

Comparative Study of nJ- and μ J-Energy Level Femtosecond Lasers: Evaluation of Flap Adhesion Strength, Stromal Bed Quality, and Tissue Responses

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PURPOSE. To compare flap adhesion strength, stromal bed quality, and tissue responses after flap preparation using nJ- and μ J-energy level femtosecond lasers.

METHODS. All corneal flaps were created by either VisuMax laser (μ J-energy level) or femto-LDV (nJ-energy level). Flap adhesion strength in the rabbits was measured with a tension meter 1 and 2 months postoperatively. To investigate tissue responses to laser delivery, immunofluorescence staining and TUNEL assay were performed 4 and 24 hours postoperatively. To assess flap bed smoothness, human donor corneas were used. Surface irregularities were graded based on scanning electron microscopy results.

RESULTS. The flap adhesion strength in the VisuMax group at month 1 and 2 was 16.95 ± 1.45 kPa and 18.33 ± 1.81 kPa, respectively; and 12.31 ± 4.15 kPa and 13.85 ± 4.78 kPa in the LDV group, respectively. No significant difference was found between the groups. Fibronectin and apoptotic cells were largely absent at the central incision site in the LDV group, but were present in the VisuMax group. The smoothness of flap beds appeared similar for both groups. An observer scored the VisuMax group 8.00 ± 1.00 and the LDV group 7.33 ± 0.58 ($P = 0.387$).

CONCLUSIONS. The flap adhesion strength increased over time after treatment with both lasers. The nJ-energy pulses produced minimal wound healing reaction and apoptotic cells along the incision plane. The application of an nJ-energy laser, which can incise the cornea without inducing significant damage to cells and wound healing reaction, offers great potential at reducing scarring following incisional laser stromal surgery.

Keywords: LASIK, femtosecond laser, energy level, flap adhesion, wound healing

LASIK is the most commonly performed corneal refractive surgery worldwide.^{1,2} The procedure involves the creation of a hinged flap followed by excimer stromal ablation to correct the refractive error. In the early years of the procedure, the corneal flaps were created using a microkeratome blade. Despite considerable refinements in mechanical microkeratome technology, complications such as incomplete flaps, buttonholes, and irregular flap incision planes can affect the outcome of the procedure.^{3,4} With recent advancements of laser optics and emergence of reports of safer and more predictable flaps fashioned by femtosecond lasers, microkeratome use has gradually declined.^{5,6}

In the United States, nearly half of LASIK patients had their flap created with femtosecond laser (IntraLase; Abbott Medical Optics, Irvine, CA, USA) in 2007.⁷ However, the use of early generation femtosecond lasers, that operated with lower repetition rates and higher laser energy, led to previously unknown complications, such as transient light sensitivity syndrome, collateral tissue damage, and vertical gas breakthrough.^{8,9} The introduction of newer laser systems operating

with high frequency and lower energy pulses has nearly eliminated these problems. An nJ-energy range laser (Femto LDV model Z6; Ziemer Ophthalmic Systems, Port, Switzerland) and a μ J energy range laser (VisuMax 500kHz femtosecond laser system; Carl Zeiss Meditec, Jena, Germany) are two examples of the latest commercially available machines that employ a high frequency, low energy photodisruptive process. The modern laser systems have improved the safety of LASIK and also reduced the intra- and postoperative complications of flap creation.¹⁰⁻¹³ Furthermore, the use of lower energy level femtosecond lasers has also significantly attenuated the inflammation and cell death adjacent to the photodisruptive site,¹⁴ which has allowed the laser to be used for other applications, such as ultra-thin posterior lamellar graft preparation for Descemet's stripping automated endothelial keratoplasty (DSAEK),¹⁵ and creation of refractive lenticule for correction of myopia and/or astigmatism.^{16,17}

The majority of published studies detailing the interaction between femtosecond laser and corneal tissue are using the IntraLase.^{14,18-20} There are only a few studies that have

elucidated the tissue responses to lower energy level lasers (e.g., VisuMax),^{21–23} and none on the LDV. There are several differences between femto-LDV and VisuMax femtosecond lasers, namely the scanning pattern, laser spot distance, pulse energy level, and interface of suction cone. These differences may have different implications on postoperative flap morphology, wound healing and inflammatory reactions, and visual recovery. Strohmaier et al.⁷ and Vetter et al.²⁴ compared the intraocular pressure (IOP) change during corneal flap preparation by both the VisuMax and LDV femtosecond lasers. They found that the flat suction cone interface of the LDV induced a significantly higher IOP than the curved cone interface of the VisuMax. In another study, Ahn et al.²⁵ showed that both femtosecond lasers produced similar flap morphology. In the current study, we compared the flap adhesion strength, stromal bed quality, and early corneal wound healing responses after flap preparation using the Femto LDV model Z6 and VisuMax 500kHz femtosecond laser systems.

MATERIALS AND METHODS

Rabbit and Human Samples

Fourteen 12- to 15-week-old New Zealand White rabbits (3–4 kg body weight) were procured from the National University of Singapore and housed in the SingHealth Experimental Medicine Center, Singapore. Animals were anesthetized with xylazine hydrochloride (5 mg/kg intramuscularly; Troy Laboratories, Smithfield, Australia) and ketamine hydrochloride (50 mg/kg intramuscularly; Parnell Laboratories, Alexandria, Australia) before surgeries and postoperative examinations. The rabbits were euthanized under anesthesia by overdose intracardiac injection of sodium pentobarbitone (Jurox, Ruthersford, Australia) at predetermined study time points (Supplementary Figs. S1–S3).

Flowcharts of the rabbits' treatment allocations in each study objective are depicted in Supplementary Figures S1 through S3. To evaluate the flap adhesion strength, six rabbits were used. They were divided equally into VisuMax and LDV groups, where right eyes were used to evaluate the flap adhesion strength 2 months post surgery and left eyes 1 month post surgery (Supplementary Fig. S1). To investigate the tissue responses to femtosecond laser incision, eight rabbits were used. Six rabbits were divided equally into VisuMax and LDV groups, where right eyes were assigned for the study of the corneal tissue responses 24 hours post surgery and left eyes 4 hours post surgery (Supplementary Fig. S2). The remainder rabbits were used as a positive control, whose right eyes were subjected to flap creation and excimer laser ablation subsequently and left eyes were used as negative controls. To assess the flap bed smoothness, six cadaveric human corneas aged 50 to 74 years were procured from the Lions Eye Institute for Transplant and Research, Inc. (Tampa, FL, USA) and preserved in Optisol-GS at 4°C. Mean death to enucleation was 6.7 ± 1.5 hours and mean death to experiment was 8.0 ± 2.4 days. Three eyes were used to assess the flap bed quality created by VisuMax and the other three by LDV.

All animals were treated according to the guidelines of the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institutional Animal Care and Use Committee of SingHealth. The use of human donor corneas conformed to the tenets of the Declaration of Helsinki and approved by the institutional review board of the Singapore Eye Research Institute. Written consent was acquired from the next of kin of all deceased donors regarding eye donation for research.

Femtosecond Laser-Assisted LASIK Procedure

VisuMax Group. The rabbit experimental model for LASIK was created as previously described and all procedures were performed by JSM.²¹ LASIK flaps were created by using a VisuMax femtosecond laser. The laser parameters were set as follows: 110- μ m flap thickness; 7.9-mm flap diameter; 170-nJ power; 90° side cut angle; and spot distance and tracking spacing of 4.8 μ m/4.8 μ m for lamellar and 2 μ m/2 μ m for flap side cuts. In order to evaluate tissue responses to the femtosecond laser delivery, the flaps were left not lifted. Displacing the flap requires manipulation of corneal tissue and may affect the roughness of flap interface. In addition, it may introduce other experimental variables, such as possible flap dislocation, epithelial ingrowth, inflammatory reaction, and bacterial infection.^{21,26,27} The positive control was created as previously described by our earlier study,²¹ whereby after the flap was lifted, the stromal bed received a 6.5-mm optical zone ablation using an excimer laser (Technolas; Bausch & Lomb, Rochester, NY, USA) to correct for –6-diopter (D) spherical error. The laser parameters were set as follows: spot size of 2.0-mm diameter, fluence rate of 120 mJ/cm², and repetition rate of 50 Hz. Once the flap was repositioned, a bandage contact lens (Bausch & Lomb) was applied and the eyelid was closed with a temporary tarsorrhaphy using a 6-0 silk suture. For the investigation of flap adhesion strength, the flaps were lifted and replaced immediately. Once the flap was repositioned, a bandage contact lens (Bausch & Lomb) was applied and the rabbit eyelid was closed with a temporary tarsorrhaphy using a 6-0 silk suture for 3 days.

To study the flap bed smoothness, the human cadaveric corneas were treated with femtosecond laser parameters similar to those used in our clinical patients: 110- μ m flap thickness, 8.2-mm flap diameter, 160-nJ power, and spot distance and tracking spacing of 4.8 μ m/4.8 μ m for lamellar and 1.5 μ m/1.5 μ m for flap side cuts.⁴ After the flap was lifted and repositioned immediately, the corneas were then immersed in a fixative and processed for scanning electron microscopy (SEM).

LDV Group. The LASIK flaps were created by using a Femto LDV model Z6 and performed by JSM. In brief, the handheld laser delivery system was advanced forward and placed onto the cornea. After contact was made between the 9.5-mm suction ring and the corneal surface, suction was applied to the interface and the flap incision procedure was then performed. The femtosecond laser parameters were set as follows: energy level of 110%, flap diameter of 8.5 mm, flap thickness of 110 μ m, hinge size of 3.45 mm; and side cut angle of 90°. Similar to the VisuMax group described above, to evaluate tissue responses to the femtosecond laser delivery, the flaps were not lifted. To investigate the flap adhesion strength, the flaps were lifted and replaced immediately. Once the flap was repositioned, a bandage contact lens (Bausch & Lomb) was applied and the rabbit eyelid was closed with a temporary tarsorrhaphy using a 6-0 silk suture for 3 days. To study the flap bed smoothness, the human cadaveric corneas were treated with femtosecond laser parameter similar to that used in the animal study described above.

Flap Adhesion Strength Test

The edge of the flap, opposite to the hinge was first identified under a surgical microscope and then lifted with a Sinskey hook (Rhein Medical, Inc., Petersburg, FL, USA) just enough to pass through a 6-0 silk loop suture. The length of the loop suture was maintained constant at 10 cm for all tests. The rabbit was then laid on the working area of the tension meter (Chatillon LTCM100; Ametek, Largo, FL, USA) and the loop

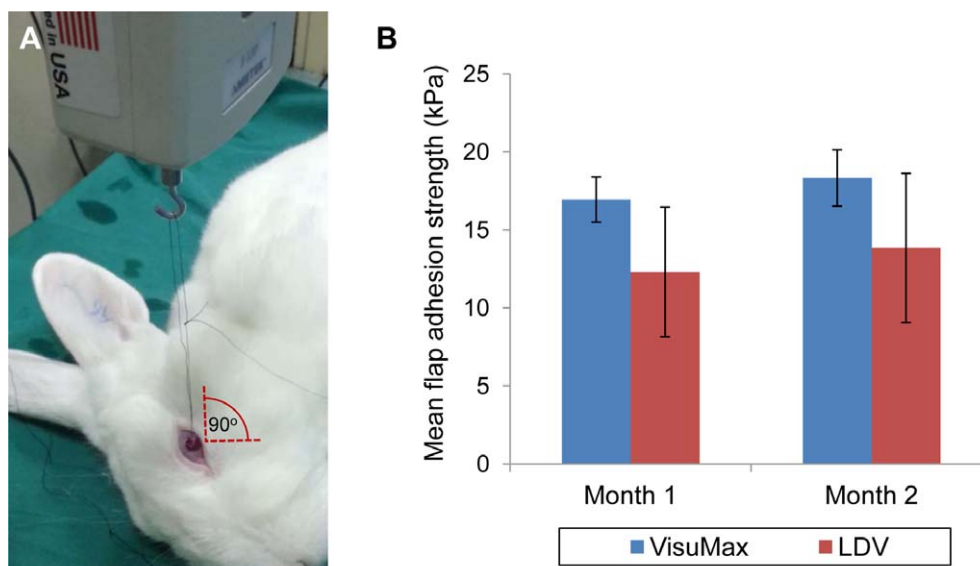


FIGURE 1. Adhesion strength of corneal flap prepared with VisuMax and Femto LDV femtosecond lasers. **(A)** Snapshot of the rabbit lying on the working area of the tension meter. The position of the rabbit was adjusted until the loop suture and the corneal surface formed a 90° angle. The flap was pulled with a speed of 5 mm/min and stopped when the flap became detached from the stromal bed. **(B)** Bar graph showing the mean flap adhesion strength at months 1 and 2 after treatment with VisuMax or Femto LDV femtosecond lasers. Height of error bars represents the standard deviation of mean flap adhesion strength.

suture was hooked to the load cell. The rabbit was positioned until the loop suture and the corneal surface formed a 90° angle (Fig. 1A). The flap was pulled with a speed of 5 mm/min. The pulling was stopped when the flap became detached from the stromal bed. The maximum load to detach the flap was recorded. The recorded values in Newton (N) were converted to unit of strength in kilo Pascal (kPa) to take into account the difference in the diameter of flaps between those created by VisuMax (7.9 mm) and Femto LDV (8.5 mm).

In Vivo Confocal Microscopy

In vivo confocal microscopy with corneal module (HRT3; Heidelberg Engineering GmbH, Heidelberg, Germany) was performed on the rabbit corneas before surgery, and 4 and 24 hours after surgery. A carbomer gel (Vidisc; Mann Pharma, Berlin, Germany) was applied on the confocal lens and used as the immersion fluid. The central aspect of the corneas was examined with a minimum of three z-axis scans, consisting of the entire corneal thickness. These micrographs were analyzed and three scans of flap interface from each cornea were selected for further analysis. Semiquantitative analysis of the flap interfacial reflectivity (haze) was performed using ImageJ (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) by measuring the mean gray value of the reflective layer.

In order to assess the effect of laser delivery on the keratocytes that lied adjacent to the central incision plane at postoperative hour 24, the number of keratocytes captured by 400 × 400 μ m confocal scans at 10 μ m anterior and posterior from the reflective keratotomy plane was counted.

Tissue Fixation and Sectioning

After the rabbits were euthanized, the eyes were enucleated and the corneas were then excised. These corneas were embedded in an optimal cutting temperature (OCT) compound (Leica Microsystems, Nussloch, Germany) and stored at

−80°C until sectioning. Using a cryostat (Microm HM550; Microm, Walldorf, Germany), serial sagittal sections (8- μ m thick) were cut and placed on polylysine-coated glass slides. These slides were stored at −80°C until immunofluorescence staining was performed.

Immunofluorescence Staining

After the slides were retrieved from −80°C storage, the sections were air dried and fixed with 4% neutral buffered paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). Following which, they were washed with 1× PBS (first BASE, Singapore), and incubated in 1× PBS containing 0.15% Triton X-100 (Sigma-Aldrich) to increase cellular permeability. These slides were then incubated in 4% bovine serum albumin (Sigma-Aldrich), a blocking reagent, and incubated overnight at 4°C with primary antibodies thereafter. The antibodies used were either mouse monoclonal antibody against cellular fibronectin (5 μ g/mL; Millipore Corp., Billerica, MA, USA), rat monoclonal antibody against CD11b (20 μ g/mL; BD Pharmingen, Franklin Lakes, NJ, USA), or mouse monoclonal antibody against Ki-67 (20 μ g/mL; Dako, Glostrup, Denmark). After washing the slides with copious 1× PBS the next day, the sections were incubated with either a goat anti-mouse or goat anti-rat AlexaFluor 488-conjugated secondary antibody (Life Technologies, Carlsbad, CA, USA) for 1 hour in room temperature. The slides were then washed with copious 1× PBS before being mounted with UltraCruz Mounting Medium containing DAPI (Santa Cruz Biotechnology, Dallas, TX, USA). The images of sections were visualized and captured using a fluorescence microscope (AxioImager Z1; Carl Zeiss, Oberkochen, Germany).

TUNEL Assay

A TMR red-based TUNEL assay (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN, USA) was used to detect apoptotic cells in the sections. This assay was performed according to the manufacturer's instructions with

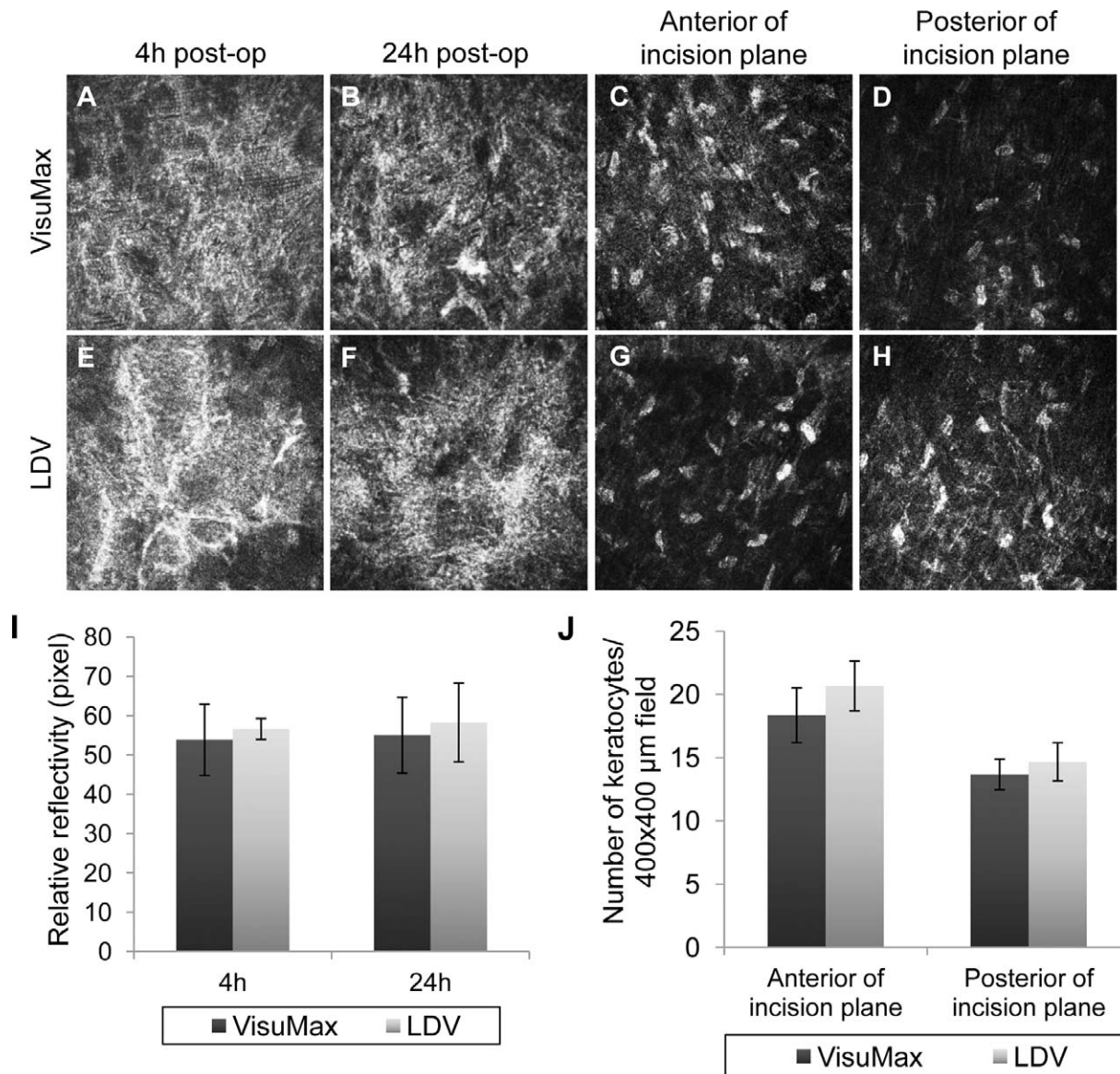


FIGURE 2. In vivo confocal micrographs of flap interface and its adjacent planes created by VisuMax and Femto LDV femtosecond lasers. Flap interface was easily identified by the presence of a light reflective layer. Representative images of flap interface 4 (A) and 24 hours (B) postoperatively, and anterior (C) and posterior (D) planes adjacent to the flap interface 24 hours after treatment with VisuMax. Images of flap interface 4 (E) and 24 hours (F) postoperatively, and anterior (G) and posterior (H) planes adjacent to the flap incision at postoperative hour 24 in the LDV group. (I) Bar graph showing relative reflectivity level of the flap interface at 4 and 24 hours after treatment with VisuMax or Femto LDV femtosecond lasers. (J) Bar graph showing average number of keratocytes appeared at the anterior and posterior planes adjacent to the flap interface at postoperative hour 24. Height of error bars in both bar graphs represents standard deviation of mean.

a minor modification, where the incubation of sections in the mixture solution was reduced to 30 minutes at 37°C.

Scanning Electron Microscopy

After the flaps of human cadaveric corneas were lifted and replaced, they were fixed in 2% paraformaldehyde (Sigma-Aldrich), 2% glutaraldehyde (Sigma-Aldrich) and 0.1 M sodium cacodylate (pH 7.4; Millipore Corp.) overnight at 4°C. The corneas were then washed twice in 1× PBS for 10 minutes each before being immersed in 1% aqueous solution of osmium tetroxide (FMB, Singapore) for 2 hours at room temperature. Following this, the samples were dehydrated in increasing concentrations of ethanol (25%, 50%, 75%, 95% to 100% ethanol, with 95% and 100% concentrations being performed

twice). The samples were then critical point dried using Bal-Tec dryer (Balzers, Liechtenstein) and mounted on stubs secured by carbon adhesive tapes. They were then sputter coated with a 10-nm thick layer of gold (Bal-Tec) and examined with a JSM-5600 scanning electron microscope (JEOL, Tokyo, Japan).

Evaluation of stromal bed morphology was performed based on a scoring system described in earlier studies.^{16,28} The scoring system was based on four criteria (11 points in total): surface relief (2 points maximum); regularity of surface structure (3 points); extent of surface irregularities (3 points); and position of irregular area (3 points). A higher score indicates a smoother bed surface. Surface relief was scored based on SEM images taken at $\times 25$ magnification and the other criteria on images captured at $\times 50$ magnification. Two

unmasked observers (AKR, YCL) were assigned to grade the images and the results were compared.

Statistical Analysis

Data were expressed as mean \pm SD. The *P* value was determined using one-way ANOVA test with statistical software (SPSS version 17.0; SPSS, Inc., Chicago, IL, USA). A value of *P* < 0.05 was considered to be statistically significant. Intraclass correlation coefficient (ICC) between two observers, who graded the flap bed irregularity, was determined using statistical software (SPSS, Inc.). Intraclass correlation coefficient \geq 0.7 indicates a good agreement between the observers.

RESULTS

Flap Adhesion Strength

The adhesion strength in the VisuMax and Femto LDV groups at postoperative month 1 was 16.95 ± 1.45 kPa and 12.31 ± 4.15 kPa, respectively (*P* = 0.184). There was a marginal increase in the adhesion strength from postoperative month 1 to month 2 in both groups, where the VisuMax group recorded 18.33 ± 1.81 kPa and the Femto LDV group 13.85 ± 4.78 kPa (*P* = 0.240). There was no significant difference between the adhesion strength recorded at month 1 and 2 in the VisuMax group (*P* = 0.361) and also in the LDV group (*P* = 0.696). The flap adhesion strength test results are depicted as a bar graph in Figure 1B.

Flap Interface Visualization by In Vivo Confocal Microscopy

The interface of the flap at fourth hour after treatment with VisuMax was characterized by the distinct presence of regularly aligned dots, which was most likely the impact of laser pulses on the stroma (Fig. 2A). This feature was less obvious at hour 24 after flap creation by VisuMax (Fig. 2B). The regularly aligned dots were not detected at 4 hours (Fig. 2E) and 24 hours (Fig. 2F) after flap preparation by Femto LDV, most likely due to the laser spots being placed directly adjacent to each other. At the level of keratotomy plane, some keratocytes could be observed at all time points in both the VisuMax and LDV groups (Figs. 2A, 2B, 2E, 2F). The relative reflectivity of the LASIK flap interface was depicted in a bar graph (Fig. 2I). The flap interfacial reflectance in the VisuMax and Femto LDV groups at postoperative hour 4 was 53.85 ± 9.11 pixels and 56.63 ± 2.67 pixels, respectively (*P* = 0.402). The reflectivity level in the VisuMax group was measured at 55.02 ± 9.66 pixels and the Femto LDV group 58.26 ± 10.03 pixels (*P* = 0.496) 24 hours after surgeries. There was no significant difference found at hours 4 and 24 postoperatively in the VisuMax group (*P* = 0.794) and also in the LDV group (*P* = 0.649).

There were keratocytes observed at the anterior plane adjacent to the flap interface in both the VisuMax (Fig. 2C) and LDV (Fig. 2G) groups 24 hours postoperatively. Slightly less keratocytes could be seen at the posterior plane adjacent to the flap incision plane in both groups (Figs. 2D, 2H). The mean number of keratocytes captured in 400×400 μ m confocal images in each group was depicted in a bar graph (Fig. 2J). No significant difference in cell count at both planes of both groups was found. At the anterior plane, there was 18.33 ± 2.16 cells in the VisuMax group and 20.67 ± 1.96 cells in the LDV group (*P* = 0.079). Posterior of the incision plane, there was 13.67 ± 1.21 cells in the VisuMax group and 14.67 ± 1.50 cells in the LDV group (*P* = 0.235).

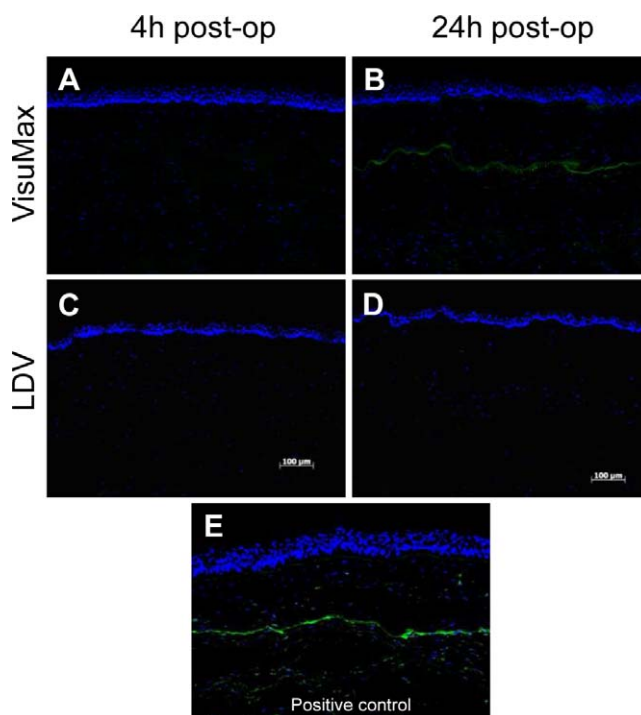


FIGURE 3. Expression of fibronectin in the central cornea after flap creation with VisuMax and Femto LDV femtosecond lasers. Fibronectin was not present in the cornea 4 hours after treatment with VisuMax (A), but was expressed relatively weak along the laser incision plane 24 hours postoperatively (B). In the Femto LDV group, fibronectin was absent in the cornea 4 (C) and 24 hours (D) after flap preparation. (E) Fibronectin was observed along the excimer-ablated stromal plane and in weaker intensity posterior to the ablation plane 24 hours after surgery in the positive control cornea. Positive control cornea received a –6-D excimer stromal ablation after the flap was lifted. Scale bar: 100 μ m.

Tissue Responses Following Flap Creation

Fibronectin was not detected in the central cornea at 4 hours after treatment with VisuMax (Fig. 3A), but was present along the flap interface 24 hours postoperatively (Fig. 3B). The staining intensity was, however, substantially weaker than that seen in the positive control cornea (Fig. 3E). In the positive control cornea, weak staining could also be seen posterior to the keratotomy plane (Fig. 3E). In the Femto LDV group, fibronectin was absent in the cornea at 4 (Fig. 3C) and 24 hours (Fig. 3D) after flap preparation.

CD11b, an inflammatory cell marker, was not detected in the central cornea at 4 (Fig. 4A) and 24 hours (Fig. 4B) after treatment with VisuMax. CD11b was also absent in the cornea at 4 (Fig. 4C) and 24 hours (Fig. 4D) after flap preparation in the LDV group. In contrast, inflammatory cells could be observed along the excimer-ablated stromal plane at 24 hours postoperatively in the positive control cornea (Fig. 4E).

TUNEL-positive cells were present along the laser incision plane in the central corneal stroma at 4 (Fig. 5A) and 24 hours (Fig. 5B) after treatment with VisuMax, but were largely absent at both time points (Figs. 5C, 5D) after flap preparation with Femto LDV. At postoperative hour 4, there was a significant difference between the VisuMax (17.33 ± 1.53 cells) and LDV (0.67 ± 0.58 cells) groups (*P* < 0.001). A significant difference was also found at postoperative hour 24 between the VisuMax (12.67 ± 1.53 cells) and LDV (0.33 ± 0.58 cells) groups (*P* = 0.002). In contrast, TUNEL-positive cells were almost negligible in central corneal epithelium at 4 (Fig. 5A) and 24 hours (Fig.

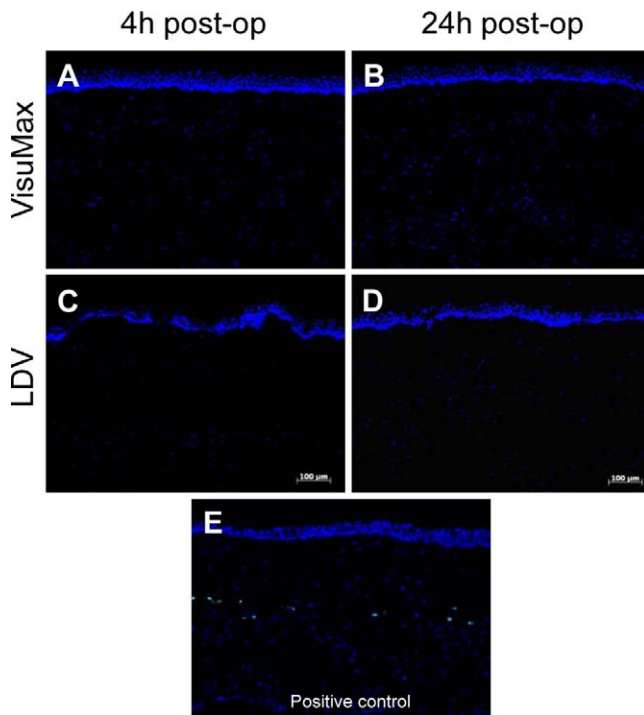


FIGURE 4. Expression of CD11b in the central cornea after flap creation with VisuMax and Femto LDV femtosecond lasers. CD11b was not present in the cornea 4 (A) and 24 hours (B) after treatment with VisuMax. In the Femto LDV group, CD11b was also absent in the cornea 4 (C) and 24 hours (D) after flap preparation. (E) Inflammatory cells were observed along the excimer-ablated stromal plane 24 hours after surgery in the positive control cornea. Positive control cornea received a -6-D excimer stromal ablation after the flap was lifted. Scale bar: 100 μ m.

5B) after treatment with VisuMax, but were abundant at 4 (Fig. 5C) and 24 hours (Fig. 5D) after flap preparation with Femto LDV. At postoperative hour 4, there was a significant difference between the VisuMax (3.67 ± 1.53 cells) and LDV (18.33 ± 5.51 cells) groups ($P = 0.036$). Significant difference was also found at postoperative hour 24 between the VisuMax (2.33 ± 0.58 cells) and LDV (11.00 ± 3.61 cells) groups ($P = 0.041$). Apoptotic cells could be observed along the excimer-ablated stromal plane at 24 hours after surgery in the positive control cornea (Fig. 5E). The mean number of apoptotic cells in the stroma and epithelium of corneas treated with VisuMax and LDV was depicted in a bar graph (Fig. 5F).

Ki-67, a proliferative cell marker, was not expressed in the central corneal stroma at 4 (Supplementary Fig. S4A) and 24 hours (Supplementary Fig. S4B) after treatment with VisuMax. There were some Ki-67-positive cells detected in the basal layer of epithelium, where the number of proliferative cells increased from 8.33 ± 1.15 cells at hour 4 to 19.00 ± 3.60 cells at hour 24 after surgery. In the Femto LDV group, Ki-67 was also absent in the corneal stroma at 4 (Supplementary Fig. S4C) and 24 hours (Supplementary Fig. S4D) after flap preparation. Similar to the VisuMax group, some basal epithelial cells expressed Ki-67 at 4 hours (8.67 ± 3.05 cells) and 24 hours (19.33 ± 5.86 cells) after treatment with Femto LDV. Proliferative cells could be observed in the basal epithelial cells at 24 hours after surgery in the positive control cornea (Supplementary Fig. S4E). The mean number of proliferative cells in the epithelium of corneas treated with VisuMax and LDV was depicted in a bar graph (Supplementary Fig. S4F).

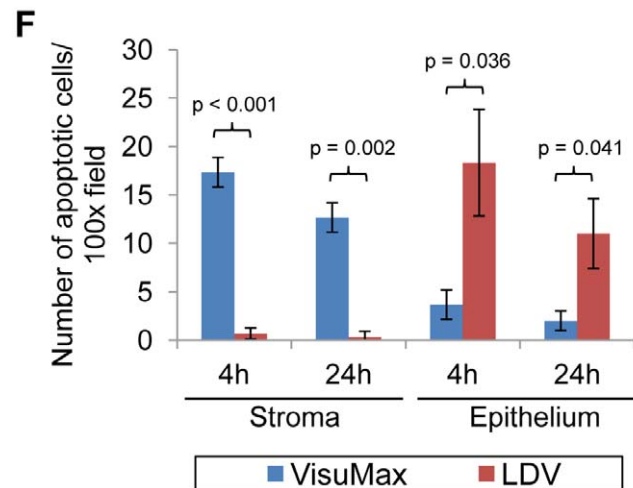
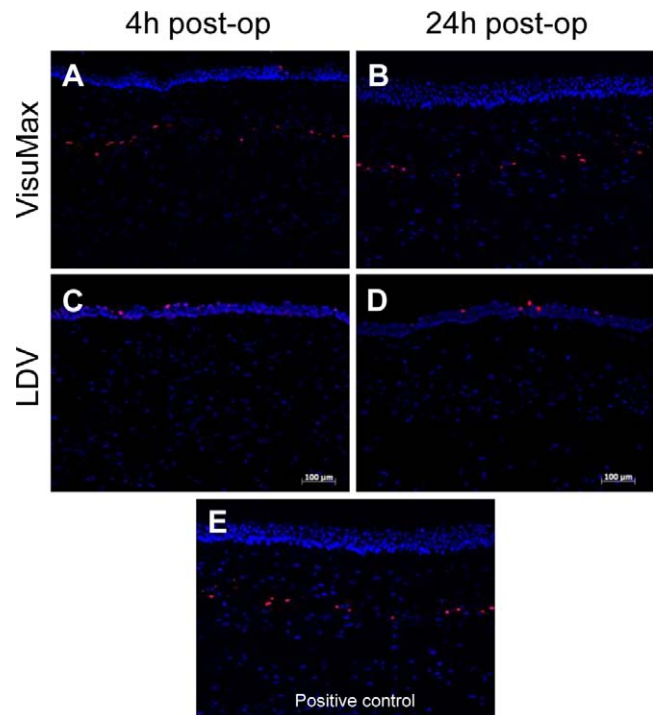


FIGURE 5. Detection of apoptotic cells by TUNEL assay in the central cornea after flap creation with VisuMax and Femto LDV femtosecond lasers. TUNEL-positive cells were present along the laser incision plane in the corneal stroma 4 (A) and 24 hours (B) after treatment with VisuMax, but were largely absent in the epithelium 4 (A) and 24 hours (B) after surgery. In the Femto LDV group, TUNEL-positive cells were absent in the cornea 4 (C) and 24 hours (D) after flap preparation. However, abundant apoptotic cells could be seen in the superficial epithelial layer both 4 (C) and 24 hours (D) after treatment with Femto LDV. (E) Apoptotic cells could be observed in along the excimer-ablated stromal plane 24 hours after surgery in the positive control cornea. Positive control cornea received a -6-D excimer stromal ablation after the flap was lifted. Scale bar: 100 μ m. (F) Bar graph showing number of apoptotic cells in the epithelium and stroma 4 and 24 hours after flap preparation with the VisuMax and Femto LDV femtosecond lasers. Height of error bars represents standard deviation of mean number of TUNEL-positive cells/ $\times 100$ viewing field.

Flap Bed Quality

The smoothness of human corneal central flap beds visualized by SEM appeared similar for both VisuMax (Figs. 6A, 6B) and LDV (Figs. 6C, 6D) groups. The flap-side cut created by

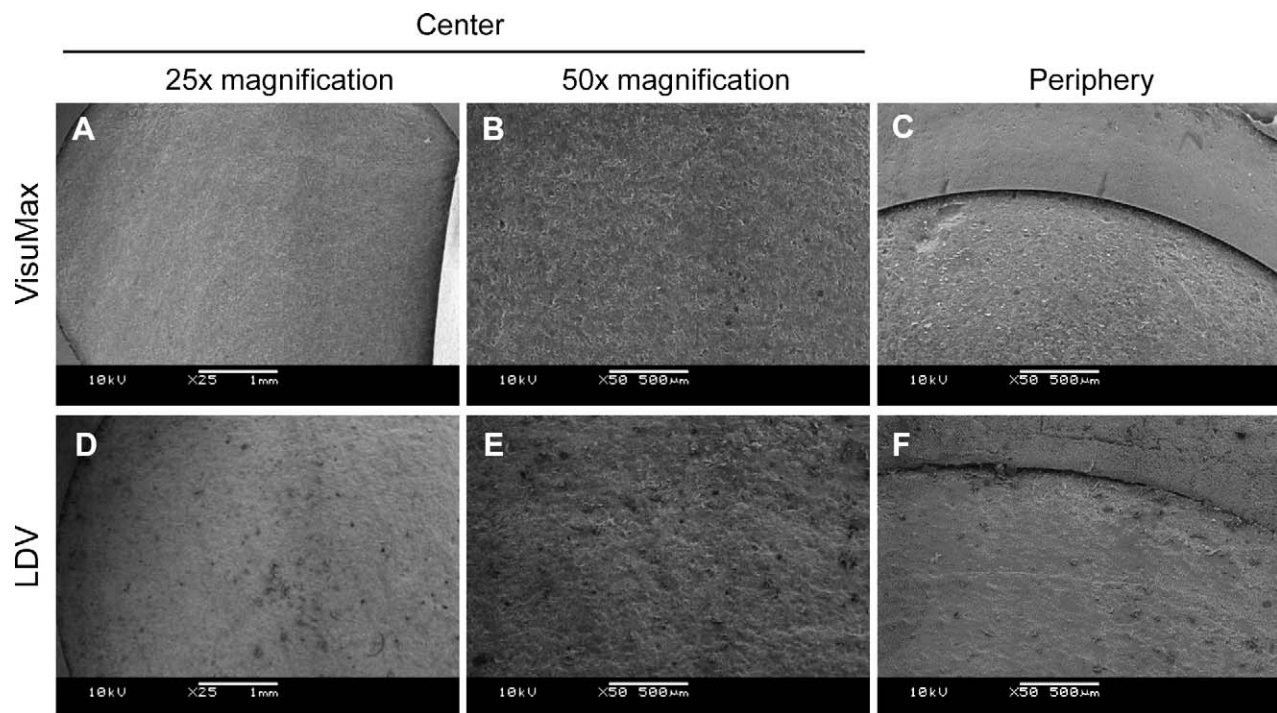


FIGURE 6. Assessment of smoothness of corneal flap bed created by VisuMax and Femto LDV femtosecond lasers. Scanning electron microscopy of central flap bed prepared with VisuMax femtosecond laser in $\times 25$ magnification (A) and $\times 50$ magnification (B), as well as of peripheral flap bed in $\times 50$ magnification (C). Scanning electron microscopy of central flap bed created by Femto LDV shown in lower magnification (D) and higher magnification (E). Scanning electron microscopy of peripheral flap bed prepared with Femto LDV in $\times 50$ magnification, showing a rough flap side cut (F).

VisuMax appeared smooth and uniform, and the transition from the peripheral flap bed to the corneal surface unaffected by the laser incision was distinct (Fig. 6C). However, the flap-side cut observed in the LDV group appeared less smooth and the depth of the side cut was less uniform (Fig. 6E).

There was a good agreement between the observers for the VisuMax group (ICC = 0.8) and LDV group (ICC = 0.7), where observer 1 scored the VisuMax group 8.00 ± 1.00 and the LDV group 7.33 ± 0.58 ($P = 0.387$); and observer 2 scored the VisuMax group 8.33 ± 0.58 and the LDV group 7.67 ± 0.58 ($P = 0.230$). Both observers agreed that there was no significant difference in the mean irregularity score of the flap bed comparing the VisuMax and LDV groups.

DISCUSSION

There are two basic paradigms in the mechanism of femtosecond laser-assisted corneal incision. One relies on low frequency with high energy laser pulses and the other one uses high frequency with low energy laser pulses to create the intrastromal incision. The earlier models of the femtosecond laser (e.g., IntraLase FS30) utilizes low frequency, high energy pulses where plasma is formed leading to the expansion of multiple cavitation bubbles that form the incision plane. The later models of femtosecond lasers (e.g., Femto LDV Z6) tested in the current study, employ high frequency, low energy pulses (in nJ range). In this cutting mechanism, the plasma is the primary driving force in the tissue dissection process. Hence, to create a complete and easy to lift flap, the laser spots have to be placed directly adjacent to each other. Because the Femto LDV Z6 does not rely on the formation of cavitation bubbles, the cutting result closely resembles that of a microkeratome blade. The VisuMax 500kHz femtosecond laser, operating in μ J energy range, employs a cutting mechanism somewhere in the

middle of the paradigms. In the current study, we found that there was a tendency toward stronger flap adhesion strength and smoother stromal bed following flap preparation with a μ J-energy level laser (VisuMax) relative to an nJ-energy level laser (Femto LDV). We also found that the lower energy pulses produced by the Femto LDV may have attributed to the lack of wound healing reaction and the absence of apoptotic cells along the flap incision plane.

From the results of our fibronectin staining, there was an absence of wound healing reaction at hour 4 and even 24 hours after flap incision with the Femto LDV. However, at hour 24 post treatment with VisuMax, fibronectin was expressed along the incision plane. In addition, we found that Femto LDV did not induce as many apoptotic cells as the VisuMax along the intrastromal incision plane. It could be possible that the lower energy pulses (nJ range) produced by the Femto LDV attributed to the above observations. In our previous work, we showed that the formation of cavitation bubbles is still the main driving force of intrastromal incision of the VisuMax femtosecond laser.²² Hence, in addition to the effect of the formation of plasma, the following rapid expansion and subsequent collapse of the cavitation bubbles might have amplified the intrastromal wound and the number of apoptotic cells. In contrast, the intrastromal injury was only inflicted by the formation of plasma at the incision plane by laser pulses produced by Femto LDV.

From the absence of CD11b staining in both study groups, it appeared that laser-induced apoptosis in the stroma occurred independent of inflammation. We concluded that modern femtosecond lasers operating with low energy level induce negligible corneal inflammation. Our results contrasted the finding by Kim et al.¹⁸ and Netto et al.,²⁰ who showed the presence of inflammatory and apoptotic cells not only along the incision plane, but also at the anterior and posterior region to this plane with the IntraLase laser.

We observed significantly more apoptotic cells in the superficial layer of the corneal epithelium treated with the Femto LDV compared with the VisuMax. This may be explained by the different interface curvature of the suction cone used to fixate the patients' eye during the laser procedure. The Femto LDV uses a flat interface cone, which created more pressure and stress on the corneal surface during the flap cutting procedure than the curved interface cone employed by the VisuMax, leading to more postoperative cell death in the epithelium.

Using an in vivo confocal microscope, the flap interface can be easily identified as a light reflective layer. The derangement of collagen lattice arrangement induced by the laser delivery to the cornea most likely causes the light to scatter and appear reflective on confocal scans. In contrast, this phenomenon is not seen in the unoperated, highly transparent corneas because the light can penetrate unobstructively.^{21,29} The reflectivity appears to be reduced over time as the corneas recovered and the collagen lattice formation restored.³⁰ Here, we did not find any significant difference in the reflectivity of the flap interface between the VisuMax and LDV groups. We also counted the keratocytes that were present at 10 μ m anterior and posterior from the flap interface to assess the collateral effect of the laser delivery to the cornea. Although we found more keratocytes in the LDV group than in the VisuMax group, the difference did not reach any statistical significance. The confocal results suggest that both laser systems do not cause excessive collateral damage.

Although no significant difference was found in the smoothness of the flap bed produced by either the Femto LDV or VisuMax, the two observers agreed that there was a tendency of a smoother stromal bed in the VisuMax group. Similarly, we found a tendency of a stronger flap adhesion strength in the VisuMax group, although no significant difference was demonstrated. Knorz et al.³¹ found that the energy levels employed to create the flap side cut did not affect the flap adhesion strength; therefore, we can rule out that the different energy level employed by the VisuMax and LDV attributing to our observation. Another factor that can potentially improve the strength of flap adhesion is flap side cut angulations. Knorz et al.³¹ and Knox Cartwright et al.³² have demonstrated that the inverted side cut angles significantly increase the flap adhesion strength and the overall corneal biomechanical strength. Since the side cut angle in this study was maintained constant at 90° for both study groups, we rule out that the side cut angulation had attributed to our adhesion strength test results.

Studies have shown that fibroblast growth factor (FGF), transforming growth factor (TGF)- β and epidermal growth factor (EGF), released during tissue injury, can stimulate the chemotaxis of corneal fibroblasts, mitosis of fibroblasts, and increase the production of collagen and fibronectin at the wound site.^{33,34} These growth factors and cytokines enhance corneal wound healing process and may also increase the tensile strength of corneal incisions by triggering key pathways of scar formation. The fact that fibronectin, CD11b, and TUNEL stainings were mostly absent at the incision plane in the LDV group means that the flaps may not adhere to the stromal bed as well as those prepared by the VisuMax.

Although our findings—especially of the flap adhesion strength and flap bed smoothness—are microscopically relevant, subsequent excimer stromal ablation in the LASIK procedure would alter the surface of the flap bed in an equal manner regardless of the femtosecond laser used to cut the flap. However, our findings have an implication on the potency of extremely low energy level femtosecond lasers and its advantages in many femtosecond laser only clinical applications, such as the possibility to create an ultrathin posterior

lamellar disc (50–70- μ m thickness) without affecting the viability of corneal endothelial cells in cases of DSAEK. It may also improve the safety and visual recovery of refractive lenticule extraction procedure even further by eliminating the apoptosis of keratocytes and excessive tissue injury.

In conclusion, we have shown that there was a tendency toward a stronger flap adhesion strength and smoother stromal bed following flap preparation with the VisuMax compared with the Femto LDV, although the differences were not statistically significant. We have also shown that the lower energy pulses produced by the Femto LDV may attribute to the absence of wound healing reaction and lack of apoptotic cells along the flap incision plane.

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