**Morphologic and Histopathologic Changes in the Rabbit Cornea Produced by Femtosecond Laser–Assisted Multilayer Intrastromal Ablation**

Zhen-Yong Zhang,1 Ren-Yuan Chu,1 Xing-Tao Zhou,1 Jin-Hui Dai,1 Xing-Huai Sun,1 Matthew R. Hoffman,2 and Xing-Ru Zhang3

**PURPOSE.** To observe morphologic and histopathologic changes in the midperiphery of the rabbit cornea produced by femtosecond laser–assisted multilayer intrastromal ablation, determine whether this method may be used to correct myopia, and study how the cornea heals when the epithelium is not injured.

**METHODS.** The right eyes of 10 New Zealand White rabbits were used for the experiments. A 60-kHz femtosecond laser delivery system was used, and three lamellar layers of laser pulses were focused starting at a corneal depth of 180 µm and ending at 90 µm from the surface, with each successive layer placed 45 µm anterior to the previous layer. In the interface of the application contact lens cone, a 6-mm diameter aluminum circle was placed at the center to block the laser, and ablation was limited to the midperiphery of the cornea. The laser settings were spot/line separation, 10 µm; diameter, 8.5 mm; energy for ablating the stroma, 1.3 µJ. Topography examination was used to document changes in corneal power. Light microscopy, transmission electron microscopy (TEM), and confocal microscopy in vivo were applied to observe changes in the cornea.

**RESULTS.** There was significant change in mean corneal power between baseline and postoperative month 3 (n = 8; P = 0.0001), with a decrease from 46.82 D to 44.42 D. There was no haze formation or refractive regression throughout the follow-up. There were no corneal structural abnormalities under light microscopy. Activated keratocytes and necrotic debris were visible under confocal microscopy. Fibroblasts were observed, and no myofibroblasts appeared under TEM.

**CONCLUSIONS.** Multilayer intrastromal ablation by the femtosecond laser with intact epithelium in the midperiphery of the corneal stroma can flatten the cornea without causing haze formation or refractive regression. This procedure allows the cornea to heal differently than when traditional corneal refractive surgery is performed and the epithelium is damaged. (Invest Ophthalmol Vis Sci. 2009;50:2147-2153) DOI:10.1167/iovs.08-2400

The major vision-threatening step in laser-assisted keratomileusis (LASIK) is the creation of the corneal flap1-2 for surface laser vision correction, it is the formation of corneal scar as a result of damage to the epithelium.3-5 It would be preferable if one were able to achieve predictable refractive error change without the creation of a corneal flap and possible injury to the epithelium. Such a method could replace currently performed refractive surgical procedures, with a theory-based expectation of good clinical outcomes.

The idea of intrastromal surgery is not new. Sato6 theorized that incising the cornea from the posterior surface to avoid cutting the Bowman layer and the epithelium would achieve corneal flattening. Unfortunately, this procedure led to corneal edema in most patients who had posterior corneal incisions caused by damage to the corneal endothelium. Krawicz7 attempted to change refractive errors by using scissors to remove corneal stroma through a limbal incision. Combining the techniques of lamellar and excimer surgery in 1989, Peyman et al.8 used a laser to remove corneal stroma from a lamellar bed in animals. Both these approaches, as well as others, injured the corneal epithelium. However, with the application of a femtosecond laser, Ratkay-Traub et al.9 introduced an approach they termed intrastromal photorefractive keratectomy by which 7 to 10 lamellar layers were ablated with a truncated cone-shaped pattern for myopia correction without damage to the epithelium and Bowman layer.

The introduction of the femtosecond laser provides a new tool to ablate stromal tissue without severing the Bowman layer or Descemet membrane. The femtosecond laser is a mode-locked, diode-pumped, and neodymium/glass laser that produces near-infrared (1053 nm) pulses and cannot be absorbed by optically clear tissue.10 The laser can, therefore, be focused at any point to produce tissue disruption at a specified and precise level within the corneal stroma. This makes it possible to relax the cornea by ablating it with no injury to epithelium, with a change in corneal power occurring because of the intraocular pressure. We present the morphologic and histopathologic changes to the rabbit cornea after multilayer ablation of the stroma with the femtosecond laser (Intralase FS; Advanced Medical Optics, Irvine, CA) at different depths in the midperiphery of the cornea.

**MATERIALS AND METHODS**

**Animals**

The right eyes of 10 New Zealand White rabbits, each weighing 1.5 to 2.0 kg, were used for the experiments. All animals were healthy and free of clinically observable ocular disease. All animals used in these studies were treated in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.
Clinical Examination Procedures

Before the experiments, the animals were examined with a slit lamp to determine whether they were free of clinically observable ocular diseases. Corneal thickness was found (Pentacam Typ70700; Oculus; Wetzlar, Germany), and corneal power was determined with a corneal topography system (model 995; Carl Zeiss, Inc., Thornwood, NY). Changes in the corneal stroma after laser ablation were documented with a confocal microscope (Confoscan 3; Nidek, Fremont, CA), and gel (Vidisic 0.2%; Carbomer 940; Bausch & Lomb, Feldkirchen, Germany) was used as a coupling agent between the front lens and the surface of the cornea. Automatic and manual modes were used to capture images of interest in the ablated area. Selected images of confocal microscopy at different time points were taken at a corneal depth of approximately 125 to 145 μm from the surface. Each image represented a corneal section measuring approximately 300 × 400 μm (horizontal × vertical), and the average z-depth separation (Δz) between optical centers of adjacent images was 3.2 μm. Follow-up examinations were performed with topical anesthesia 7, 30, and 180 days after surgery.

Femtosecond Laser Procedure

Animals were premedicated intramuscularly with injection of diazepam (1 mg). For general anesthesia, 10% ketamine hydrochloride (35 mg/kg body weight) was injected intramuscularly. For additional local anesthesia, 0.5% dicaine eyedrops was applied to the right eyes. Eyes were fixed with a suction ring (Fig. 1A) connected to the microkeratome system (LSK M2; Moria, Inc, Doylestown, PA) used to produce suction pressure. The cornea was applanated with the disposable applanating contact lens and a 6-mm diameter aluminum circle was placed at the center of the interface of the lens cone to block the laser (Figs. 1A, 1B). This design ensured that only the central corneal stroma in the midperiphery was ablated by the laser. For intrastromal ablation, three lamellar layers of laser pulses were focused starting at a corneal depth of 180 μm and ending at 90 μm from the surface, with each successive layer placed 45 μm anterior to the previous layer. No edge cuts were performed. The laser settings were spot-line separation, 10 μm; diameter, 8.5 mm; and energy for ablating the stroma, 1.3 μJ. A topical antibiotic agent (2.5% gentamicin eyedrops; EENT Hospital, Fudan University, Shanghai) was administered to the eyes three times on the first day after the surgery.

Histologic Methods

After the animals were anesthetized, corneoscleral tissues were dissected and fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for light microscopy and transmission electron microscopy (TEM).11 For light microscopy, the samples were embedded in paraffin. Serial sections 4-μm thick were stained with hematoxylin and eosin. TEM was performed in accordance with the standard method.12,13 Samples were fixed with osmium tetroxide and dehydrated in a graded ethanol series, rinsed with propylene oxide, and sectioned for observation. A compass was used to center the fixed cornea and to orient the treatment area. Histologic outcomes were masked during analysis.

Statistical Analysis

All statistical analyses were performed with a statistics program (Stata 10.0; Stata Corp., College Station, TX). Statistical significance was evaluated using one-way ANOVA followed by independent-samples t-test. P < 0.05 was considered significant.

RESULTS

Clinical Outcomes

Mean central cornea thickness was 296.23 ± 29.28 μm (n = 10), slightly lower than the mean value of 325 to 350 μm obtained when using ultrasound. The difference can likely be attributed to imaging (Pentacam; Oculus) not requiring topical anesthetic, which is known to increase corneal thickness.14 The disparity could also be attributed to the small sample size. The time required for each lamellar layer laser ablation was 9 seconds. Microbubbles accompanied each lamellar layer ablation and appeared within the scope of 6 to 8.5 mm in the midperiphery of the corneal stroma (Fig. 2A). The microbubbles would last for 25 to 30 minutes, after which the cornea became transparent again (Fig. 2B). No bubbles were found in the anterior chamber (Fig. 2C). During the 3-month

FIGURE 1. (A) Suction ring and applanating contact lens with a 6-mm diameter aluminum circle at its center (arrow). (B) Contact lens docking into the suction ring.

FIGURE 2. Images of the rabbit cornea. (A) Immediately after surgery, the bubbles appeared within an area of 6 to 8.5 mm in the midperiphery of the corneal stroma (arrow). (B, C) Thirty minutes after surgery, the cornea became transparent again, and no bubble was found in the anterior chamber.
follow-up, no haze formed, and the cornea was always transparent.

Corneal power was obtained from the topography examinations conducted at 0 week (baseline, before surgery), 1 week, 1 month, and 3 months after surgery. Mean corneal power (MCP; mean value of corneal power shown by topography at two directions) decreased significantly in postoperative week 1 ($P = 0.0137$), month 1 ($P = 0.0002$), and month 3 ($P = 0.0001$) compared with baseline ($n = 8$). MCP was 46.82 D at baseline, 45.64 D at 1 week, 45.43 D at 1 month, and 44.42 D at 3 months after surgery (Fig. 3). MCP on postoperative month 3 also decreased significantly compared with month 1 ($n = 8; P = 0.0083$). MCP showed no significant change between postoperative week 1 and month 1 ($n = 8; P = 0.6221$). Topographic examinations not only provided the value of corneal power, they documented corneal flattening (Fig. 4).

**Confocal Microscopy**

Within the corneal stroma of the normal control eye, only keratocyte nuclei were reflective and well contrasted against a dark background (Fig. 5A); on postoperative day 3, the background of the image of corneal stroma in the ablated area appeared to be hyperreflective. The reflective particles and hyperreflective objects with visible cytoplasmic processes representing the necrotic debris and activated keratocytes, respectively, were visible (Fig. 5B). In postoperative week 1, the background was a little darker than on postoperative day 3, and the activated keratocytes were also visible (Fig. 5C). In postoperative months 1 and 3, the background became dark, and the activated keratocytes could occasionally be observed (Figs. 5D–F). At all indicated time points, no endothelial cell abnormalities appeared.

**Light Microscopy**

The cornea exhibited no obvious changes after surgery at any time point. After surgery, inflammatory cells were not observed. No structural abnormalities were observed in the corneal epithelium, stroma, Descemet membrane, or endothelium. The three to four layers of epithelium were well preserved, and no epithelial hyperplasia was detected (Figs. 6A–C).
Transmission Electron Microscopy

Normal corneal keratocytes presented with oval nuclei, long processes, abundant heterochromatin, and few organelles (Fig. 7A). At 1 week after surgery, some of the keratocytes were larger and had larger nuclei, more organelles and euchromatin, bigger nucleoli, fairly apparent mitochondria, and enlarged rough endoplasmic reticulum (RER; Figs. 7B, 7C). These data suggest that keratocytes were activated and that fibroblasts were generated. At postoperative month 1, many more fibroblasts appeared. Their organelles and Golgi body were more abundant and better developed (Figs. 7D, 7E). At 3 months, fibroblasts tended to decrease in number and were morphologically characterized by a decrease in organelles such as the RER, enlargement of mitochondria, and collapse of mitochon- drial cristae (Fig. 7F). No myofibroblasts with actin microfilament bundles were observed, and the collagen was well organized throughout follow-up.

**DISCUSSION**

To the best of our knowledge, there are no reports on femtosecond laser-assisted multilayer intrastromal ablation in the midperiphery of the cornea and no demonstration of the morphologic and histopathologic characteristics of a cornea with uninjured epithelium.

Many studies have shown that the femtosecond laser, as used for LASIK, significantly reduces the complications that have plagued mechanical microkeratome technology.\(^{15-20}\) Because of its rare incidence of flap-related complications, the femtosecond laser is becoming recognized as a safer method of flap creation.\(^{19}\) Its excellent efficiency and predictability and its reduced incidence of inducing surgical astigmatism and higher-order aberration make it a beneficial tool in refractive surgery.\(^{19}\) However, femtosecond laser-assisted LASIK and other currently performed corneal refractive surgery procedures inevitably injure the corneal epithelium. It is also well accepted that complications and clinical outcomes of the current corneal refractive surgical techniques are primarily subject to flap creation or subsequent corneal wound healing.\(^{22,23}\) The key to reducing the complications and producing better clinical outcomes could be to avoid flap creation, thus keeping the corneal epithelium intact.

The 60-kHz femtosecond laser permits the energy per spot down to the submicrojoule level, thereby reducing the dimensions of the gas bubbles.\(^{24}\) Using the previous version of the femtosecond laser at a repetition of the 3- to 5-kHz rate produced a cavitation bubble with a diameter of 3 to 15 μm and a shock wave with a range of approximately 20 μm.\(^{25}\) Although reports on the 60-kHz femtosecond laser used in this study are not available, this laser permits a spot/line separation as low as 4 × 4 μm and a pulse energy less than 1 μl to create a superior stromal bed.\(^{25}\) As is suggested by previous results,\(^{36}\) corneal tissue is removed because of the effect of the laser plasma, and the most efficient tissue removal can be achieved by placing the approximately spherical microplasms adjacent to each other. Therefore, we can infer that the gas microbubble observed in this study would be less than or equal to approximately 4 μm in diameter. If it were larger, the microbubble would substantially affect the beam path of the next laser pulse. As a result, the 10-μm spot/line separation and 45-μm layer separation settings in our experiment would make the femtosecond laser generate thousands of microcavitations that separate from each other within and among the three different lamellar layers. The shock wave with which the plasma expands is not sufficient to dissect the lamella. Even the corneal tissue removal caused by microbubble generation is negligible because the unconnected microbubbles serve to disrupt the integrity of the midperipheral cornea and relax it. The greatest strength of the cornea lies within the anterior stroma\(^{27}\) and in the periphery, where the lamellae are more tightly packed. Accordingly, ablation of the anterior stromal layer would lead to the midperipheral cornea relaxing to some extent and the cornea flattening under the intraocular pressure. Additionally, because corneal keratocytes are connected by functional gap junctions,\(^{28}\) disruptions of the communication with posterior keratocytes may affect the integrity of the anterior keratocyte layer.\(^{29}\) Further studies are necessary to investigate how the changes in corneal integrity affect corneal biomechanical prop-
properties and whether the biomechanical effects of intrastromal ablation contribute to corneal relaxation.

Femtec (Heidelberg, Germany), a laser company, has introduced an approach termed presbycor by which circumferential side cut-only ablations are made within the human cornea to create central ectasia (Luiz Ruiz, unpublished data, 2007). The reported outcome sounds contradictory to ours; however, the theory behind this approach and the study conducted by Ratkay-Traub et al.9 is based on corneal tissue removal, whereas ours is based on tissue relaxation. When the midperipheral corneal tissue is relaxed by intrastromal ablation, the cornea flattens under intraocular pressure.

Gas bubbles occasionally appear in the anterior chamber during corneal flap creation with a femtosecond laser, and it is believed that pressure from the suction device and docking system forces bubbles under the flap to subsequently escape through the peripheral corneal stroma and trabecular meshwork into the anterior chamber.30 Less energy, which is the advantage of the 60-kHz system, is needed to cause the photodisruption, leading to an expected absence of anterior chamber gas bubbles. It was also noted in our experiment that the cornea was highly transparent again approximately 30 minutes after surgery. Although there is no uniform agreement regarding how the bubbles were absorbed, it is speculated that the cavitation bubbles, consisting of water and carbon dioxide, are ultimately absorbed through the corneal endothelium23 or, alternatively, by means of the trabecular meshwork in the peripheral cornea.

Netto et al.31 demonstrated that without side cut or injury to the epithelium, intrastromal ablation with the 30-kHz femtosecond laser produced necrosis identified by typical randomly disrupted cellular morphology without the characteristics of apoptosis. In their experiment, the necrotic debris that was presumably a direct energy-related effect of the laser was observed at 24 hours after ablation with a higher than normal energy of 2.7 μJ. This was confirmed by our study in which the intrastromal ablation produced necrotic debris identified morphologically by in vivo confocal microscopy on postoperative day 3. Although necrotic debris is a far greater stimulus to inflammatory cell infiltration than apoptotic bodies and other remnants of apoptotic cells, Netto et al.31 did not report inflammatory cell infiltration in the cornea with the epithelium intact. This correlates well with our observations on light microscopy throughout the follow-up. Netto et al.31 also observed that monocytes would infiltrate the cornea when the epithelium was damaged, regardless of whether a 15-kHz, 30-kHz, or 60-kHz femtosecond laser (Intralase; Advanced Medical Optics, Santa Ana, CA) was applied to create the corneal flap. These findings support the finding that corneal repair reaction to intrastromal ablation with epithelium intact, as in our approach, is different from currently performed refractive procedures with injured epithelium. According to light microscopy and confocal microscopy, the endothelium appeared to be normal at all examination times. TEM revealed that the collagen fibrils were still well organized after femtosecond laser ablation. Overall, ablation had little visible effect on the collagen fibrils, indicating that the femtosecond laser offers safe ablation.

On confocal microscopy, the keratocytes of the ablated area appeared as hyperreflective objects with visible cytoplasmic
processes; these are the same characteristics that have been ascribed to activated kerocytes.28,32 On TEM, myofibroblasts with actin microfilament bundles were not observed, presumably because of the absence of some of the cytokines necessary to activate the quiescent keratocyte and to differentiate fibroblasts into myofibroblasts. This assumption is supported by the evidence that the persistence of myofibroblasts over time requires cytokine input from the epithelium and disappearance of the transient α-smooth muscle actin (α-SMA)-positive cells found in the periphery, near the flap-stroma interface, in corneas treated with LASIK.33 Although it is difficult to distinguish these cells from myofibroblasts without immunocytochemical detection of α-SMA when TEM does not reveal that the myofibroblasts have elevated amounts of RER, the good correlation between the present histopathologic findings and the clinically observed absence of haze formation throughout the follow-up suggests that the cornea heals differently when it is ablated in the stroma without injury to the epithelium. When the corneal stroma is ablated with the epithelium intact, quiescent keratocytes are activated and proliferate, whereas the transformation of fibroblasts to myofibroblasts is inhibited by the absence of necessary cytokines.

Refractive regression, which limits the predictability of all currently performed corneal refractive surgical procedures, is attributable to epithelial hyperplasia and stromal remodeling.34,35 Our study demonstrated that there was no significant difference between postoperative week 1 and month 1 in the MCP, suggesting that no regression occurs. Although we did not perform ultrasound biomicroscopy or optical coherence tomography, which has the capacity to measure the thickness of each layer within the cornea, to document the thickness of the epithelium, light microscopy examinations showed no epithelial hyperplasia. In addition, if epithelial hyperplasia occurred despite the absence of some cytokines modulating the epithelial-stromal wound repair interaction, its thickening in the proximity of the midperipheral cornea would cause it to flatten. Although there was a significant decrease in MCP between month 1 and month 3, we did not attribute this to a thickening of the cornea because of epithelial hyperplasia but rather to the flattening of the corneal curvature as a rabbit grows.36 After LASIK, the hyperplasia may resolve over a period of months to years.37 Because the duration of follow-up observation for this study was 3 months, further investigation is warranted to rule out late-onset regression.

Multilayer intrastromal ablation using the femtosecond laser with intact epithelium in the midperipheral corneal stroma can flatten the cornea without causing haze formation or refractive regression. In addition, the cornea heals differently when the epithelium is not injured. Further studies are warranted to verify the feasibility, safety, and reproducibility of the results in primate animals or humans. Refining the femtosecond laser pulses at 211 nm and 263 nm. Lasers Surg Med. 2003;25:119–126.

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