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Corneal Response to Femtosecond Laser Photodisruption in the Rabbit

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Abstract

In this report we evaluated the effect of femtosecond laser energy on the development of corneal haze and keratocyte activation in rabbits following intra-stromal photodisruption to create LASIK flaps using a modified commercial femtosecond surgical laser. Three groups of flap parameters were studied: 1.5 $\mu\text{J}/\text{pulse}$ with 10 μm spot separation and complete side cut (Group 1), 3.5 $\mu\text{J}/\text{pulse}$ with 14 μm spot separation and complete side cut (Group 2), 3.5 $\mu\text{J}/\text{pulse}$ with 14 μm spot separation and partial (50 μm) side cut (Group 3). All flaps were left attached without lifting to avoid epithelial contamination. Rabbits were then evaluated pre- and post-operatively by quantitative in vivo and ex vivo confocal microscopy. The achieved flap thickness 1 week after surgery averaged 88.9 ± 12.8 , 90.8 ± 6.9 and 86.5 ± 6.8 μm for Groups 1–3 respectively ($p = \text{NS}$). Interface thickness was significantly greater ($p < 0.05$) in the higher energy groups averaging 40.0 ± 11.2 and 37.7 ± 5.7 μm for Groups 2–3 compared to 28.6 ± 4.5 μm for Group 1. Corneal haze was barely detectable and not significantly different between groups, although haze was detected in the region of the side-cuts in groups 1 and 2. No clinically significant changes in stromal or epithelial thickness were noted. Laser confocal microscopy showed the presence of small diameter cells within the flap interface that resided within disrupted regions of the corneal collagen lamellae. Keratocyte activation was only detected in regions of the 100% side cut and not over the flap interface. In conclusion, the results of this study indicate that photodisruption of the corneal stroma alone without flap elevation regardless of laser energy does not induce significant corneal haze in the rabbit. However, a thicker stromal interface was seen with the higher energy suggesting greater stromal damage.

Introduction

LASIK is currently the most common form of corneal surgery performed to correct refractive errors because of the rapid visual recovery and decreased risk of developing central corneal haze and scarring. (Wilson, 2004) Since LASIK requires the initial surgical creation of an anterior corneal flap using a mechanical microkeratome, complications during surgery include the incomplete or complete flap removal, uneven flap thickness, and the formation of central

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holes in the flap. (Stonecipher, et al., 2006) Recently, femtosecond lasers have been introduced to cut corneal LASIK flaps. (Nordan, et al., 2003) Femtosecond lasers pulses are focused into the cornea to create a plasma generated cavitation bubble by laser induced optical breakdown (LIOB) of the tissue. Scanning of the corneal stroma using either a spiral or raster pattern at the same focal plane then produces a cleavage plane that can be separated to produce a LASIK flap prior to surgery that has apparently fewer complications compared to the mechanical microkeratome. In general, femtosecond laser generated flaps are more advantageous than mechanical microkeratome generated flaps because of improved uniformity and better predictability of flap thickness. (Kezirian, et al., 2004; Stonecipher, et al., 2006)

While recent laboratory animal studies suggest that femtosecond laser created flaps show stronger adhesion to the corneal bed, LIOB of the stroma appears to lead to significantly greater inflammation at 4 and 24 hours after surgery compared to mechanical microkeratomes. (Kim, et al., 2006) Clinical experience also suggests that eyes undergoing femtosecond flap dissection experience increased interface inflammation for the first 2 days after surgery. (Binder, 2004) Interestingly, decreasing the laser energy from 8 μJ to 2.8 μJ per pulse eliminated this postoperative complication suggesting that high laser energy may induce greater damage to the corneal stroma. Transient light sensitivity in patients following femtosecond laser flap creation has also been linked to the use of high laser energy levels which may induce keratocyte activation and increased light scattering. (Petroll, et al., 2006; Stonecipher, et al., 2006) Together these findings suggest that femtosecond laser photodisruption at higher energy levels may produce greater damage to keratocytes and stromal nerves producing these complications.

In order to address these questions, we have evaluated the effects of femtosecond laser flap creation on development of keratocyte activation and corneal haze in a rabbit model using two different energy and pulse separation algorithms. Since flap elevation has been proposed as possibly inducing additional corneal damage, (Binder, 2004) flaps were left attached to identify only the effects of laser photodisruption to the corneal stroma. Furthermore, to isolate any effects of corneal epithelial damage contributing to the activation of keratocytes, one group of eyes received only partial flap edge cuts that did not extend to the epithelium. In this report we show that femtosecond laser photodisruption alone does not appear to induce keratocyte activation or corneal haze at either low or high energy levels regardless of epithelial damage.

Materials and Methods

Rabbits

A total of 15 New Zealand white rabbits were used in this study. Before in vivo confocal microscopy and surgery, each rabbit was anesthetized with an intramuscular injection of zylazine (10 mg/kg) and ketamine hydrochloride (50 mg/kg). After induction of anesthesia, one drop of 0.5% proparacaine hydrochloride was applied to the eye. All procedures were approved by the UCI IACUC and conducted in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. One animal in Group 3 died after anesthesia and was eliminated from the study.

Femtosecond Laser Photodisruption

One eye of each rabbit received a 7.5 mm diameter, 110 μm thick central corneal flap using a prototype commercial femtosecond surgical laser that had been modified for use on experimental tissue. Rabbits were divided into 3 groups depending on energy, spot size, and flap side cut parameters: Group 1 (5 Rabbits): 1.5 μJ /pulse-10 μm spot size with complete side cut, Group 2 (5 Rabbits): 3.5 μJ /pulse-14 μm spot size with complete side cut, Group 3 (5 Rabbits): 3.5 mJ/pulse-14 μm spot size with partial (50 μm) side cut.

In Vivo Confocal Microscopy

In vivo confocal microscopy was performed under anesthesia preoperatively and at 1, 2, 4, 6 and 8 weeks postoperatively. Corneal interface haze, corneal epithelial thickness, stromal thickness, stromal flap thickness and interface thickness were measured using a method previously described (Jester, et al., 1999). Briefly, in vivo confocal microscope examinations were performed using a Tandem Scanning Confocal Microscope (TSCM, Tandem Scanning Corporation, Reston, VA) with a 24x surface-contact objective (numerical aperture, 0.6; working distance, 1.5 mm), Oriel 18011 Encoder Mike Controller (Oriel Corp., Stratford, CT) for focal plane control, and Dage MTI VE-1000 camera (Dage MTI, Michigan City, IN). One drop of 2.5% hydromethylcellulose (Gonak, Akorn Inc., Buffalo Grove, IL) was placed on the tip of the objective as a coupling gel. All camera settings were kept constant throughout the experiment. For each examination, at least 4 through-focus data sets extending from the anterior surface to the corneal endothelium was obtained from the central cornea.

Digital Image Analysis and 3-Dimensional Display

The images were analyzed using a TSCM software developed for in vivo CM. (Li, et al., 2000) Measurement of epithelial thickness (ET), stromal thickness (ST) and flap thickness were as previously described. (Gokmen, et al., 2002) In this study the flap interface was clearly identified as an optical plane in the 3-D data set that contained indistinct light scattering structures, possibly related to regions of collagen disruption associated with LIOB, and was located in the middle of an acellular region lacking normal keratocyte nuclei. The distance between this interface plane and the surface of the corneal epithelium was then recorded as the stromal flap thickness. To measure interface thickness and haze the anterior and posterior optical plane containing normal keratocyte nuclear density that bordered the acellular zone was identified and the distance (interface thickness) or light scattering (interface haze) was then measured between these two planes.

Ex Vivo Confocal Microscopy

At 8 weeks after surgery, rabbits were euthanized and the anterior chamber perfused with 2% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4. Corneas were removed, and kept in 2% paraformaldehyde/PBS overnight. Corneas were then cut into 2mm square tissue blocks and stained *en bloc* with Syto® 59 (Molecular Probes, Eugene, OR) and phalloidin (Alexa Fluor® 488 phalloidin, Molecular Probes) in 50% TD buffer (0.5% DMSO, 0.5% Triton X, 2.5% Dextran 40 in PBS, pH 7.4) overnight to identify nuclei and cell cytoskeleton, respectively. Specimens were placed on an Axiovert 200 microscope (Zeiss, Jena, Germany) and imaged by using laser confocal microscope system (LSM 510 META, Carl Zeiss), which is equipped with a mode-locked Titanium:Sapphire laser (Chameleon®, Coherent Inc., Santa Clara, CA). The fluorescent signal detection from phalloidin and Syto 59 were obtained using the 488 nm and 633 nm laser lines of the argon and red helium-neon lasers, respectively. From the same optical plane, collagen organization of the corneas was visualized by second harmonic imaging microscopy (SHIM). Imaging of second harmonic generated (SHG) signals from collagen has been previously used to identify the three-dimensional organization of the stromal collagen lamellae in humans, rabbits and mice. (Morishige, et al., 2006; Morishige, et al., 2007) Briefly, SHG signals were generated using the mode-locked Titanium:Sapphire laser tuned to 800 nm to obtain maximum SHG emission signal. SHG forward scattered signals passing through the tissue were collected using a 0.8 NA condenser lens with a narrow band-pass filter (400/50) placed in front of the transmission light detector. Backward scattered SHG signals were detected using 370 to 440 nm band filter of the Meta detector on the Zeiss 510 META (Zeiss).

Statistics

Analysis of the in vivo confocal microscope data included the averaging of measurements from the 4 through-focus data sets for each eye at each time point. Group averages were then calculated and recorded. The data for each group was then reported as mean $\pm$ standard deviation. For statistical analysis a Mann-Whitney and a two way and one way repeat measures ANOVA for each measurements was applied using Sigma Stat (Systat Software, Inc., Point Richmond, CA).

Results

In Vivo Confocal Microscopy

Figure 1 shows in vivo confocal microscope images from rabbit corneas before (Figure 1, A and B) and 7 days after (Figure 1, C and D) femtosecond laser generate flap dissection. Before surgery, a uniform layer of squamous surface epithelial cells was detected overlying the normal corneal stroma that contained detectible keratocyte nuclei (B, arrows). One week after surgery, the overlying central corneal epithelium appeared normal (C), however, the interface between the anterior corneal flap and the posterior corneal bed was identified as an acellular region lacking normal keratocyte nuclei with occasional isolated nuclei (D, arrows) and indistinct, light scattering areas suggesting regions of disrupted collagen. At the edge of the flap, disruption of keratocyte density could be observed within the corneal stroma, which appeared mild at lower (1.5 μ J, E) and more marked at higher (3.5 μ J, F) laser energy levels. Higher energy levels were also associated with earlier activation of keratocytes adjacent to the flap edge (F, arrows); although activation and fibrosis at the flap edge was also detected with the lower laser energy at later times.

The flap interface at 1 week was also identified in XZ slices through the 3-D data sets as an acellular region showing slight increased haze (Figure 2A, arrow). Interactively scrolling through the 3-D data set confirmed that the region detected in the XZ slice lacked any intact keratocyte nuclei (Figure 2B), thus allowing for measurement of the anterior corneal flap thickness. In general the flap interface appeared more pronounced in corneas cut using the higher laser energy levels (Figure 2C) and showed a wider region of stromal acellularity and increased haze (arrow). The slight increase in haze associated with higher energy flap dissection appeared to be related to increased light scattering from indistinct structures at the interface, possibly associated with the increase size of the cavitation bubbles that may lead to disruption of the collagen organization (Figure 2D). After 8 weeks post-surgery, no increase in cell density or keratocyte activation within the flap interface was detected in either the 1.5 μ J (Group 1) or 3.5 μ J (Group 2 and 3) laser energy treated groups.

At the edge of the flap, keratocyte activation and migration into the wound was noted by 1 and 2 weeks after injury for both Group 1 and 2 that received 100% side cuts (Figure 3A, data only shown for 3.5 μ J laser cuts). No detectible change in cell density was observed in Group 3 with 3.5 μ J laser energy but only 50% side cuts (Figure 3B). This finding suggested that keratocyte activation after femtosecond laser flap generation may be associated with epithelial damage. By 8 weeks after injury, keratocyte activation at the flap margin appeared to markedly diminish in both Groups 1 and 2, although cell density remained elevated at the edge of the flap (Figure 3C). By contrast, eyes receiving only 50% side cuts showed no clear evidence of keratocyte repopulation into the wound (Figure 3D).

Epithelial, Stromal, Flap, Interface Thickness and Haze Measurements

Changes in epithelial, stromal, flap, interface thickness and haze are summarized in Table 1 and Figure 4 and Figure 5. Epithelial thickness (Figure 4A) showed no significant differences between groups, although thickness at 8 weeks post-surgery was significantly decreased

compared to the thickness after 1 week for all groups ($p < 0.05$) but not to baseline indicating that this difference was not clinically relevant. Stromal thickness appeared to gradually increase after surgery (Figure 4B) for all groups, however no significant difference was detected between groups. Stromal flap thickness was significantly decreased for all groups ($p < 0.05$) an average of $13 \mu\text{m}$ by 8 weeks after surgery (Figure 4C). Achieved stromal flap thickness averaged $88 \mu\text{m}$ for all groups at 1 week based on a predicted surgical flap thickness of $110 \mu\text{m}$ as calibrated for human cornea. The finding that flaps were thinner than expected may be related to differences between rabbit and human cornea, possibly regarding the tissue refractive index and the focus of the laser beam within the tissue. Interface thickness as measured by determining the distance between the normal keratocyte nuclear density above and below the interface, hence the thickness of the 'acellular' zone, was significantly thinner in Group 1 compared to Group 2, $p < 0.05$, at the first week after surgery (Figure 5A). Interface haze was generally unremarkable in all groups and no significant differences were detected (Figure 5B).

Ex Vivo Microscopy

The response of the cornea to femtosecond laser photodisruption was evaluated for all groups after collection of the 8 week in vivo confocal data. Laser confocal microscopy of corneas confirmed the decrease in keratocyte density at the flap interface as indicated by the marked decrease in nuclei (Figure 6A, arrow) and the appearance of a few scattered rounded nuclei (arrowheads). Images taken at the flap interface (B and C) showed that these round nuclei were organized along uniform rows or furrows within the flap margins. The spacing of these rows were different between the low and high energy generated flaps averaging $\sim 27 \mu\text{m}$ with $1.5 \mu\text{J/pulse}$ (Figure 6B) and $\sim 37 \mu\text{m}$ with $3.5 \mu\text{J/pulse}$ (Figure 6C). However, given the small sample size, this difference was not significant. Compared to normal keratocytes within the intact stroma (Figure 7A–C), cells residing at the flap interface appeared smaller with smaller nuclear diameters, reduced cell spreading, and less cell interconnectivity for both the low (Figure 7D–F) and high energy (Figure 7G–I) generated flaps. Using second harmonic imaging microscopy to identify collagen organization, small diameter defects or disruptions in the collagen lamellae were noted to be present at the same optical plane as the cells at the interface (Figure 7E and 7H, arrows). These defects were approximately $6\text{--}7 \mu\text{m}$ in diameter, appeared slightly but not significantly larger in the higher energy group, and formed along distinct lines creating grooves within the collagen lamellae. Interestingly, the smaller cells at the interface appeared to reside preferentially at or within these defects (Figure 7F and 7I).

Discussion

This study demonstrates that femtosecond photodisruption of the corneal stroma without flap elevation does not produce significant keratocyte activation leading to corneal haze at the flap interface using laser energies between 1.5 and $3.5 \mu\text{J/pulse}$. Since previous electron microscopic studies have shown that LASIK induces changes in keratocyte cellular activity that go undetected by in vivo confocal microscopy (Dawson, et al., 2005), it is possible that the different energy levels may have induced different keratocyte responses that went undetected. Regardless of the potential differences in keratocyte responses at the ultrastructural level, the current study could not detect any difference in the corneal light scattering. Since in vivo confocal microscopy is a sensitive technique for objectively measuring corneal haze, our findings suggest that laser energies below $3.5 \mu\text{J/pulse}$ do not produce clinically significant haze when flaps remain attached.

Keratocyte activation was noted at the flap margins when the laser cuts extended vertically through the corneal epithelial surface. No keratocyte activation was detected when photodisruption was limited to only 50% vertical side cuts, stopping short of the corneal epithelium indicating that damage to the stroma in either the parallel or vertical plane that

avoids the epithelium does not induce corneal haze. These findings are consistent with a previous report indicating that femtosecond laser photodisruption to create a 100 μm thick midstromal pyramid without epithelial involvement results in corneal thinning without haze. (Sletten, et al., 1999) Taken together these findings suggest that keratocyte activation leading to corneal haze requires stromal and epithelial injury, although development of haze following LIOB of the stroma using higher energy levels than those used in the current study cannot be ruled out. This conclusion is in general agreement with the widely proposed cellular mechanism for keratocyte activation involving combined epithelial and stromal damage leading to the release of growth factors and cytokines, which stimulate keratocyte migration, proliferation and differentiation to corneal fibroblasts and myofibroblasts that ultimately result in corneal haze. (Jester, et al., 1999; Jester, et al., 1999; Moller-Pedersen, et al., 1998; Netto, et al., 2005)

Recent clinical reports, however, have drawn increasing attention to the relationship between laser power and the development of interface haze and transient light sensitivity (TLS), proposing that higher energy femtosecond laser photodisruption produces keratocyte activation. (Binder, 2004; Kim, et al., 2006; Petroll, et al., 2006; Stonecipher, et al., 2006) This association has been supported by the clinical impression that lower energy levels in general have reduced these complications. As noted in a recent report, 6 out of 20 cases of Intralase LASIK surgery experiencing interface haze or TLS occurred only in cases with higher energy pulses of 2.7 μJ compared to lower energy group of 1.8 μJ . (Petroll, et al., 2006) Since in our study similar energy levels of 1.5 μJ and 3.5 μJ were used, it is not likely that the stromal damage induced by femtosecond laser photodisruption results in development of interface haze in the rabbit. While some haze was detected in our study, there was no significant difference between the two energies used in this study. Haze that was detected was not associated with activation of keratocytes and was more likely due to disruption of collagen lamellae. These findings, however, are in general disagreement with a recent confocal study in a human patient that reported activation of keratocytes 24 hours and 2 months following Interlase flap creation without flap elevation (Kitzmann, et al., 2007). While it is possible that human keratocytes that underlie Bowman's layer are more sensitive to femtosecond LIOB than rabbit keratocytes, this study did not measure haze and therefore it is not clear if the changes in light scattering that were identified were associated with clinical haze. Furthermore, since LIOB of the stroma results in the formation of an acellular zone, the increased light scattering from keratocytes observed at 24 hours most likely was associated with keratocyte cell death and not keratocyte activation as suggested by the authors. Also noted in the paper, keratocyte activation at 2 months in the patient was limited to increased light scattering from cell nuclei, similar to what was detected in the rabbit and shown to be from isolated cells residing in regions of disrupted collagen and not associated with activated keratocytes.

While the interface thickness or the acellular zone was significantly thicker in the higher energy groups, suggesting greater stromal damage, this damage was not sufficient to lead to keratocyte activation and haze in this study. It should be noted that in our study flaps remained attached in order to limit the stromal damage to femtosecond photodisruption. Elevation of the flap may cause additional damage to the interface, that when compounded with the initial photodisruption results in keratocyte activation. While this is possible, flap elevation also exposes the stromal surface to factors released from the damaged corneal epithelium. Although we did not measure the width of epithelial damage immediately after femtosecond photodisruption, it is likely that the higher energy pulses produce greater epithelial injury as well. This increased epithelial injury may then lead to higher levels of growth factors and cytokines that are released from the epithelium or tears. (Stramer, et al., 2003; Vesaluoma, et al., 1997) Elevation of the flap may then allow for the access of these growth factors to stromal keratocytes leading to cell activation. Without elevation, only those keratocytes immediately adjacent to the damaged corneal epithelium become activated explaining our observations that

haze and keratocyte activation was observed only at the flap margins and only when the epithelium was damaged.

In addition to exposure of the stromal interface to factors from the epithelium and tears, flap elevation also exposes the stromal surface to iatrogenically introduced factors, such as particulate debris from surgical gloves, instruments, and airborne particles. Small, highly reflective particles at the flap interface are a common finding by *in vivo* confocal microscopy, the origin of which remains uncertain. These interface particles may have different origins that were originally thought to be associated with the use of metal blades in the microkeratomes. More recent studies have suggested that these particles are associated with plastic particles, ocular surface debris, implanted necrotic epithelium or other synthetic products. (Ivarsen, et al., 2004; Perez-Gomez, et al., 2003) Histopathologic studies have also implicated cellular reactions leading to formation of vacuoles within keratocytes or histiocytes as well as disruptions in the extracellular matrix. (Dawson, et al., 2005) The recent findings of particles associated with Intralase generated LASIK flaps indicate that these particles are not limited to the use of microkeratomes, and probably have other origins (Hu, et al., 2007; Petroll, et al., 2006; Ramirez, et al., 2007; Sonigo, et al., 2006). In our study, no particles were detected at the interface suggesting that interface deposits are associated with flap elevation and exposure of the stromal interface to damaged epithelial, tear film or surgical airborne debris. Clearly, further study using this model with and without flap elevation may provide further insight into the origins of interface flap debris and the possible relationship to keratocyte activation.

An interesting finding in our study was the detection of small disruptions in the corneal collagen lamellae that were identified using second harmonic imaging. These disruptions appear similar to the “hyper-reflective zone” identified by Sonigo et al using *in vivo* confocal microscopy that contained small equidistant punctate defects within the flap interface outside the region of excimer laser photoablation. (Sonigo, et al., 2006) As suggested by Sonigo et al, these defects may be associated with the cavitation bubbles produced by the femtosecond laser photodisruption and the consequent disorganization of the collagen. While *in vivo* confocal microscopy did not detect these structures beyond 7 days after surgery, small discrete regions within the lamellae that lacked SHG signals were detected 8 week after surgery in our study. The loss of SHG signal may indicate that photodisruption of the collagen distorts the normal fibrillar organization required for SHG signal generation. These may also be voids in the collagen lamellae produced by the cavitation bubbles. In favor of the later possibility is the finding that small, disconnected cells appear to preferentially reside at these sites. Since flap creation and elevation is clinically followed by excimer laser photoablation, these voids would clinically be observed only in the periphery of the flap interface as noted in the *in vivo* confocal studies. (Sonigo, et al., 2006) Whether these disruptions have any longer term clinical implications remain to be identified.

In conclusion, we have evaluated the response of corneal keratocytes to different energy levels of femtosecond laser photodisruption. When corneal flaps are not lifted, corneal haze and fibrosis is limited to the flap margin only if laser cuts extend full thickness through the corneal epithelium. These findings suggest that disruption of the corneal epithelium is a critical prerequisite to the induction of keratocyte activation leading to stromal haze. Such a mechanism suggests that access of epithelial debris or growth factors from damaged epithelium and tears to the interface stroma may be a leading cause for interface stromal haze, and TLS after LASIK.

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Figure 1.

In vivo confocal microscopic images of normal rabbit (epithelium: A, stroma: B, arrows: keratocyte nuclei) and 1 week after Interlase surgery (epithelium: C, interface with bright condensed nuclei (arrows): D, flap edge of low power: E and flap edge of high power showing activated keratocytes (arrows): F).

Figure 2. Cross sectional image reconstruction from 3-dimensional data set (A and C) and XY image of stromal interface (B and D) taken 1 week after Intralase surgery (low power: A and B; high power: C and D). Arrows in A and C indicate flap interface.

Figure 3.

In vivo confocal microscopic images 2 weeks of Intralase flap margin 2 weeks (A and B) and 8 weeks (C and D) after Intralase surgery using high energy with 100% side cuts through the corneal epithelium (A and C) or 50% side cuts that did not damage the corneal epithelium (B and D).

Figure 4.
Changes in epithelial thickness, stromal thickness and flap thickness.

Figure 5.
Changes in interface thickness and interface haze.

Figure 6.

Ex vivo confocal microscopy of stained corneas (green: actin, red: nuclei). A: cross sectional reconstruction from 3-dimensional data set taken from a cornea 8 weeks after Intralase surgery. Arrows indicate the acellular interface containing a few small round nuclei (arrowheads). B: image of interface generated by 1.5 $\mu\text{J}/\text{pulse}$ laser, C: image of interface generated by 3.5 $\mu\text{J}/\text{pulse}$ laser. Note that cells appear to align along rows

Figure 7.

Ex vivo laser confocal microscopy (A, D and G), SHIM (B, E, and H) and colocalization (C, F, and I) of normal rabbit cornea (A–C) and Intralase flap generated with 1.5 μJ (D–F) or 3.5 μJ /pulse (G–I). Laser confocal microscopic images (A, D and G, green: actin, red: nuclei) shows different morphology of keratocytes. SHIM images (B, E and H, cyan: SHG forward signal, magenta: SHG backward signal) shows disruptions in the collagen lamellae. Note that small cells at interface appear to reside within the disruptions of the collagen lamellae.

Table 1

Group	Number	Epithelium		Stroma		Flap		Interface		Haze	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group 1											
Pre-OP	5	44.2	4.2	315.1	25.3						
Week 1	5	47.4	2.0	332.5	17.5	88.9	12.8	28.6	4.5	29	43
Week 2	5	42.9	3.1	322.2	39.0	79.1	6.7	36.8	8.3	13	20
Week 4	5	45.2	4.6	324.5	38.2	83.9	13.4	38.1	2.8	-15	44
Week 6	5	42.7	3.8	334.1	11.1	87.2	8.1	38.4	9.7	9	5
Week 8	5	41.3	1.0	335.6	21.7	75.3	13.9	43.6*	7.0	3	13
Group 2											
Pre-OP	5	42.7	2.7	320.0	25.8	90.8	6.9	40.0	11.2	44.5	18.7
Week 1	5	46.2	2.1	339.4	12.3	85.7	3.8	45.1	7.5	24.8	21.2
Week 2	5	41.5	1.7	335.9	18.5	90.1	6.2	47.6	5.4	37.9	28.9
Week 4	5	42.0	1.3	339.7	12.7	81.6	9.0	39.1	12.5	28.9	33.1
Week 6	5	40.3	4.3	344.6	9.0	76.6	12.5	44.1	5.7	20.8	19.0
Week 8	5	40.8	3.1	346.3	4.2						
Group 3*											
Pre-OP	4	44.1	3.4	305.6	15.6						
Week 1	4	45.7	3.3	314.8	20.1	86.5	6.8	37.7	5.7	14.9	10.4
Week 2	4	43.5	1.1	321.4	23.9	88.0	2.0	44.4	2.6	16.8	11.8
Week 4	4	44.9	2.5	312.1	17.5	88.6	7.4	42.9	5.8	-1.2	6.1
Week 6	4	42.8	1.0	319.4	22.9	90.3	4.0	39.6	3.1	9.0	21.6
Week 8	4	38.4	6.7	331.4	18.0	74.8	8.7	48.1	2.3	11.0	14.0

Bold = Significantly different compared to Group 1 (p<0.05)

* Group 3 = One animal died after anesthesia and was eliminated from the study.