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Wound healing in rabbit corneas after flapless refractive lenticule extraction with a 345 nm ultraviolet femtosecond laser

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Purpose: To characterize corneal wound healing in a rabbit model after flapless refractive lenticule extraction with a 345 nm ultraviolet femtosecond laser.

Setting: Departments of Ophthalmology and Anatomy II, University of Erlangen-Nürnberg and Wavelight GmbH, Erlangen, Germany.

Design: Experimental study.

Methods: Flapless refractive lenticule extraction was performed in 1 eye each of 20 New Zealand white rabbits (~5.0 diopters). Groups of 4 animals were euthanized after 48 hours, 1 week, 2 weeks, 4 weeks, and 3 months, respectively. Corneal samples were prepared for histology and fluorescence microscopy. To assess corneal cell death, proliferation, and myofibroblastic transdifferentiation, terminal uridine deoxynucleotidyl nick end-labeling (TUNEL) assay as well as immunostaining for Ki67 and α-smooth muscle actin (αSMA) were performed on sagittal cryosections.

Results: Histology revealed a zone of keratocyte depletion with a thickness of approximately 50 μm around the extraction site. At 48 hours, pronounced TUNEL staining of keratocytes was detected at the interface (159.9 cells/mm ± 18.4 [SD]), which steadily decreased to 74.9 ± 19.8 cells/mm at 1 week and 5.7 ± 4.8 cells/mm at 2 weeks. Ki67 staining of keratocytes was evident at 48 hours (10.0 ± 3.8 cells/mm), which then decreased at 1 week (5.2 ± 1.7 cells/mm) and 2 weeks (0.4 ± 0.5 cells/mm). From 4 weeks onward, no TUNEL or Ki67 staining was detected. The corneal stroma was αSMA-negative at all timepoints.

Conclusion: Application of the 345 nm laser showed no signs of problematic repair processes in the cornea, which supports the initiation of the clinical phase.

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In recent years, small-incision lenticule extraction (SMILE, Carl Zeiss Meditec AG) has been established as a flapless alternative to laser in situ keratomileusis (LASIK) and other flap-reliant refractive surgery procedures.1–3 In the small-incision lenticule extraction procedure, a femtosecond laser is used to create a refractive lenticule in the depth of the corneal stroma without creating a flap or otherwise damaging the corneal epithelium. To achieve a refractive change, the lenticule is extracted via 1 or 2 small peripheral incisions of only a few millimeters in length. With this method, the epithelial wound remains markedly smaller than with LASIK or photorefractive keratectomy (PRK). The development of LASIK in the 1990s had already represented a step forward in terms of patient comfort because it created a smaller epithelial wound than PRK. However, it also introduced new sources of complications that are directly or indirectly related to flap creation.4–10 Refractive surgery procedures involving flap formation (eg, LASIK) have been discussed to destabilize the corneal stroma to a higher degree than flapless interventions such as PRK or small-incision lenticule extraction because the stromal bed is markedly reduced in thickness because of the biomechanically inactive flap.11–13 Some studies claim this represents a risk...
factor for iatrogenic ectasia, which can be considered an indirectly flap-related complication according to this theory. The small-incision lenticule extraction procedure, on the other hand, renders the corneal portions anterior to the refractive lenticule (cap) largely intact, with the exception of 1 or 2 extraction canals. Therefore, the corneal cap might still contribute to the biomechanical strength of the cornea after surgery, although it has not yet been clarified whether this is of actual clinical relevance. Another advantage of small-incision lenticule extraction is the much smaller epithelial wound when compared with PRK or LASIK. Moreover, the postoperative incidence and severity of dry-eye syndrome are significantly lower after small-incision lenticule extraction than after LASIK. Taken together, this combines markedly increased postoperative patient comfort with decreased corneal destabilization in the small-incision lenticule extraction procedure. To our knowledge, at present, small-incision lenticule extraction is achieved only with the Visumax infrared (IR) femtosecond laser system (Carl Zeiss Meditec AG). In 2 previous studies, we described a new 345 nm ultraviolet (UV) femtosecond laser capable of facilitating flapless lenticule extractions similar to the small-incision lenticule extraction procedure. The shorter wavelength of UV light allows a UV femtosecond laser to create a more accurate laser focus when compared with IR laser systems. This corresponds with less pulse energy necessary to cut corneal tissue and should therefore increase the maximum level of cutting precision and safety achievable with a UV platform.

In the present study, the 345 nm laser system was applied for the flapless extraction of intrastromal refractive lenticules from rabbit eyes in vivo to obtain preclinical wound-healing data. Corneal cell death, keratocyte proliferation, and myofibroblastic transdifferentiation were evaluated over a follow-up of 3 months to assess the laser’s readiness for the clinical phase.

**MATERIALS AND METHODS**

**Study Design and Laser Parameters**

Twenty female New Zealand white rabbits weighing between 2.5 kg and 3.5 kg were subjected to flapless refractive lenticule extractions in 1 eye, whereas the contralateral eyes served as untreated controls. Before surgery, the animals were kept in colonies comprising a maximum of 5 animals. After surgery, the animals were kept in individual boxes until they were killed humanely. For laser treatment and lenticule extraction, the animals were anesthetized with 50 mg/kg ketamine (Ketavet) and 10.5 mg/kg xylazine (Rompun 2.0%). For additional anesthesia of the cornea, oxypurine hydrochloride eyedrops (Conjuncaen EDO) were given. Animals were killed humanely by intravenous injection of 300 mg pentobarbital (Narcoren).

Within 4 hours of enucleation, the corneas were dissected and processed subsequently for light and fluorescence microscopy. Corneal cell death, proliferation, and myofibroblastic transdifferentiation were determined using a terminal uridine dioxynucleotidyl nick end-labeling (TUNEL) assay and immunostaining for Ki67 (proliferation) and α-smooth muscle actin (SMA) (transdifferentiation). Furthermore, standard histology was used to morphologically characterize the corneas after lenticule extraction.

**Experimental Animals**

Twenty New Zealand white rabbits were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept in the professional care of the university’s experimental animal facility for basic and preclinical research (Franz-Penzoldt-Center, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany). The animals were housed in rooms with controlled temperature and 12-hour light-dark cycles. Food and water were always provided ad libitum. For laser treatment and lenticule extraction, the animals were anesthetized with 30 mg/kg ketamine (Ketavet) and 10.5 mg/kg xylazine (Rompun 2.0%). For additional anesthesia of the cornea, oxypurine hydrochloride eyedrops (Conjuncaen EDO) were given. Animals were killed humanely by intravenous injection of 300 mg pentobarbital (Narcoren).

**Light and Fluorescence Microscopy**

After lenticule extraction and a postoperative wound-healing period, the corneas were dissected and bisected across the interface. One half was fixed at room temperature for 24 hours with a solution containing glutaraldehyde 2.5% in Sörensen’s phosphate buffer (0.1 M monopotassium phosphate, 0.1 M disodium phosphate × 2 H2O) and embedded in epoxy resin (Epon) as described previously. Subsequently, 1.0 μm sagittal semi-thin sections were cut and stained with toluidine blue for histological analysis. The other half was embedded in an optimum cutting temperature compound (Tissueux, Sakura Finetek Europe B.V.) and cryosectioned sagittally (5.0 μm) for TUNEL assay and immunofluorescence. Two samples containing an extraction canal were also dissected from every treated cornea. Again, 1 sample was embedded in epoxy resin and the other in an optimum cutting temperature compound for the same analyses as described for the specimens containing the interface. After the samples were cryosectioned, TUNEL assays were performed according to the manufacturer’s recommendations (In Situ Cell Death Detection Kit, Roche Diagnostics).

For immunofluorescence, the samples were fixed in cold acetone for 10 minutes and incubated in a solution containing 10% normal goat serum (Sigma-Aldrich Chemie GmbH), 1.0% bovine serum albumin (Sigma-Aldrich Chemie GmbH), and 0.5% Triton-X-100 (Merck KGaA) in phosphate-buffered saline (PBS) (0.01 M, pH 7.4) for 1 hour at room temperature to block unspecific binding of the antibodies. Then, the samples were incubated overnight at 4°C with the primary antibody diluted with a solution of 3% normal goat serum (Sigma-Aldrich Chemie GmbH), 1.0% bovine serum albumin (Sigma-Aldrich Chemie GmbH), and 0.5% Triton-X-100 (Merck KGaA) in PBS (0.01 M, pH 7.4). A mouse anti-human Ki67 (Dako Deutschland GmbH, cat. no. M7240029) and a mouse anti-human α-SMA (cat. no. 36.000, Novocastra Laboratories Ltd.) were used as primary antibodies at dilutions of 1:100 and 1:50, respectively. After 3 rinses in PBS, the samples were incubated for...
1 hour at room temperature with the secondary antibody (goat anti-mouse immunoglobulin G Alexa 555, cat. no. A21422, Invitrogen Co.) diluted 1:500 with a solution of 3.0% normal goat serum (Sigma-Aldrich Chemie GmbH), 1.0% bovine serum albumin (Sigma-Aldrich Chemie GmbH), and 0.5% Triton-X-100 (Merck kGaA) in PBS (0.01 M, pH 7.4). After 3 further rinses with PBS, nuclei were stained using 4,6-diamidino-2-phenylindole (Sigma-Aldrich Chemie GmbH), coverslipped with

Figure 1. Histology of rabbit corneas after UV femtosecond laser flapless lenticule extraction (scale bar = 200 μm). Left column: Central region of the extraction site at different postoperative timepoints. At 48 hours (A) a keratocyte-free zone with a thickness of approximately 50 μm is discernible around the cutting line (asterisk). Partial and gradual repopulation of the depleted stroma (C, E, G, I) without having reached the original keratocyte density at 3 months (I). Right column: Epithelial incision at different postoperative timepoints. At 48 hours, the epithelial wound was already closed by an epithelial cell callus (B) which decreased in size over time (D, F, H, J). Semi-thin sections stained with toluidine blue.
fluorescent mounting medium (Dako Deutschland GmbH), and viewed with a fluorescence microscope (BX-51TF; Olympus Corp.).

RESULTS
Standard Histology
Two days after lenticule extraction, a zone of keratocyte depletion was detected at the extraction site (Figure 1, A). The keratocyte-free region had a sagittal extension of approximately 50 μm. Over time, this region was more and more repopulated by keratocytes, which was already evident 2 weeks after lenticule extraction (Figure 1, C). The repopulation process had further intensified at 4 weeks and 3 months (Figure 1, G and I). However, even after 3 months, the depletion zone had not yet reached a normal keratocyte count (Figure 1, I). The epithelial and endothelial cells appeared unharmed. At the sites of epithelial incision, a callus of epithelial cells formed and then steadily decreased in size over time (Figure 1, right column). After 3 months, no callus was detected. Instead, the epithelium appeared slightly thickened at the incision site in 1 of 4 specimens (Figure 1, J).

Keratocyte Death
At 48 hours, TUNEL-assay analysis showed pronounced staining of keratocytes near the extraction site around the interface (Figure 2, A). Morphometric analysis revealed a cell death count of 159.9 cells/mm ± 18.4 (SD), which steadily decreased to 74.9 ± 19.8 cells/mm at 1 week and 5.7 ± 4.8 cells/mm at 2 weeks (Figure 2, A to C). At 4 weeks and 3 months, no TUNEL-positive keratocytes were detected (Figure 2, D). The corneal endothelium was TUNEL-negative at all timepoints. All differences between groups in TUNEL-positive cell counts were statistically significant (P < .05, 2-tailed Student t test).

Keratocyte Proliferation
Marked Ki67 staining of keratocytes was evident at 48 hours (10.0 ± 3.8 cells/mm) at the border of the keratocyte depletion zone (Figure 3, A). The Ki67-positive cell count had decreased conspicuously after 1 week (5.2 ± 1.7 cells/mm; P > .05, 2-tailed Student t test) and 2 weeks (0.4 ± 0.5 cells/mm, P < .05, 2-tailed Student t test) (Figure 3, B and C). After 4 weeks and 3 months, no Ki67-positive keratocytes were found (Figure 3, D). The corneal endothelium was Ki67-negative at all timepoints.

Keratocyte Transdifferentiation
The central corneal stroma was free of zSMA-positive cells at all timepoints (Figure 4, A to E). The pronounced staining of numerous zSMA-positive keratocytes found after 48 hours was confined to the close proximity of the epithelial callus (Figure 5, A). At 1 week and 2 weeks, the staining around the callus had markedly decreased, with only some isolated zSMA-positive keratocytes still being observed (Figure 5, B and C, respectively). From 4 weeks onward, no zSMA-positive keratocytes were detected (Figure 5, D).

DISCUSSION
The long-term clinical success of refractive surgery procedures hinges on the subsequent wound-healing response in the cornea. It is of critical importance that the stromal tissue repair processes remain largely regenerative in nature, without disturbing corneal transparency and geometry.

If, however, a fibrotic repair process is induced, corneal haze and scar contractions might develop and impair the visual outcome. Fibrotic wound healing is associated with proliferating keratocytes (Ki67-positive) that are thought to partially represent activated cells further transdifferentiating into stromal myofibroblasts (zSMA-positive). Because stromal myofibroblasts are responsible for the deposition of scar tissue, their presence is a pivotal indicator for the development of corneal fibrosis. In this respect, it is a promising sign for the new 345 nm femtosecond laser that no zSMA-positive cells were found in rabbit corneas after lenticule extraction within the first 3 months of wound healing, except for some
isolated cells around the epithelial incision. The local presence of myofibroblasts in proximity of the epithelial wound is not surprising because epithelium-derived cytokines such as transforming growth factor-β2 were shown to elicit keratocyte transdifferentiation and stromal fibrosis in areas where the basal membrane was damaged and had lost its barrier function.28,29 Because of the peripheral location of the spatially very confined αSMA-positive cells around the small incisions, a hypothetical mild and temporary corneal haze in these regions would not be likely to cause any detectable visual impairment. This is underscored by the fact that even with PRK and LASIK, in which the epithelial damage is much more pronounced, corneal haze and other fibrotic complications normally pose no serious threat to the long-term visual outcome.8,10 Therefore, the few

Figure 3. Ki67 immunofluorescence on sagittal cryosections at different timepoints after flapless refractive lenticule extraction (−5.0 D) from rabbit corneas (scale bar = 200 μm). A: At 48 hours. B: At 1 week. C: At 2 weeks. D: At 4 weeks. Pronounced staining of Ki67-positive keratocytes around the area of keratocyte depletion (asterisk) at 48 hours (A). Steady decrease in proliferating keratocytes over time. No Ki67-positive keratocytes discernible after 4 weeks (D). Basal cells within the corneal epithelium served as internal positive control and were Ki67-positive in all corneas (blue = 4′,6-diamidino-2-phenylindole; red = Ki67).

Figure 4. The αSMA immunofluorescence on sagittal cryosections of rabbit corneas at the center of the lenticule extraction site. A: At 48 hours. B: At 1 week. C: At 2 weeks. D: At 4 weeks. E: At 3 months. No αSMA-positive keratocytes discernible near the lenticular bed at all timepoints (scale bar = 200 μm). Asterisks mark the keratocyte-free area. F: Rabbit ciliary muscle (CM) and iris muscle (IM) serving as positive controls (‘ = ciliary process; blue = 4′,6-diamidino-2-phenylindole; red = α-smooth muscle actin).
αSMA-positive cells detected around the small incisions within the first 2 weeks after surgery should be of no pathologic relevance.

The absence of αSMA-positive cells along the lenticule beds and caps suggests that the Ki67-positive keratocytes detected at 48 hours and 1 week postoperatively do not represent cells activated in the context of stromal fibrosis. They might simply constitute cells that were induced to proliferate by the laser or by the manual extraction of the lenticules. That almost no Ki67-positive keratocytes were found 2 weeks after surgery (and none at all from 4 weeks onward) in this study corresponds with previous findings after PRK and LASIK in rabbit eyes, and seems to reflect a normal regenerative wound-healing pattern. Among other aspects, Dong et al. examined the development of Ki67-positive keratocyte counts after small-incision lenticule extraction and LASIK (also in rabbit eyes). After 1 week, they found only one half as many Ki67-positive cells in the corneal stroma than those found 3 days postoperatively. This is almost exactly what we found after UV femtosecond laser flapless refractive lenticule extraction, that is, our Ki67-positive cell count at 1 week after surgery was also halved in comparison to 2 days postoperatively. Furthermore, Dong et al. found almost no proliferating keratocytes after 1 month. Again, this corresponds with our data because we did not detect Ki67-positive keratocytes from 4 weeks postoperatively and onward. Therefore, regarding keratocyte proliferation, there was a marked conformity of our data with that derived from wound-healing experiments after the standard small-incision lenticule extraction procedure.

In a previous study, we found that the keratocyte death rate after formation of a cutting plane was significantly higher with the 345 nm laser than with a common IR femtosecond laser (Wavelight FS 200, Wavelight GmbH). However, keratocyte death is a common and harmless feature of femtosecond laser refractive surgery if it remains confined to the area around the interface and if the death rate decreases continuously after surgery. Because this was the case in our previous study as well as after flapless lenticule extraction in the present study, the postoperative TUNEL-positive cell counts gave us no reason for concern. The number of TUNEL-positive keratocytes after UV laser flapless refractive lenticule extraction declined dramatically within the first 2 weeks after surgery. No TUNEL-positive cells were found after 4 weeks and 3 months. This is in line with the findings by Mohan et al., who also found an absence of TUNEL-positive stromal cells in rabbit corneas after PRK and LASIK from 4 weeks postoperatively and onward. Our TUNEL assay-derived data also corresponded with our histological findings. In this study, an approximately 50 μm thick zone devoid of keratocytes was shown around the cutting line 48 hours postoperatively. Over time, this area had been partially repopulated by keratocytes, although the stromal cell count had not returned to normal 3 months after lenticule extraction. This, however, was not interpreted as an alarming sign because Erie et al. have shown that the stromal cell count remains significantly decreased, even years after successful PRK and LASIK, without having a detectable adverse effect on the visual outcome.

Loss of endothelial cells, on the other hand, must always be considered a serious side effect. Although the corneal
endothelium plays a functional key role for corneal transparency, it has an extremely low capacity to regenerate in the human system.\textsuperscript{35,36} In a previous study,\textsuperscript{23} the 345 nm femtosecond laser was already shown to pose no threat to endothelial cells in enucleated porcine eyes, even if the stromal cut was placed as near as 50 \textmu m from the endothelium. However, to our knowledge, the present study is the first to provide long-term data after flapless refractive lenticule extraction from an in vivo rabbit model. Judging from the inconspicuous histology and from the absence of TUNEL-positive endothelial cells at all timepoints evaluated, it seems appropriate to conclude that the new 345 nm femtosecond laser poses no apparent danger for the corneal endothelium.

The present study was limited by several aspects. First, the refractive outcome of the flapless lenticule extraction procedures performed was not measured and no wavefront was determined in the rabbits used. Because the primary focus of this study was on the corneal wound-healing processes around the cutting planes, measurements of refractive outcomes and wavefronts were not considered to be of pivotal importance for the key variables analyzed (ie, corneal cell death, keratocyte proliferation, and myofioblastic transdifferentiation). However, future preclinical and clinical studies focusing on the precision and accuracy of UV femtosecond laser lenticule extraction should provide precise refractive outcome data as well as preoperative and postoperative wavefront measurements. The extent of corneal nerve loss from the surgical procedure and during the postoperative recovery was also not evaluated in the present study. Surgery-induced damage to the corneal nerve plexus is thought to be a major cause of post-LASIK and post-small-incision lenticule extraction dry eye.\textsuperscript{19–22} It has been reported that the postoperative incidence and severity of dry-eye syndrome as well as the degree of corneal nerve loss are markedly lower after small-incision lenticule extraction than after LASIK.\textsuperscript{19–22} However, at present, there are no postoperative data available on UV femtosecond laser flapless lenticule extraction. Therefore, future studies to determine the extent of corneal nerve loss after UV laser flapless refractive lenticule extraction as well as its recovery and correlation with dry-eye syndrome are necessary.

Another limitation of the present study is that it was designed as a histological study that also included TUNEL assays and immunostaining for Ki67 and zSMA. This design was considered sufficient to provide an initial understanding of the nature of the postoperative wound-healing processes after UV laser flapless lenticule extraction. However, transmission electron microscopy (TEM) might provide a greater understanding of the processes. Therefore, future preclinical wound-healing studies after UV laser lenticule extraction with the 345 nm femtosecond laser would benefit from TEM as an additional method. The study design was further limited because only 1 level of correction (−5.0 D) was administered. The question of whether the postoperative wound-healing processes after a very small (eg, −1.5 D) or a very large correction (eg, −10.0 D) differ from this intermediate level is yet unanswered. Hence, the pursuit of this question might also be of interest for future studies. For further comparative reasons, it would have been useful to include a Visumax control group to directly compare the obtained UV laser-related data with the wound-healing process after standard small-incision lenticule extraction. Because this was not possible at the time the present study was performed, a thorough comparison of our results with the currently existing literature on post-small-incision lenticule extraction, post-LASIK, and post-PRK wound healing was supposed to fill this gap. However, an exact repetition of the described experiments using the Visumax laser would help to more accurately interpret the wound-healing data obtained in this study.

In conclusion, viewed in line with previous studies of the new 345 nm femtosecond laser,\textsuperscript{23–25} this study represents a further step forward on the way toward the first clinical trials. This is emphasized by the pronounced accordance of our wound-healing data after UV laser flapless lenticule extraction with previous findings after PRK, LASIK, and standard small-incision lenticule extraction.\textsuperscript{29–31} Because no corneal and extracorneal ocular hazards have become evident in the preclinical testing phase thus far, we are confident that the UV femtosecond laser is headed in the correct direction toward the clinical testing phase.

**WHAT WAS KNOWN**

- To date, small-incision lenticule extraction is performed with only 1 IR femtosecond laser system.

**WHAT THIS PAPER ADDS**

- An alternative 345 nm UV femtosecond laser prototype was successfully used to perform a flapless lenticule extraction procedure similar to small-incision lenticule extraction in rabbit eyes.
- Postoperative wound healing with the 345 nm UV femtosecond laser prototype seemed comparable to wound healing that occurred after PRK, LASIK, or femtosecond laser small-incision lenticule extraction and showed no signs of a fibrotic repair response throughout a 3-month follow-up.

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