, and positive control, 90, 70, and 70 μm with Viscoat coating groups, respectively (Fig. 5). The mean percentage of endothelial damage areas assessed by SEM with the ×50 magnification images was 9.3% ± 2.1%, 9.7% ± 3.6%, 13.1% ± 4.4%, 15.1% ± 2.9%, 30.1% ± 6.6%, and 15.4% ± 3.9% (Fig. 6), and the mean percentage of disrupted endothelial cells assessed by SEM with the ×500 magnification images was 7.2% ± 2.3%, 6.5% ± 2.7%, 9.0% ± 3.4%, 12.1% ± 4.9%, 23.4% ± 7.6%, and 10.6% ± 3.2%, for the naïve, inverted, and positive control, 90, 70, and 70 μm with Viscoat coating groups, respectively (Fig. 6). The 70-μm group had noticeably higher percentage of endothelial damage than the 90 μm group (P = 0.236, P = 0.008, and P = 0.056, evaluated by vital staining, SEM with ×50 magnification, and SEM with ×500 magnification micrographs, respectively). However, after instillation of Viscoat to the endothelium of the 70-μm grafts, there was a significant reduction of endothelial damage (the P values for the comparison of the 70 and 70 μm with Viscoat coating groups were 0.031, 0.028, 0.032, respectively, evaluated by vital staining, SEM with ×50 magnification, and SEM with ×500 magnification micrographs). There was no significant difference between the naïve control and 70 μm with Viscoat coating groups (P = 0.192, P = 0.201, P = 0.236, evaluated by vital staining, SEM with ×50 magnification, and SEM with ×500 magnification micrographs, respectively), indicating the femtosecond laser with inverse cutting technique did not significantly induce greater endothelium damage with Viscoat protection.

The mean percentage of the ECD loss evaluated by specular microscopy was 8.0% ± 4.4%, 8.5% ± 4.5%, 10.7% ± 3.7%,
8.9% ± 2.5%, and 6.5% ± 2.4%, for the naïve, inverted, and positive control, 90, 70, and 70 µm with Viscoat coating groups, respectively.

In the in vitro live/dead viability assay using calcein-AM and EthD-1, dead cells were stained red. There was no significant difference in the endothelial cell viability between the Intershield and control groups, indicating the Intershield of the laser head did not induce endothelial toxicity. The mean percentage of dead cells was 1.73% ± 1.33% for the Intershield group, and 2.17% ± 1.55% for the control group (P = 0.539; Mann–Whitney U test; Fig. 7).

**DISCUSSION**

The preparation of corneal tissue for DSAEK/EK is an important step in achieving a successful surgical outcome. An ideal graft should have a precise depth, uniform shape, good quality of stromal interface and endothelial viability. In the recent years, much effort has been made for the potential strategies to overcome the existing limitations of conventional microkeratome or femtosecond laser cutting. Our results demonstrated that combining the use of Femto LDV laser and viscoelastic materials could aid in the creation of ultrathin and planar grafts with maintaining satisfying interface smoothness and endothelial viability.
In the present study, the endothelial damage/loss was determined by vital dye staining, SEM and specular microscopy together to get more accurate results. Among them, the ECD measurements using specular microscopy may be prone to sampling errors, as this technique is dependent on extrapolation of data from an assessment of a relatively small area of the cornea. Although these three methods have different principles of assessment, their results showed similar trends. A noticeably higher percentage of endothelial damage was found in the 70 μm group than in the 90 μm group. It may be because the laser photodisruption plane was closer to the endothelial layer, and this finding was in agreement with a previous study. Kimakura et al. reported greater corneal endothelial cell damage in grafts that were 70 μm compared to 150 μm, using 150-kHz Intralase machine and cutting from the epithelial surface.

The Femto LDV laser, compared with Intralase, Femtec, or VisuMax femtosecond laser, has lower pulse energies (nanoJoule versus microJoule) and narrower spot size. Femtosecond lasers with lower pulse energy have been reported to be more suitable for cutting corneal ultrathin tissue. We demonstrated that after using Viscoat to protect the endothelial cells, the endothelial damage significantly reduced, and was comparable to the level of the naïve control corneas. There was no significant difference between the 90 μm group and positive control group, suggesting that the endothelial damage may result from the applanation, rather than the laser cutting energy, when cutting at the depth of 90 μm. The applanation of the laser application head may lead to some endothelial damage, as the positive control group had a higher percentage of damaged endothelium than the naïve control or inverted control group. This suggests the use of viscoelastic materials as

**Figure 5.** Representative photos showing the damaged endothelium evaluated by Trypan Blue and Alizarin red staining of the naïve control (A), inverted control (B), positive control (C), 90 μm (D), 70 μm (E), and 70 μm with Viscoat coating groups (F). Damaged cells were stained with Trypan blue. The mean percentage of endothelial damage is shown in the bar graph (error bars represent standard deviations) (G). There was a significant difference between the naïve control and positive control groups, and the 70 and 70 μm with Viscoat coating groups. No significant difference was noted between the other groups. *P < 0.05. Magnification: x200. Scale bars: 100 μm.
FIGURE 6. Representative SEM micrographs (magnification: ×50 and ×500) showing the areas of damaged endothelial cells of the naïve (A), inverted (B), and positive control (C), 90 μm (D), 70 μm (E), and 70 μm with Viscoat coating groups (F). For the ×50 micrographs, the areas were then semiquantified using ImageJ software (NIH), and the mean percentage of the areas of damaged endothelial cells is shown in the bar graph (G). For the ×500 micrographs, the percentage of the disrupted endothelial cells was determined by counting the number of cells with disrupted cell membranes and the total number of cells, and the mean percentage of the disrupted endothelial cells is shown in the bar graph (H). The Viscoat droplets were also observed (F, arrows). A significant difference was noted between the 70-μm group and other groups (*P < 0.05), but was not noted between the rest groups (G, H). Error bars represent standard deviations.

FIGURE 7. The live/dead viability assay by calcein-AM and EthD-1 showing there was no significant difference in the mean percentage of dead cells between the Intershield ([A], 1.73% ± 1.33%) and control groups ([B], 2.17% ± 1.55%). Cell nuclei were stained blue with DAPI. Dead cells were stained red. Magnification: ×200. Scale bar: 100 μm.
Corneal Graft by Femtosecond Laser Inverse Cutting

In conclusion, the use of femtosecond laser inverse cutting to prepare ultrathin DSEK grafts seems promising. With Viscoat coating during the cutting process, the 70 µm or 90 µm grafts had uniform shape with high accuracy, good endothelial viability, and stromal interface quality. It eliminates the current limitations of microkeratome or femtosecond laser-assisted DSAEK/DSEK, and has the potential to improve postoperative optical quality and therefore visual outcomes. A clinical trial to compare the visual outcomes of the femtosecond laser inverse cutting-assisted DSEK versus conventional microkeratome-assisted DSAEK is in progress.

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