Sequential In Vivo Confocal Microscopy Study of Corneal Wound Healing After Cross-linking in Patients With Keratoconus

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ABSTRACT

PURPOSE: To evaluate the short- and long-term sequential histological changes of the cornea in vivo after corneal collagen cross-linking (CXL) in patients with keratoconus.

METHODS: Eighteen patients with keratoconus (Amsler-Krumeich classification: stages I, II, and III) underwent CXL with riboflavin/ultraviolet A (UVA) in one eye. The corneas were examined preoperatively and within 5 hours, 7 and 14 days, and 1, 3, 6, 9, 12, 18, 24, and 36 months after the procedure using in vivo confocal microscopy.

RESULTS: Early changes included edema, superficial nerve loss, cellular modifications, and isolated endothelial damage. At intermediate time points, there was nerve fiber regeneration, increased reflectivity of the extracellular matrix, enlarged keratocytes and extracellular deposits, and remodeling of the endothelial layer (two eyes). At later time points, loss of keratocytes and remodeling of the extracellular deposits were noted.

CONCLUSIONS: Although the cornea has no significant tissue modifications clinically after CXL, this study has shown that corneal wounding by riboflavin/UVA collagen CXL induces cellular wound-healing mechanisms and alters the normal structure and cellularity of the cornea for up to 36 months. [*J Refract Surg*. 2009;xx:xxx-xxx.]

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Collagen cross-linking (CXL) of the cornea using ultraviolet A (UVA) light and photosensitized riboflavin was developed by Spoerl et al in 1998 to induce biochemical strengthening of the corneal stroma.[1] Experimental and clinical data support the hypothesis that the procedure increases the stiffness of the cornea with minimal cellular and tissue changes.[2] Although based on case reports and small clinical series, several uses have been proposed recently. The initial and main indication of this technique is to retard or halt the progression of keratoconus.[3]

In vivo confocal microscopy has provided a noninvasive mode of observation and quantification of corneal tissue in normal and abnormal conditions. In addition, in vivo confocal microscopy facilitates the sequential observation of wound healing after refractive procedures and corneal graft surgery, as well as after CXL in animal models and patients with keratoconus.[4-7] The aim of this study was to analyze the chronological microscopic changes that occur in the cornea after CXL in patients with keratoconus.

PATIENTS AND METHODS

Eighteen eyes of 18 consecutive patients with stage I (5 eyes), II (9 eyes), and III (4 eyes) keratoconus, according to the Amsler-Krumeich classification,[8] treated with CXL between November 2005 and December 2008 at the Department of Ophthalmology of the Hospital de Clínicas "José de San Martín" were enrolled in this study (Table 1). All patients were at least 14 years old. Only one eye of each patient underwent the procedure. The study was conducted according to the te-

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The authors have no commercial or proprietary interest in the materials presented herein.

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TABLE 1

Characteristics of Patients Who Underwent Corneal Collagen Cross-linking for Keratoconus

Characteristic	Value
Age (median, range) (y)	25 (16 to 49)
Sex (F/M)	7/10
Keratoconus grade (no. eyes)	I (5), II (9), III (4)
K1 (mean±SD, range) (D)	48.43±5.40 (43.35 to 63.26)
K2 (mean±SD, range) (D)	52.89±5.53 (45.27 to 67.20)
Central corneal thickness (mean±SD, range) (μm)	457.18±33.01 (414 to 519)

SD = standard deviation

nets of the Declaration of Helsinki. All patients gave written consent before entering the study and the protocol was approved by the hospital's Institutional Ethics Committee.

Preoperative examination was performed including distance (Snellen/Feet) and near (Jaeger Standard) corrected and uncorrected visual acuity; slit-lamp microscopy; keratometry (KR 3000; Topcon, Japan) and corneal topography (Kern Tech 2000 Topographer); corneal anterior surface aberrometry (Kern Tech 2000 Topographer); and pachymetry, obtained with the patient in the seated position, both with ultrasonic pachymetry (Accupach Ultrasound Pachymeter; Accutome, Malvern, Pa) and Visante Optical Coherence Tomography (OCT; Carl Zeiss Meditec, Jena, Germany).

The clinical diagnosis of keratoconus was based on topographic findings as well as on biomicroscopic signs of keratoconus. Preoperative keratoconus progression was confirmed by corneal topography and clinical history of the patient with 6-month follow-up.

Exclusion criteria were patients having additional corneal diseases (herpes, scars, infections, etc), thinnest point pachymetry <400 μm, or concomitant autoimmune diseases as well as pregnant or nursing women. Patients wearing any kind of contact lenses 4 weeks before baseline examination were also excluded.

Treatment Procedure

All surgical procedures were conducted under sterile conditions in an operating room. The epithelium was removed mechanically in an area of 7-mm diameter with Merocel sponge (Medtronic Xomed Inc; Jacksonville, Fla) to facilitate riboflavin diffusion into the stroma. After the debridement, riboflavin photosensitizer solution containing 0.1% riboflavin-5-phosphate and 20% dextran T-500 was administered onto the cornea every 3 minutes, beginning 30 minutes before irradiation until the end of the treatment. Ultraviolet A irradiation (370 nm) was applied for 30 minutes using a UVA emitter (CL II TM; Pentium Laboratories, Argentina) with a surface irradiance of 3 mW/cm^2. Total radiant exposure to the cornea was 5.4 J/cm^2. The UVA source consisted of two light-emitting diodes (UV-A 370 nm; Roithner Lasertechnik, Vienna, Austria). Before the procedure, the energy irradiated was controlled with a power meter (Thorlabs, Newton, NJ). During the procedure, the cornea was hydrated with saline. Upon treatment (UVA irradiation) completion, the cornea was copiously irrigated with abundant saline and a therapeutic contact lens was placed for 4 days until the abrasion healed.

Postoperative medications prescribed were antibiotic eye drops ofloxacin 0.3% (Oflox) four times daily and anti-inflammatory ketorolac tromethamine 5 mg/mL (Acular) twice daily until corneal abrasion healed, as well as 1% prednisolone acetate (Prednefrin Forte) four times daily for 30 days and then changed to fluorometholone 0.250 g three times daily for 30 days and twice daily for an additional 30 days. The use of artificial tears was indicated if needed. Furthermore, patients were prescribed vitamin C (Redoxon) 2 g daily and L-cystine 500 mg, pyridoxine hydrochloride 100 mg (vitamin B6, Megacistin) 1 tablet twice daily for 3 months, according to the Superficial Treatment Protocol established by our institution.[9,10]

Confocal Microscopy

In vivo confocal microscopy of the cornea was performed with the Rostock Cornea Module/HRT II (Heidelberg Engineering GmbH, Dossenheim, Germany). Two-dimensional black and white images representing an area of 400×400 μm were captured using an immersion objective lens (63×) and a 400× field of view (FOV) lens. The resultant digital resolution is approximately 1 μm/pixel (transversal) and 2 μm/pixel (longitudinal). Additional images were obtained with a 300× FOV lens for larger original view and capture without increased resolution. Preoperative in vivo confocal microscopy examination was performed between 7 and 15 days before the procedure. Postoperative examinations were initially performed within 5 hours after the procedure while the patients were wearing a bandage soft contact lens, followed by sequential examination at 7 days, 2 weeks, and 1, 3, 6, 9, 12, 18, 24, and 36 months after CXL. Both eyes of the patients, treated and untreated, were evaluated each time.

Evaluations included corneal nerves (presence, absence, and regeneration), keratocytes (density and

TABLE 2

Time Correlation of Corneal Tissue Findings Using In Vivo Confocal Microscopy After Corneal Collagen Cross-linking in Patients With Keratoconus

Time	Epithelium	Nerves*	Stroma	Endothelium
5 hours	Absent	Not seen	Hyperreflective	Normal
7-15 days	Irregular inflammation	Absent	Granular deposits, stellate keratocytes	Normal and hyperreflective†
30 days	Normal	Regeneration	Enlarged keratocyes, rod-like deposits	Normal and isolated loss†
3 months	Normal	Regeneration	Enlarged keratocytes, hyperreflective ECM, fibrillar deposits	Normal
6-9 months	Normal	Regeneration	Idem (3 months)	Normal
12 months	Normal	Regeneration	Hypocellular and hyper-reflective fibrillar deposits	Normal
24-36 months	Normal	Barely normal, regeneration	Hypocellular and hyper-reflective remodeling deposits	Normal

ECM = extracellular matrix
**Sub-basal nerve plexus.*
†2 cases.

morphological changes), extracellular matrix (deposits, lines or striae, and reflectivity), endothelium (density and morphological changes), as well as the presence of inflammatory cells, dendritic cells, and stromal edema.[11-13]

RESULTS

Corneal tissue findings at each evaluation time point after CXL are presented in Table 2.

Preoperative examination disclosed a normal epithelium with isolated focal scars in Bowman's layer in one case. The sub-basal nerve plexus was well developed with occasional thick nerve fiber bundles. Beneath Bowman's layer, a sheet of increased density of keratocytes nuclei and punctiform deposits was observed. Striae, seen as hyporeflective lines running parallel or oblique to each other, were observed in the mid and deep corneal stroma. The endothelium showed mild variation in size and cell density.

Immediately after the procedure (Fig 1), the corneal epithelium was absent and the bandage soft contact lens was in contact with a hyperreflective layer that obscured cellular details and nerve fiber bundles. Down to mid stroma (200 to 250 µm), keratocytes nuclei were barely visible and empty spaces that may represent extracellular edema were randomly distributed among the shadows of cell boundaries. The deep stroma and the endothelium were normal.

At days 7 and 15 after CXL, the corneal epithelium had full-thickness development with irregular-sized epithelial cells and a few isolated inflammatory cells. The superficial and sub-basal nerve plexus were absent from the site of UVA radiation. Hyperreflective granular deposits were observed beneath Bowman's layer (Fig 2A). The anterior stroma revealed persistent corneal edema and star-shaped cells representing keratocytes with barely seen nucleus (Fig 2B). At a depth of 240 µm, keratocytes displayed fine punctiform cytoplasmic particles and highly reflective small nuclei (Fig 2C). Next to the damaged area, there were keratocytes with short processes and large uniform reflective nuclei that may represent activated keratocytes (Fig 3A). The mid stroma corneal nerve plexus was present. The deep stroma and the endothelium were without significant changes. In two eyes, the stromal changes extended to the deep corneal layers and the endothelium showed an irregular reflective appearance with isolated inflammatory cells (Fig 3B).

One month after CXL, isolated "wandering" inflammatory cells were present in the epithelium. Bordering the radiated area, short and fine regenerated nerve fiber bundles were observed (Fig 4A). The superficial stroma showed incipient repopulation by enlarged keratocytes with short cellular processes (Fig 4B). In the mid stroma, the keratocytes with punctiform granular cytoplasm were embedded in an extracellular matrix containing rod-like structures. Between 230- and 400-µm depth, the stroma showed hyperreflective sheets of longitudinally aligned filaments (Fig 4C). The deep stroma and the endothelium were normal. In two cases,

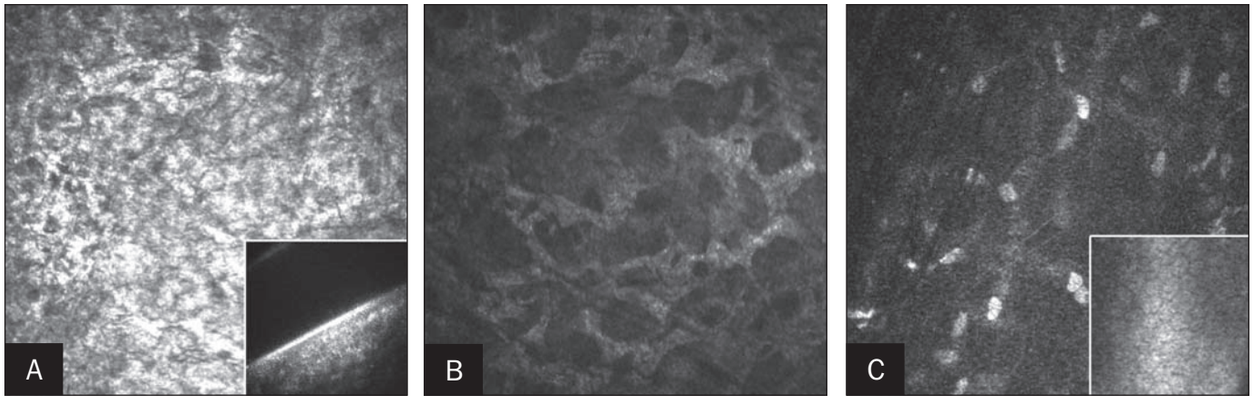

Figure 1. Confocal microscopy images 5 hours after cross-linking. **A)** Hyperreflective superficial stroma without cellular details. Inset shows the oblique image of the contact between the bandage contact lens and the cornea. **B)** Stromal edema. **C)** Posterior stroma and endothelium (inset) without significant changes.

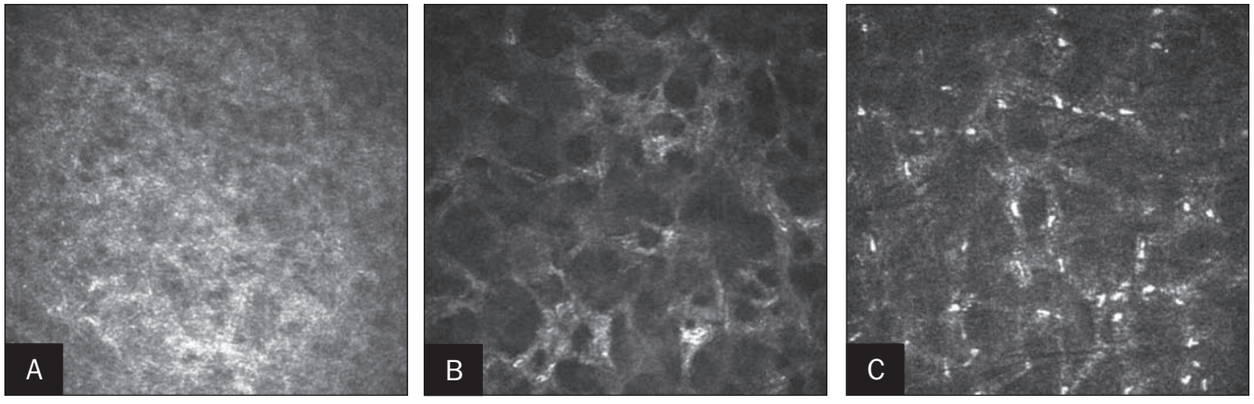

Figure 2. Confocal microscopy images 7 days after cross-linking. **A)** Granular appearance of the superficial stroma. **B)** Persistent stromal edema. **C)** Corneal stroma at 290-μm depth with punctiform highly reflective nuclei.

the endothelium showed small hyporeflective patches and retraction of the cytoplasm in a few endothelial cells (Fig 4D).

Three months after CXL, regeneration of the sub-basal nerves was evident. The superficial stroma showed large keratocytes with long cytoplasmic extensions. The striae disclosed a more compact arrangement interspersed by a highly reflective extracellular matrix (Figs 5A and B). Numerous rod-like reflective structures were seen. At 240- to 270-µm depth, the stroma showed hyperreflective compactly arranged filaments and sheets of hyperreflective cells (Fig 6A). Keratocytes at the border of the irradiated area disclosed a star-like appearance (Fig 6B). The endothelium was normal, except in the two cases with endothelial damage, which did not change in appearance.

At 6 and 9 months after CXL, nerves continued to regenerate. No significant changes were observed within the stroma regarding the findings at 3 months, with individual variation in the size of keratocytes and extracellular fibrillar deposits. The two eyes with endothelial damage showed a normal appearance with mild to moderate polymegathism.

Examination at 12 months disclosed persistent active nerve regeneration (Fig 7A), hyperreflective extracellular matrix beneath Bowman's layer, hypocellular superficial stroma with rod-like structures, and multiple fine dots. The mid and deep stroma showed reflective lines and sheets of irregularly arranged keratocytes with broad cytoplasmic extensions (Figs 7B and 7C). The endothelium was normal except in one case that contained inflammatory cells. At 24 and 36 months, the sub-basal nerve plexus was barely normal except for focal areas devoid of nerves (Fig 8A). The anterior and mid stroma remained hypocellular with mildly increased reflectivity of the extracellular matrix when compared with preoperative findings (Figs 8B and 8C). Active remodeling of the fibrillar deposits was noted. The endothelial layer was unchanged.

The severity of stromal changes observed after the

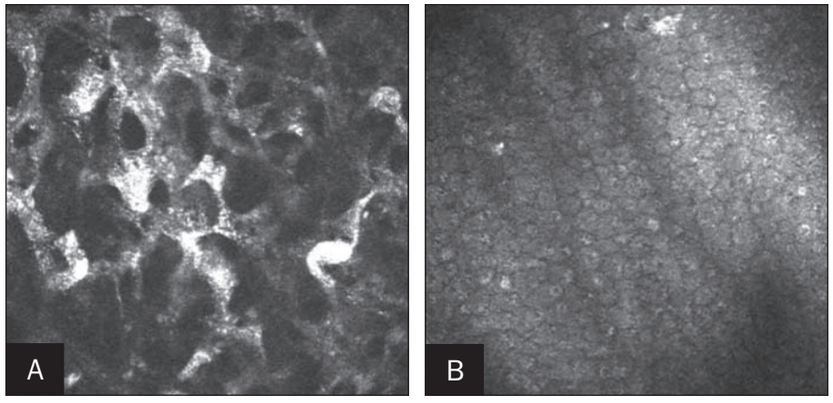

Figure 3. Confocal microscopy images 15 days after cross-linking. **A)** Enlarged keratocytes at the edge of the irradiated area. **B)** Hyperreflective endothelial surface and isolated inflammatory cells.

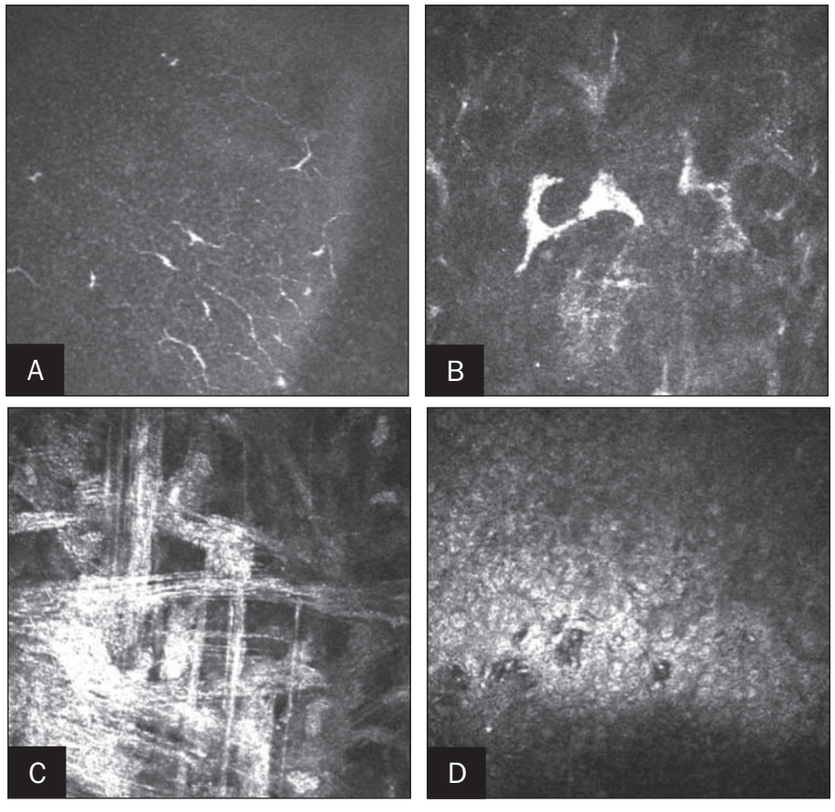

Figure 4. Confocal microscopy images 1 month after cross-linking. **A)** Incipient regeneration of the sub-basal nerve fiber bundles. **B)** Enlarged keratocytes in the anterior stroma. **C)** Fibrillar deposits seen at 260- to 350-μm depth. **D)** Damage of the endothelial cell layer.

first week varied widely among the patients and was not related to the stage of keratoconus (Amsler-Krumeich classification).

No significant changes or deviation from the initial morphological findings were observed in the non-irradiated eyes.

DISCUSSION

In vivo confocal microscopy of the cornea has provided a method for visualization of normal and pathological changes without the need of tissue samples, fixation, and staining. One of the limitations of this method is that interpretation of the images is mainly based on pattern recognition of morphological features and reflectivity. Several studies have shown a correlation between the results of this procedure with histopathological findings in inflammatory and infectious diseases, corneal degenerations and dystrophies, metabolic diseases, scarring after surgical procedures, and experimental models. The reliability of the findings in the present study was sustained by observing similar features in different patients at the same time points.[11-13]

In this series, the procedure of corneal collagen CXL created a central area of cellular damage, extending

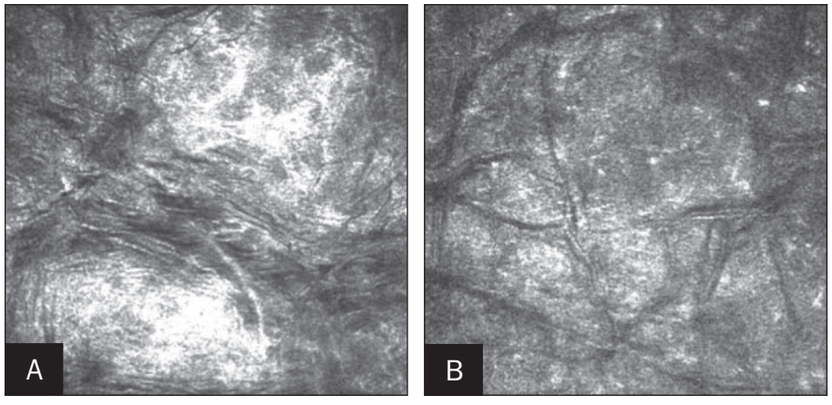

Figure 5. Confocal microscopy images 3 months after cross-linking. Highly reflective appearance of the extracellular matrix of the **A)** anterior and **B)** posterior stroma.

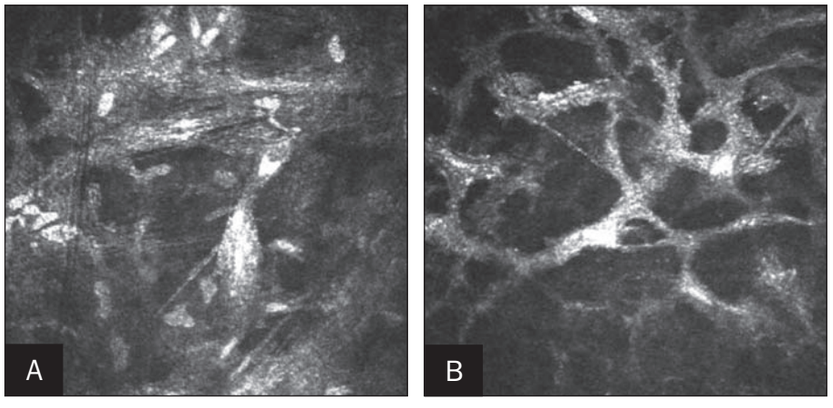

Figure 6. Confocal microscopy images 3 months after cross-linking. **A)** Syncytial-like keratocytes observed at 320-μm depth. **B)** Enlarged high-reflective keratocytes at the edge of the irradiated area.

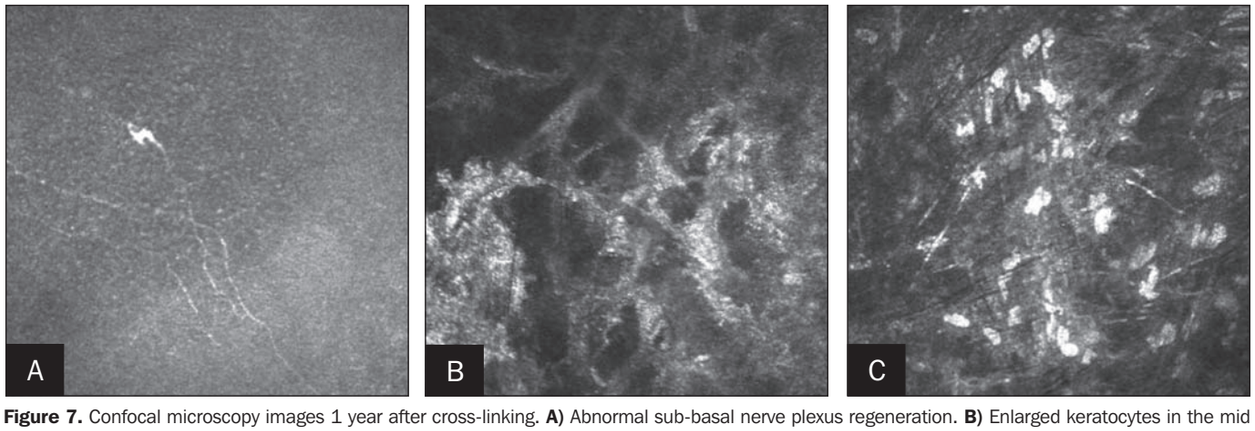

Figure 7. Confocal microscopy images 1 year after cross-linking. **A)** Abnormal sub-basal nerve plexus regeneration. **B)** Enlarged keratocytes in the mid stroma. **C)** Keratocytes with enlarged nuclei.

from the superficial corneal nerve plexus to approximately 350-µm depth. The initial damage after the procedure included superficial nerve fiber loss and disappearance of keratocytes, associated with hyperreflective dots and extracellular stromal edema. The UVA-irradiated keratocytes may be destroyed, undergo apoptosis, or suffer delayed damage of a lesser degree.[14,15]

Depending on the extent of injury, stromal cells may react with cellular changes, giving the appearance of enlarged keratocytes. Two main types of scarring were observed, both associated with enlarged keratocytes. One is the presence of extracellular deposits of hyperreflective, long, thin, individual fibers forming bands or sheets. The other, resembling cellular scarring, appears to occur by enlargement of adjacent keratocytes, which may become evident as patches or sheets of cells with abundant cytoplasm. These activated kera-

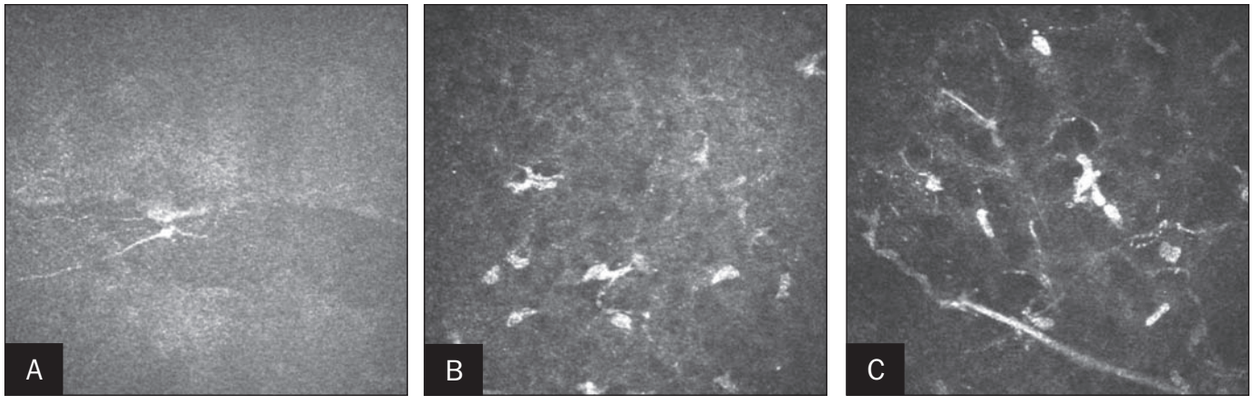

Figure 8. Confocal microscopy images 3 years after cross-linking in the same patient. **A)** Incomplete regeneration of the sub-basal nerve fiber bundles. **B)** Low density of keratocytes in the anterior stroma. **C)** Abnormal nerve fiber bundles and disarray of keratocytes in the mid stroma.

tocytes may represent myofibroblasts, as has been demonstrated in corneal wound healing and after refractive surgery.[16] The arrangement of extracellular fibrillar deposits suggests that they are composed of new synthesized collagen by activated keratocytes. It is interesting to note that these deposits were almost exclusively located at the interphase between the deepest penetration of UVA radiation and the preserved posterior corneal stroma where classical demarcation lines are observed.[17] Enlarged keratocytes from the edge of the irradiated zone contribute to repopulation of the damaged area.[6] However, the density of keratocytes in the central area remained extremely low even at 36 months after CXL. The thin corneal nerve branches of the superficial and sub-basal plexus are not visualized or identified immediately after the procedure, whereas the thick stromal nerves remain visible. The first evidence of corneal nerve regeneration was observed at 1 month after the procedure, and this process was still active or incomplete at 36 months.

Some collateral effect on the corneal endothelium was observed in two cases with central corneal thicknesses of 419 µm and 420 µm. The initial finding was increasing reflectivity of the endothelial cell layer 7 to 15 days after the procedure that evolved to individual cell loss at 1 month, suggesting mild initial and delayed damage. The abnormal endothelium regained a normal appearance with residual polymegathism after 3 months and a regular appearance without significant change in endothelial cell density at 1 year. Endothelial damage after CXL has been demonstrated in rabbits.[18,19]

Regarding the extracellular matrix, aside from the deposits mentioned above, there was increased reflectivity and the stromal striae appeared to adopt a more irregular and compact configuration. Three eyes showed a marked increase of reflectivity of the stroma resembling the description of haze found in patients after CXL.[20] A review of preoperative images in these cases showed more irregular and dense striae in comparison with eyes without histological haze as was mentioned previously.

After complete full thickness regeneration of the removed epithelium, the superficial epithelial layer disclosed abnormal desquamation as seen in other corneal procedures that may be related to some degree of loss in sensitivity due to nerve damage.

No evidence of inflammatory cell reaction was observed within the corneal stroma. Experimental studies in rats and rabbits have demonstrated that cells died by apoptosis, a mechanism of cell death that does not lead to inflammation.[2,14,21] Wandering, isolated, mononuclear inflammatory cells were seen within the epithelium at all times in which examinations were performed. In one case, mononuclear cells were observed in the corneal endothelium not related to endothelial or deep stromal damage. Except for one eye in which dendritic cells were already present in the corneal epithelium during the preoperative examination, only two other eyes showed migration of dendritic cells into the paracentral cornea.

The current sequential study of corneal wound healing after CXL in patients with keratoconus provides new in vivo confocal microscopy data regarding the damage immediately following CXL, activation of cellular reparative processes, and potential development of delayed endothelial cell damage.

AUTHOR CONTRIBUTIONS

Study concept and design (J.O.C., A.E.T., C.J.A.); data collection (A.E.T., C.J.A.); interpretation and analysis of data (J.O.C., A.E.T.); drafting of the manuscript (J.O.C., A.E.T.); critical revision of the manuscript (J.O.C., A.E.T., C.J.A.); statistical expertise (J.O.C., A.E.T.); obtained funding (J.O.C., A.E.T.); administrative, technical, or material support (J.O.C., A.E.T.); supervision (J.O.C., A.E.T.)

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AUTHOR QUERIES

According to the Author Contributions, Drs Croxatto and Tytiun obtained funding; however, this is not mentioned in the author information box on page 1. Please clarify.

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Page 3, right column: Please verify the range of depth, as it differs in the text and figure caption.
Text: Between **230- and 400-µm d**epth, the stroma showed hyperreflective sheets of longitudinally aligned filaments (Fig 4C).
Figure 4C: **260- to 350-µm** depth

Page 4, left column: Please verify the depth value, as it differs in the text and figure caption.
Text: At **240- to 270-µm** depth, the stroma showed hyperreflective compact arranged filaments and sheets of hyperreflective cells (Fig 6A).
Figure 6A: **320-µm**

Page 4, right column and Table 2: Please clarify the following. What is meant by "barely normal?"
At 24 and 36 months, the sub-basal nerve plexus was **barely normal** except for focal areas devoid of nerves (Fig 8A).

Page 7, right column: Please provide a reference(s) for the following statement.
After complete full thickness regeneration of the removed epithelium, the superficial epithelial layer disclosed abnormal desquamation as seen in other corneal procedures that may be related to some degree of loss in sensitivity due to nerve damage.

In Table 2, please clarify "idem (3 months)" (row 6-9 months, column stroma). Should it be identical, or same as at 3 months?