

Morphological and Immunohistochemical Changes After Corneal Cross-Linking

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Purpose: To present light and electron microscopic as well as immunohistochemical findings after corneal cross-linking (CXL).

Methods: Six keratoconus corneas after CXL, 12 keratoconus corneas without CXL, and 7 normal corneas were examined by light microscopy, indirect immunohistochemistry using antibodies against proapoptotic BAX, antiapoptotic survivin, and BCL-2, and anti-smooth muscle actin and, in part, by transmission electron microscopy. Direct immunofluorescence with 4'6-diamidino-2-phenylindole was performed to analyze keratocytes/area in the anterior, middle, posterior, peripheral, and central corneal stroma.

Results: The period between CXL and keratoplasty ranged from 5 to 30 months. All keratoconus corneas showed the typical histological changes. Increased proapoptotic BAX expression and/or antiapoptotic survivin expression were noticed in keratocytes and endothelium in 2 keratoconus specimens after CXL. Smooth muscle actin was only observed in subepithelial scar tissue of 2 keratoconus corneas without CXL. Keratoconus corneas after CXL revealed a significant reduction in keratocyte counts in the entire cornea ($P = 0.003$) compared with keratoconus corneas without CXL and normal corneas. This difference was because of a loss of keratocytes in the anterior ($P = 0.014$) and middle ($P = 0.024$) corneal stroma. Keratocytes in CXL corneas were reduced in the center ($P = 0.028$) and the periphery ($P = 0.047$).

Conclusions: CXL in human keratoconus can cause considerable morphologic corneal changes up to 30 months postoperatively. Especially noteworthy is a long-lasting, maybe permanent, keratocyte loss in the anterior and middle corneal stroma involving the central and peripheral cornea. As long-term corneal damage after CXL is of genuine concern, particular care should be taken to perform this procedure only in accordance with investigational protocols.

Key Words: keratoconus, corneal cross-linking, keratocytes

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Keratoconus is a noninflammatory ectasia of the cornea with documented changes in corneal epithelial basement membrane structure^{1–3}; in stromal collagen composition, distribution, and organization leading to lamellar and/or fibrillar slippage^{2,4–7}; in extracellular matrix components³; and in keratocyte morphology and cell–matrix interactions.^{8–10}

In addition, collagen loss, proteoglycan changes, and the upregulation of degradative enzymes have been shown in keratoconus and support the occurrence of additional biochemical changes.^{11–14} Histopathologic studies of keratoconus corneas reported fragmentation of the epithelial basement membrane, superficial linear ruptures in Bowman layer filled with fibrotic connective tissue, and folds in Descemet membrane.¹⁵

Therapeutic options for patients with keratoconus include rigid gas permeable contact lenses, intracorneal ring segments, and topography-guided photorefractive keratectomy.^{16,17} In approximately 20% of cases, keratoconus progresses to the extent that corneal transplantation is unavoidable.¹⁸

In 2003, corneal cross-linking (CXL) using the photosensitizer riboflavin and UVA light to increase the biomechanical stability of the cornea and the resistance to collagenases was shown to halt progression of keratoconus.¹⁹ In CXL-treated eyes, maximal keratometry readings and refractive errors remained stable or decreased, whereas visual acuity improved. Corneal transparency and endothelial cell density remained unchanged by CXL.¹⁹ These findings in keratoconus were confirmed by others,^{20–22} and the indication for CXL was extended to corneal melting,^{23,24} keratitis refractive to therapy,²⁵ and laser in situ keratomileusis–induced keratectasia.^{26,27} Extrapolating from rabbit experiments, a minimal corneal thickness of 400 μm was postulated also in human CXL by Wollensak et al^{28,29} to prevent endothelial damage.

In vivo confocal microscopic examination after CXL showed a loss of keratocytes in the anterior and intermediate stroma, associated with stromal edema immediately after treatment. Gradual keratocyte repopulation of the corneal stroma, starting between the second and third postoperative month, becoming complete after 6 months, and continuing thereafter was reported by Mazotta et al.³⁰ Between the third and the sixth months after CXL, increased density with hyper-reflectivity of the extracellular matrix combined with activated keratocyte nuclei and elongated cell processes was seen. In late scans 2 years after the procedure, “bridge”- and “needle”-shaped hyperreflective bands compatible with newly

replaced collagen and different lamellar interconnections were documented.^{31,32} This is in accordance with the histological findings in rabbit corneas after CXL, where repopulating keratocytes were observed to repair the cytotoxic effects in 6-weeks time.³³ Recently, histology of one normal human cornea 24 hours after CXL was reported. It confirmed the complete loss of keratocytes down to a depth of 250 to 280 μm with normal keratocyte densities beneath in the immediate postoperative course.³⁴ We report for the first time histological, immunohistological, and electron microscopic findings in 6 corneas treated with CXL 5 to 30 months before keratoplasty and compare these findings with normal corneas and keratoconic corneas without CXL treatment.

PATIENTS AND METHODS

All 6 CXL procedures were performed elsewhere, and preoperative data were provided retrospectively by the corresponding surgeons. Clinical patients' characteristics are given in Table 1. CXL was performed using a standard protocol with 0.1% riboflavin application every 2 minutes for 60 minutes and concomitant UVA irradiation (370 nm; 3 mW/cm²) for the last 30 minutes in all patients. In 2 patients, hyposmolar riboflavin was added to increase corneal thickness intraoperatively to a minimum of 400 μm . Additional procedures were performed in 2 patients. One patient underwent photorefractive keratectomy 21 months after CXL, and a second CXL procedure was performed 4 months later (patient 4). In a second patient, corneal ring implantation was performed 12 months after CXL (patient 6). However, a small corneal perforation intraoperatively necessitated the immediate explantation of the ring segment. Corneal transplantation was required in all 6 patients because of further decrease in visual acuity associated with increased astigmatism and/or worsened corneal scarring 5 to 30 months after CXL. For comparison, 12 keratoconic corneas without CXL were obtained after keratoplasty for progressive disease. In addition, 7 normal corneas were taken from eyes enucleated for intraocular melanoma.

Specimens were fixed in 4% formaldehyde in 0.075 M phosphate buffer for 24 hours, dehydrated in increasing concentrations of ethanol (70%–99%), and infiltrated with paraffin (Merck, Darmstadt, Germany) at 60°C. Sections with a thickness of 3 μm were cut and floated on deionized water

at 45°C and mounted as single sections on Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany). Slides were subsequently dried at 60°C for 1 hour and stained with hematoxylin and eosin, periodic-acid Schiff, and Alcian blue.

For immunohistochemistry, formalin-fixed corneal buttons were mounted on Super Frost Plus slides, deparaffinized and steamed in target retrieval solution for 30 minutes. The tissue was immunostained using the alkaline phosphatase–antialkaline phosphatase method (APAAP). Primary antibodies and dilutions used are listed in Table 2. Briefly, the primary antibody was applied for 30 minutes. Thereafter, the tissue was incubated with biotinylated secondary antibodies (Dako ChemMate Detection Kit APAAP, Mouse) and APAAP complex (Dako ChemMate Detection Kit APAAP) as a tertiary reagent for 20 minutes each. The slides were developed for 10 minutes in a solution of chromogen Fast Red substrate (DakoCytomation, Hamburg, Germany). Tris-buffered normal saline (pH 7.6) was used as wash buffer between steps and as negative control. Antibody dilutions were made with ready-made antibody diluent (DakoCytomation) for primary antibodies. All slides were counterstained with hemalaun and mounted in Malinol (Waldeck GmbH & Co Division Chroma, Münster, Germany). Tissue from a conjunctival intraepithelial carcinoma and a basal cell carcinoma was used as a positive control.

For direct immunofluorescence, 3- μm unstained sections were deparaffinized and immunostained with 4'6-diamidino-2-phenylindole (Vector Laboratories Ltd, Peterborough, UK) as a nuclear stain to analyze keratocyte densities. A JenOptic ProGress camera attached to a Leica Fluorescence DM 2500 microscope was used to document sections. Images were processed further with Adobe Photoshop CS5.1 to split corneal stromal images into 9 areas: 2 peripheral corneal sections, 1 central corneal section, anterior corneal stroma, middle corneal stroma, and posterior corneal stroma. Adobe Photoshop CS5.1 software was also used to count keratocytes manually and analyze referring corneal stromal areas to obtain keratocyte counts per area (1 mio pixel).

Transmission Electron Microscopy

Tissue specimens of 3 keratoconus patients after CXL were postfixated in 2% osmium tetroxide, processed through

TABLE 1. Clinical Characteristics of Patients Undergoing CXL

Patient Number	Age (yr)	Sex	Preoperative Corneal Thickness (μm)	Preoperative VA	Hypoosmolar Riboflavin	Additional Procedures	Period CXL \leftrightarrow KP (mo)	Astigmatism Postoperative	Scarring Postoperative
1	59	F	540	1/50	No	No	6	\leftrightarrow	\uparrow
2	20	M	402	20/400	No	No	9	\downarrow	\uparrow
3	15	M	461	20/50	Yes	No	5	\uparrow	\uparrow
4	24	M	n.k.	n.k.	n.k.	PRK, 2CXL	30/23	\uparrow	\uparrow
5	34	M	557	20/100	No	No	6	\uparrow	\leftrightarrow
6	53	M	344	20/40	Yes	Corneal ring implant	26	\leftrightarrow	\uparrow

2CXL, second crosslinking procedure; F, female; KP, keratoplasty; M, male; n.k., not known; PRK, photorefractive keratectomy; VA, visual acuity.

TABLE 2. Characteristics of Antibodies Used

Antibody	Company	Host	Dilution
BAX	DAKO	Polyclonal rabbit	1:50
Survivin	DAKO	Monoclonal mouse	1:10
BCL-2	DAKO	Monoclonal mouse	1:200
Smooth muscle actin	DAKO	Monoclonal mouse	1:100

alcohol and propylene oxide for embedding in Araldite or acetone–Durcupan and sectioned with a Reichert–Jung ultratome. Semithin sections were stained with toluidine blue, whereas ultrathin sections were stained with uranyl acetate and lead citrate and examined under a Zeiss transmission electron microscope. Statistics were performed using χ^2 and Kruskal–Wallis tests, as well as Spearman correlations. The results were considered statistically significant at $P < 0.05$.

RESULTS

Histology

Histological findings in all keratoconus specimens after CXL included thinning of the central corneal epithelium with an intact epithelial basement membrane, breaks in Bowman layer, a thinned (para-) central corneal stroma with reduction of keratocytes, as well as an unaffected Descemet membrane with an intact endothelium (Fig. 1). Collagen structure did not differ between keratoconus with and without CXL by light microscopy. In 1 cornea (patient 4), which had undergone several surgical procedures, the Alcian blue stain revealed deposits of acid mucopolysaccharides in the corneal stroma, in keratocytes, and in endothelial cells.

Immunohistochemistry

BAX, Survivin, BCL-2

Proapoptotic BAX stained positive in the epithelium of 1 normal cornea, and in 8 of 12 keratoconus epithelia without CXL. Corneal epithelium of keratoconus specimens after CXL was negative for BAX. BAX did not stain the keratocytes or the endothelium in any normal cornea or any keratoconus cornea without CXL, but was positive in keratocytes and endothelium in 2 keratoconus corneas after CXL (Fig. 2A). Antiapoptotic survivin positivity was observed in the epithelium of 4 keratoconus corneas without CXL, and in 1 keratoconus cornea with CXL. In addition, survivin expression was present in the endothelium of 1 keratoconus cornea after CXL. In 1 normal cornea, antiapoptotic BCL-2 was present in several keratocytes. All other corneas were negative for this marker.

Smooth Muscle Actin

Smooth muscle actin stained positive only in subepithelial scar tissues of 2 keratoconus corneas without CXL (Fig. 2B). Neither normal corneas nor keratoconus corneas after CXL demonstrated any keratocytes positive for smooth muscle actin keratocytes.

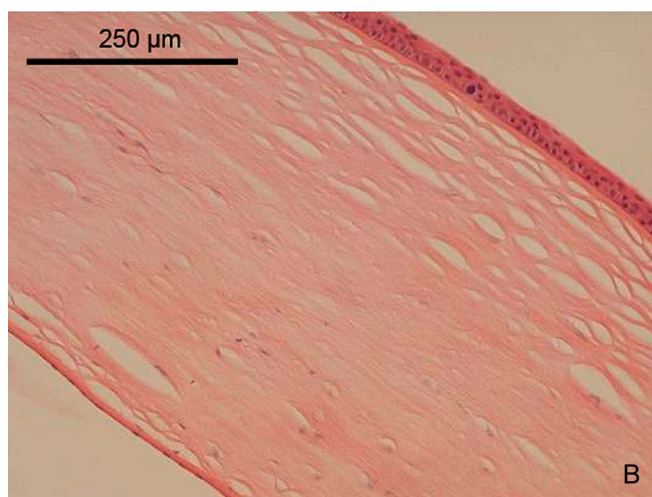
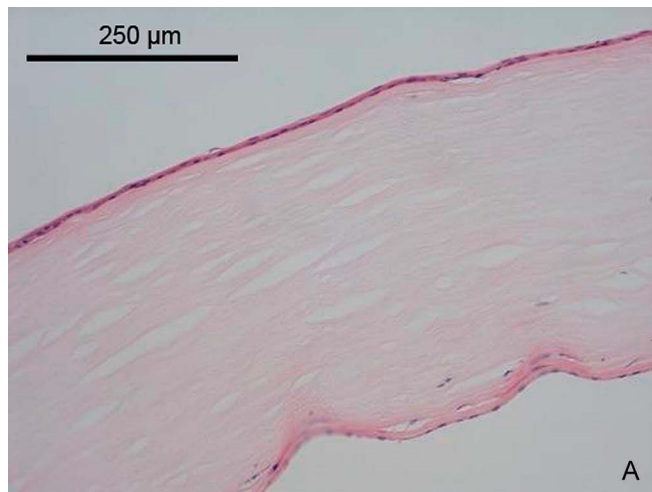


FIGURE 1. A, Keratoconus cornea 6 months after CXL with centrally thinned corneal epithelium, breaks in Bowman layer, and thinned corneal stroma with near complete loss of keratocytes. The endothelium seems normal (hematoxylin–eosin, original magnification $\times 20$). B, Keratoconus cornea 5 months after CXL. The corneal stroma is thinned centrally with a significant loss of keratocytes in the anterior stroma (hematoxylin–eosin, original magnification $\times 20$).

Transmission Electron Microscopy

Electron microscopy was performed in 3 keratoconus specimens after CXL. It showed a compact collagenous matrix containing few cellular elements in the anterior stroma with thin vacuolated cytoplasmic processes. Moreover, degenerative changes of keratocytes were observed in the anterior stroma with loss of osmiophilic staining. The posterior stroma and the endothelium seemed relatively unremarkable, but an increased number of intracellular vacuoles were present in posterior keratocytes and endothelial cells.

Keratocyte Counts

Overall keratocyte counts per area were significantly decreased in keratoconus corneas after CXL ($P = 0.033$)

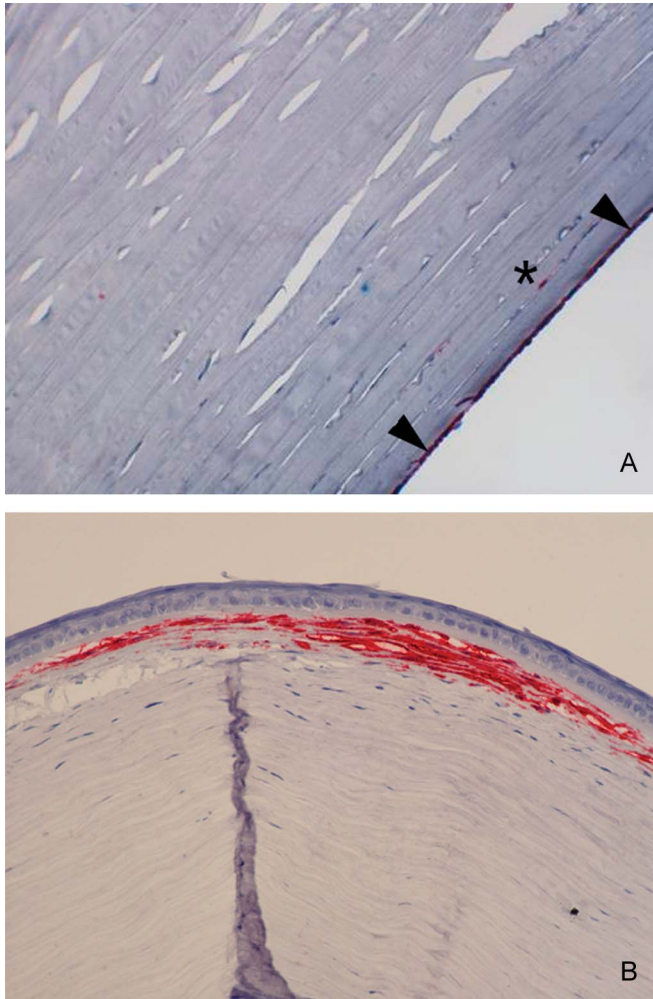


FIGURE 2. A, Keratoconus cornea after CXL. BAX-positive keratocytes in the deep corneal stroma (asterisk) and BAX-positive endothelial cells (arrow; original magnification $\times 40$). B, Smooth muscle actin-positive myofibroblasts in subepithelial scar tissue of keratoconus cornea without CXL.

compared with normal corneas and keratoconus corneas without CXL (Fig. 3). Keratocytes per 1 mio pixel were $328 (\pm 81)$ cells in normal corneas, $398 (\pm 97)$ cells in keratoconus corneas without CXL and $244 (\pm 145)$ cells in keratoconus corneas after CXL. This difference was especially obvious in the anterior ($P = 0.014$) and middle ($P = 0.024$) corneal stroma (Fig. 4). Keratocyte counts in the anterior stroma averaged $399 (\pm 119)$ in normal corneas, $378 (\pm 96)$ in keratoconus without CXL, and $187 (\pm 127)$ in keratoconus with CXL. In the middle stroma, measurements were $347 (\pm 74)$ keratocytes for normal corneas, $426 (\pm 123)$ keratocytes for keratoconus corneas without CXL, and $274 (\pm 213)$ keratocytes for keratoconus after CXL. In the posterior stroma, keratocyte counts were not significantly decreased in keratoconus corneas after CXL compared with normal corneas and keratoconus corneas without CXL. Interestingly, keratocytes were reduced after CXL in the peripheral ($P = 0.047$) and the central corneal stroma ($P = 0.028$; Fig. 5). No correlation was

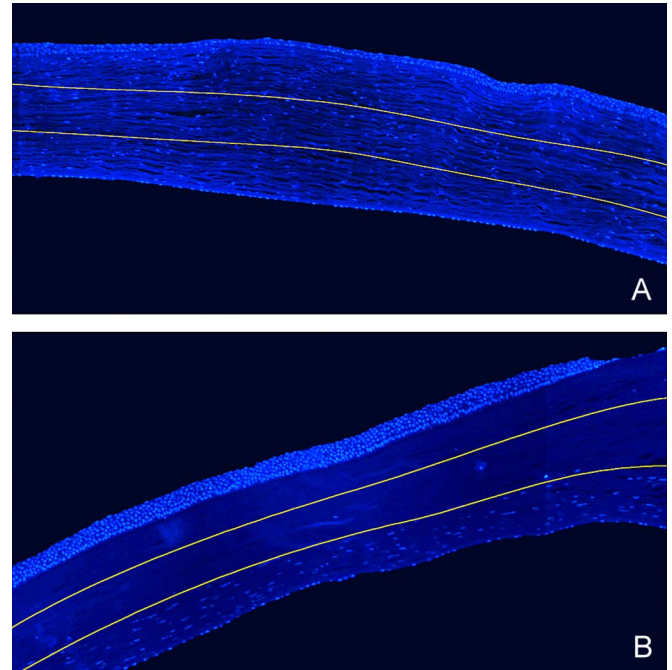


FIGURE 3. A, Keratoconus cornea without CXL. Normal distribution of keratocytes in the corneal stroma (DAPI stain, original magnification $\times 10$). B, Keratoconus cornea 6 months after CXL. Near complete loss of keratocytes in the anterior and middle stroma (DAPI stain, original magnification $\times 10$).

evident between keratocyte counts and time since CXL. CXL corneas in which hypoosmolar riboflavin was used in addition to swelling the corneal stroma did not show a significant difference in keratocyte counts compared with corneas that underwent CXL according to the standard protocol. The same was true for corneas that underwent a second procedure including photorefractive keratectomy, corneal ring implantation, or a second CXL.

DISCUSSION

CXL by combined riboflavin-UVA treatment was introduced in 2003 as a promising new therapy in keratoconus to stop progression and delay keratoplasty.¹⁹ Histological changes in keratoconus patients after this procedure have not been published. We observed long-term toxic effects of CXL treatment in keratoconus corneas.

In cell culture experiments and rabbits, riboflavin-UVA was shown to lead to dose-dependent apoptotic keratocyte damage 24 hours postoperatively by the formation of reactive oxygen species.^{33,35,36} According to Wollensak et al,^{35,36} keratocyte damage can be expected in human corneas down to a depth of $300 \mu\text{m}$ using the standard surface UVA irradiance of $3 \text{ mW}/\text{cm}^2$ ($5.4 \text{ J}/\text{cm}^2$). And indeed, complete loss of keratocytes down to a depth of 250 to $280 \mu\text{m}$ with normal keratocyte density beneath was shown in a normal human cornea 24 hours after CXL.³⁴ Endothelial necrosis and apoptosis occurred in rabbit cornea with a corneal thickness less than $400 \mu\text{m}$.²⁸ Therefore, a minimum corneal thickness of $400 \mu\text{m}$

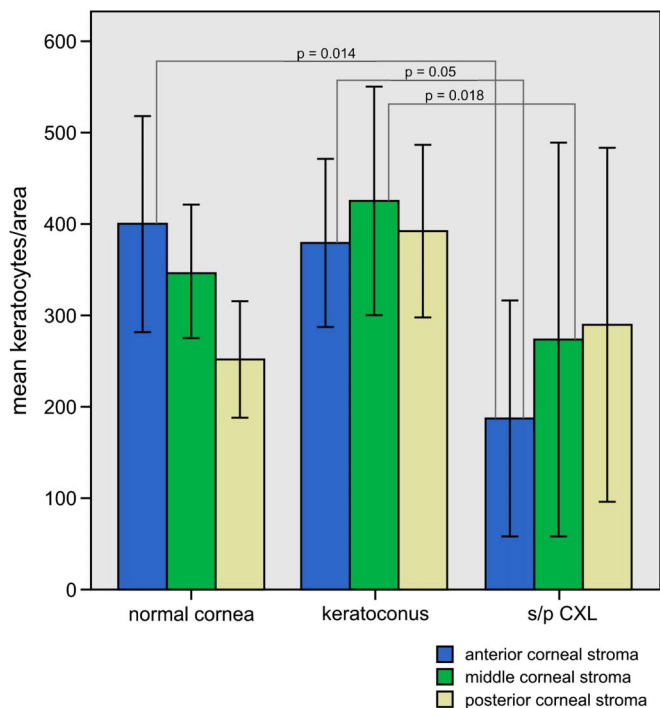


FIGURE 4. Mean keratocytes/area in anterior, middle, and posterior corneal stroma in normal corneas, keratoconus corneas without CXL, and keratoconus corneas after CXL. A significant difference was evident between keratocyte counts in keratoconus corneas with and without CXL in the anterior ($P = 0.05$) and the middle stroma ($P = 0.018$). In addition, significantly reduced keratocytes were present in the anterior stroma of keratoconus corneas after CXL compared with normal corneas ($P = 0.014$).

(without epithelium) was postulated to be safe in human CXL treatment. Preoperative swelling of the cornea with hypoosmolar riboflavin, however, has been shown to be effective and safe in keratoconus patients with thinner corneas.³⁷

Wound healing experiments in approximately 300 μm thin rabbit corneas after CXL showed a complete loss of endothelial cells and stromal keratocytes by day 3. On day 7, the endothelium was intact again, and keratocyte repopulation of the posterior stroma began. By week 4, keratocyte repopulation of the anterior stroma was observed, with normal cytoarchitecture by week 6.³³ In vivo confocal microscopy in humans after CXL also demonstrated a reduction in anterior and intermediate stromal keratocytes followed by a gradual repopulation until up to 6 months.³⁰ In our study, keratocyte loss was still evident 5 to 30 months after CXL in keratoconus patients. The cell loss was mainly confined to the anterior and middle stroma, but it involved the central and the peripheral cornea. This finding is in complete disagreement to animal studies and in vivo confocal studies in humans. Additional procedures, such as photorefractive keratectomy, corneal ring implantation, or a second CXL procedure were not responsible for this keratocyte loss. In addition, the use of hypoosmolar riboflavin to increase stromal thickness to a minimum of 400 μm for treatment was not associated with

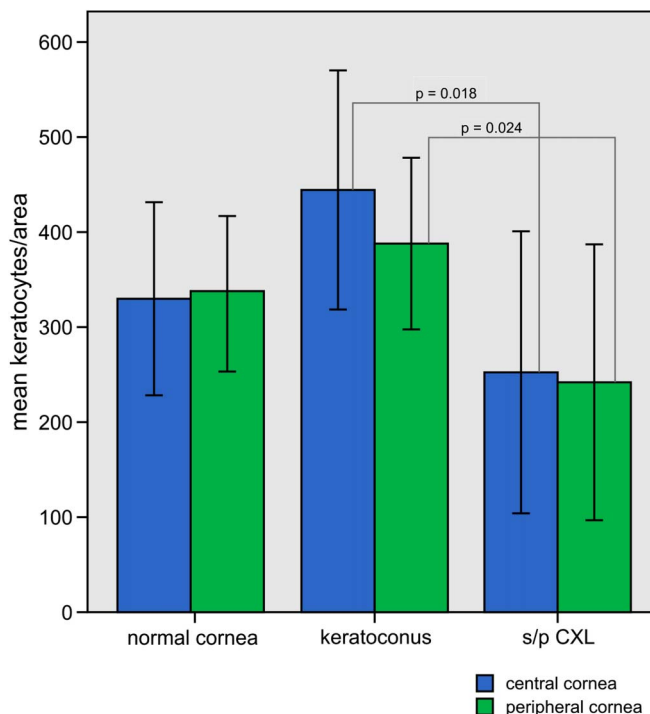


FIGURE 5. Mean keratocytes/area in the peripheral and central corneal stroma in normal corneas, keratoconus corneas without CXL, and keratoconus corneas after CXL. A significant reduction of keratocytes was observed in keratoconus corneas after CXL in the central ($P = 0.018$) and peripheral ($P = 0.024$) corneal stroma compared with keratoconus corneas without CXL.

a further keratocyte reduction compared with patients treated with the standard protocol.

We were surprised to find keratocytes and endothelial cells staining for proapoptotic BAX and antiapoptotic survivin in keratoconus specimens after CXL compared with normal corneas and keratoconus corneas without CXL. The CXL procedure itself does not seem to be responsible because these markers are typically expressed only for a short time after wounding. It may be a feature associated with keratoconus itself, as Kaldawy et al⁸ could also demonstrate apoptotic cells in all 3 layers of the keratoconus corneas. However, our keratoconus specimens without CXL did not express any apoptosis markers in the stroma or endothelium. Thus, it may be speculated that the CXL procedure may be indirectly involved. Further research is certainly warranted to explain these findings.

Keratocyte apoptosis and necrosis are also known to occur after mechanical epithelial debridement or incision through the epithelium as performed as part of the CXL procedure or in the case of keratoplasty. However, this observation is only made in the anterior 50 μm of the corneal stroma, is typically seen early after the trauma, and the subsequent repopulation by activated stromal keratocytes is complete in several days after the injury.³⁸⁻⁴² In our study, no difference was observed in keratocyte counts in the peripheral cornea in keratoconus buttons without CXL and normal corneas confirming that the trephine cut was not responsible for the loss of keratocytes in the corneal periphery after CXL.

Chronic keratocyte apoptosis associated with the ongoing epithelial injury may be an important pathophysiological factor in keratoconus mediated by interleukin-1 and Fas-ligand.^{8,41,43} But keratocyte counts were not significantly different in keratoconus specimens without CXL and normal corneas indicating that—indeed—CXL is responsible for the keratocyte loss observed in our study.

Wollensak et al³³ identified α -actin-positive myofibroblasts, especially in the periphery of the irradiated areas, by weeks 4 and 6 after CXL in rabbit corneas. The presence of these cells is interesting because myofibroblasts, presumed derivatives of keratocytes responding to transforming growth factor beta, are a critical component of the wound healing cascade.⁴⁴ They play a comprehensive role in collagen and extracellular matrix remodeling through production of collagen, glycosaminoglycans, collagenases, gelatinases, and matrix metalloproteinases.^{45–47} The regression of keratectasia in keratoconus patients after CXL might be explained in part by the action of myofibroblasts.³³ Five to 30 months after CXL, we could not identify any actin-positive myofibroblasts in the corneal stroma. The wound healing process after CXL seems to be complete by that time. It may parallel wound healing after photorefractive keratectomy, where myofibroblasts are typically observed for 3 months.⁴⁷

The increase in biomechanical stiffness in corneas after CXL is paralleled by an increase in corneal collagen diameter in animal experiments.⁴⁸ In vivo confocal microscopy confirmed an increased density of the anterior extracellular corneal matrix starting between the third and sixth month postoperatively with bridge-like and needle-shaped hyperreflective bands in humans after CXL.³¹ In our study, CXL corneas did not show any obvious changes in collagen structure by light microscopy, but the anterior corneal stroma was unusually compact by transmission electron microscopy. Special measurements to document changes in collagen diameter thickness were not performed.

Our results demonstrate that CXL can cause considerable long-term changes to keratoconus corneas. Our series of cases having consisted of surgically removed corneas for keratoconus is biased for poor outcome, but permanent keratocyte loss after CXL is a potentially serious consequence of the procedure that deserves further study. To exclude additional negative influential factors, CXL treatment should only be performed under investigational protocol.

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