Corneal Healing After Riboflavin Ultraviolet-A Collagen Cross-Linking Determined by Confocal Laser Scanning Microscopy In Vivo: Early and Late Modifications

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• PURPOSE: To assess early and late micromorphological modifications of cross-linked corneas in vivo by means of Heidelberg Retinal Tomography (HRT) II confocal microscopy.

• DESIGN: Prospective nonrandomized open trial.

• METHODS: Micromorphological examination of 44 cross-linked keratoconic corneas was performed in vivo by HRT II confocal laser scanning microscopy. Riboflavin ultraviolet (UV)-A–induced corneal collagen cross-linking (CXL) was performed according to the Siena protocol: pilocarpin 1% drops 30 minutes before, topical anesthesia with lidocaine 4% drops 15 minutes before irradiation, mechanical scraping of epithelium (9-mm-diameter area), preirradiation soaking for 10 minutes in riboflavin solution 0.1% (Ricrolin, Sooft, Italy) applied every 2.5 minutes for 30 minutes, 30 minutes exposure to solid-state UVA illuminator (Caporossi; Baiocchi; Mazzotta, X-linker, CSO, Italy), 8-mm-diameter irradiated area, energy delivered 3 mW/cm^2. All patients were examined by confocal scans preoperatively and at the following times after treatment: one, three, and six months, and one, two, and three years.

• RESULTS: No damage to the limbal region was observed. Epithelial regrowth was complete after four days of soft contact lens bandage. The anatomy of the subepithelial plexus was restored one year after the operation with full corneal sensitivity. Increased density of extracellular matrix in late postoperative period indicated cross-linked collagen to a depth of 340 μm expressed by a late demarcation line.

• CONCLUSION: In vivo confocal microscopy showed early and late modification of corneal microstructure after the treatment. The three-year stability of CXL recorded could be related to increased cross-links formation, synthesis of well-structured collagen and new lamellar interconnections. (Am J Ophthalmol 2008;146:527–533. © 2008 by Elsevier Inc. All rights reserved.)

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RIBOFLAVIN ULTRAVIOLET (UV)-A CORNEAL COLLAGEN cross-linking (CXL) is the only etiopathogenetic approach to keratoconus that can delay or block corneal ectasia,[1,2] reducing the demand for keratoplasty.[3] There is also some experimental evidence of possible benefits of this new method in post-laser-assisted in situ keratomileusis (LASIK) ectasia[4] and corneal melting, through increased corneal biomechanical resistance and reduced collagenase activity.[5] Confocal micromorphological analysis in vivo has shown early modifications in all corneal layers after therapeutic riboflavin UV-A corneal collagen CXL, penetration of the treatment in humans,[6] and good safety.[7] Here, we report follow-up of corneas by in vivo corneal micromorphological microscopy three years after corneal collagen CXL.

METHODS

SINCE SEPTEMBER 15, 2004, MICROMORPHOLOGICAL EXAMINATION of 44 cross-linked corneas has been performed at Siena University Ophthalmological Department by Heidelberg Retinal Tomography (HRT) II confocal laser scanning microscopy in vivo (Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany). We report confocal analysis of the first 10 Italian patients treated (Siena Eye Cross Project 2004 to 2007)[8] three years after the operation. All patients had clinically (uncorrected visual acuity and best spectacle-corrected visual acuity) and instrumentally (Costruzione Strumenti Oftalmici [CSO] corneal topography, optical Orbscan IIz Bausch & Lomb and ultrasound DGH pachymetry, CSO surface aberrometry, and Zeiss biomicroscopy) documented progressive keratoconus. Riboflavin UV-A–induced corneal collagen CXL was performed as follows in all cases: pilocarpin 1% drops 30 minutes preoperatively, topical anesthesia with lidocaine 4% drops 15 minutes before and once after epithelial removal, corneal mechanical (blunt metal spatula) epithelial scraping of an area 9 mm in diameter, preirradiation corneal soaking for 10 minutes in riboflavin solution (Ricrolin, Sooft, Italy) applied every 2.5

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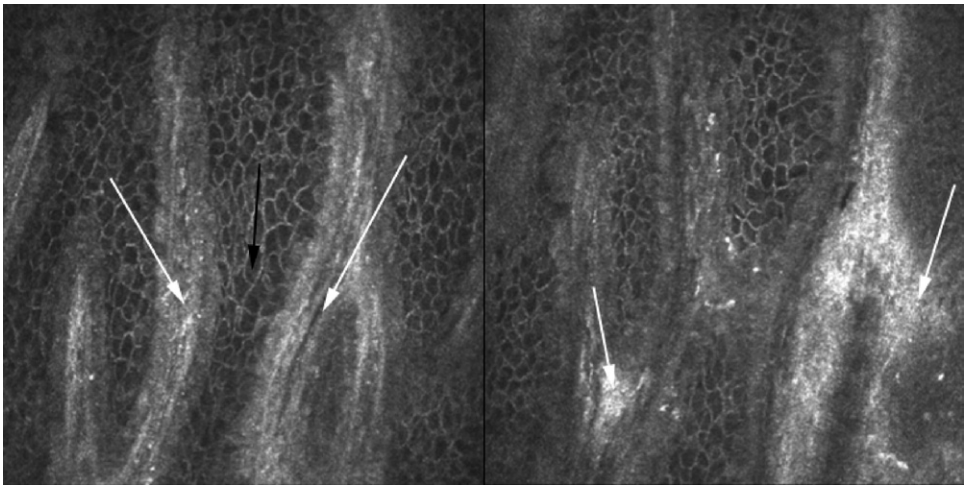

FIGURE 1. Riboflavin ultraviolet (UV)-A collagen cross-linking (CXL). Preoperative and postoperative limbal scans showing palisades of Vogt (Left, white arrows) and corneal epithelium (Left, black arrow). No loss of limbal germinal structures was observed after CXL (Right). Slightly increased reflectivity of extracellular tissue surrounding palisades is evident (Right, white arrows).

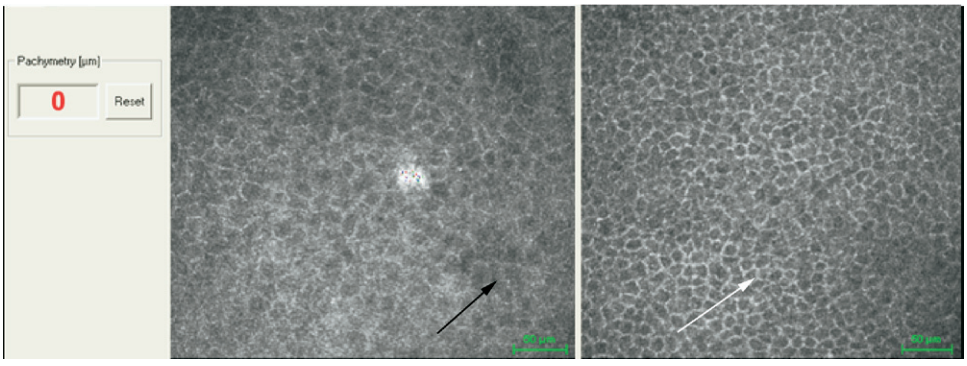

FIGURE 2. Riboflavin UV-A collagen CXL. Epithelial scans showing time-dependent qualitative improvement of cell mosaic (Right, white arrow) with respect to preoperative epithelium, especially in keratoconus apex region, where a thin epithelium with undefined basal epithelial cell borders and distortion of cell mosaic is evident (Left, black arrow).

minutes for 30 minutes, exposure to solid-state UV-A illuminator (Caporossi; Baiocchi; Mazzotta, X-linker, CSO, Italy) for 30 minutes, irradiating an area 8 mm in diameter (energy delivered 3 mW/cm^2), antibiotic medication with ofloxacin drops and flurbiprofen drops four times a day for two weeks, and therapeutic soft contact lens bandage for four days. We also used preservative-free topical steroids (dexamethasone phosphate drops) in the postoperative period in selected cases with predictive risk factors for haze development based on preoperative clinical and micromorphological evaluation[9] and for four weeks in patients with strong corneal edema or haze. All patients were examined by HRT II confocal laser scanning microscope (Rostock Cornea Module; Heidelberg Engineering) preoperatively and at the following times after treatment: one, three, and six months, and one, two, and three years. We analyzed all corneal layers, assessing stromal modifications, keratocyte repopulation and activity, and healing.

RESULTS

- **LIMBUS:** All corneal layers, especially the epithelium, regenerated rapidly (four days) and no damage to the limbal region was observed. Slightly increased reflectivity of extracellular tissue surrounding palisades of Vogt was detected without any pathologic significance and without affecting the germinal epithelium (Figure 1).

- **EPITHELIUM:** In all cases, epithelial regrowth was complete after four days of soft contact lens bandage. No growth retardation or persistent epithelial deficit was detected after corneal collagen CXL. Micromorphological analysis showed time-dependent epithelial stratification and qualitative improvement of cell mosaic compared to preoperative epithelium, especially in the keratoconus apex region where the epithelium was often thin with undefined basal epithelial cell borders and distorted cell

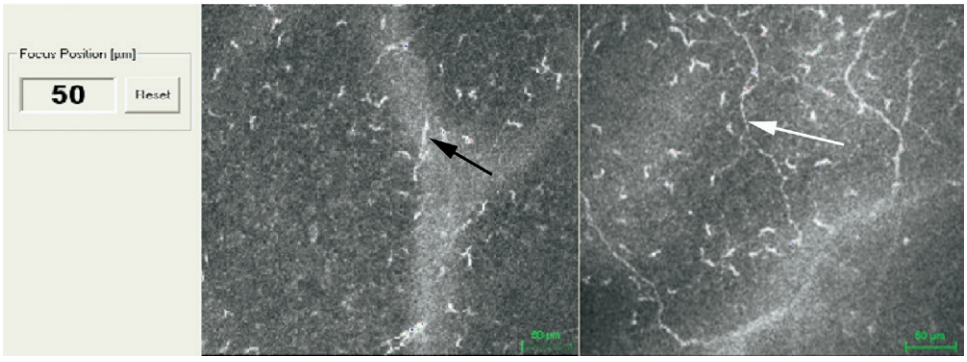

FIGURE 3. Riboflavin UV-A collagen CXL. Structure of the subepithelial plexus was not well defined until one year after CXL: note disconnected neuritic flocculations under Bowman lamina at a depth of 50 µm (Left, black arrow). After one year, the number of fibers was greater and interconnections were evident (Right, white arrow).

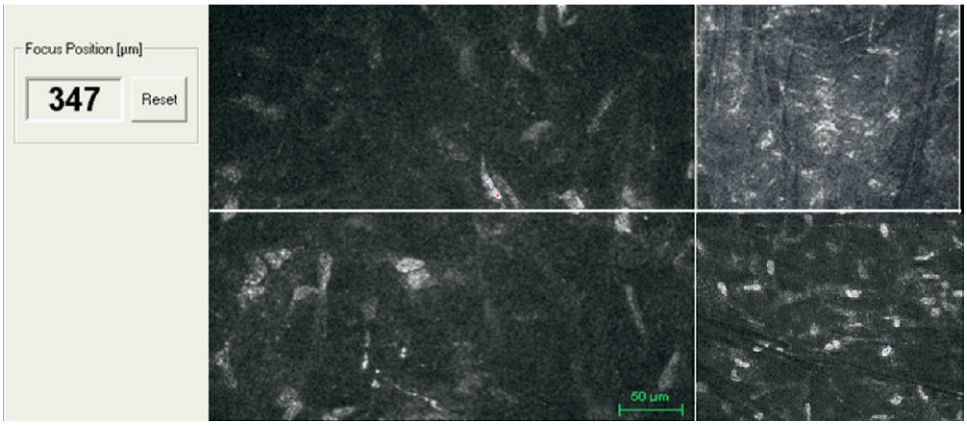

FIGURE 4. Riboflavin UV-A collagen CXL. Change in stromal reflectivity after CXL. After six months (Top right) the reflectivity (hyper) of anterior midstroma was inverted with respect to initial postoperative reflectivity (hypo) (Top left). This characteristic was expressed as early and late demarcation lines between treated and untreated stroma (Bottom right and Bottom left) to a depth of 340 µm including epithelium (white demarcation line).

mosaic. The epithelium showed good compensatory function and smoothing of corneal surface irregularities during progressive stratification, especially after the third postoperative month (Figure 2).

• **NERVES:** In all cases, early postoperative confocal analysis showed immediate disappearance of subepithelial plexus and anterior-midstromal nerve fibers. Regeneration started more rapidly in subepithelial nerve fibers, growing from the surrounding nonirradiated area just one month after the operation, whereas anterior-midstromal fibers regenerated from the deep stromal nerve plexus between the second and third months postoperative. Regeneration of nerve fibers was almost complete six months after the operation, with fully restored corneal sensitivity. Plexus structure was not well defined until one year after corneal collagen CXL, with what appeared to be disconnected neuritic flocculations under the Bowman lamina. After two years, the number of fibers increased and interconnections resembled the preoperative structure (Figure 3).

• **STROMA:** In vivo confocal analysis showed disappearance of keratocytes from the anterior midstroma to a depth of 340 µm with epithelium in situ,[6] confirming treatment penetration found in previous studies in animal models and in human corneas ex vivo, as well as standardized parameters measured and recorded in Dresden pilot studies,[10–15] for the first time directly in humans in vivo.[6] A clear vertical transition area (VTA) between the edematous hyporeflective stroma with keratocytes apoptotic bodies and normo-reflective deep stroma (beyond 340 µm, measured confocally with epithelium in situ), regularly populated by keratocytes, was established in vivo in humans by early postoperative scans after soft contact lens removal.[6] Gradual repopulation of the corneal stroma, starting between the second and third month after the operation, becoming complete after six months and continuing thereafter, was also demonstrated by confocal microscopy.[6] A major feature of in vivo confocal microscopy, starting between the third and sixth months after the operation, was increased density (with hyperreflectivity) of the extra-

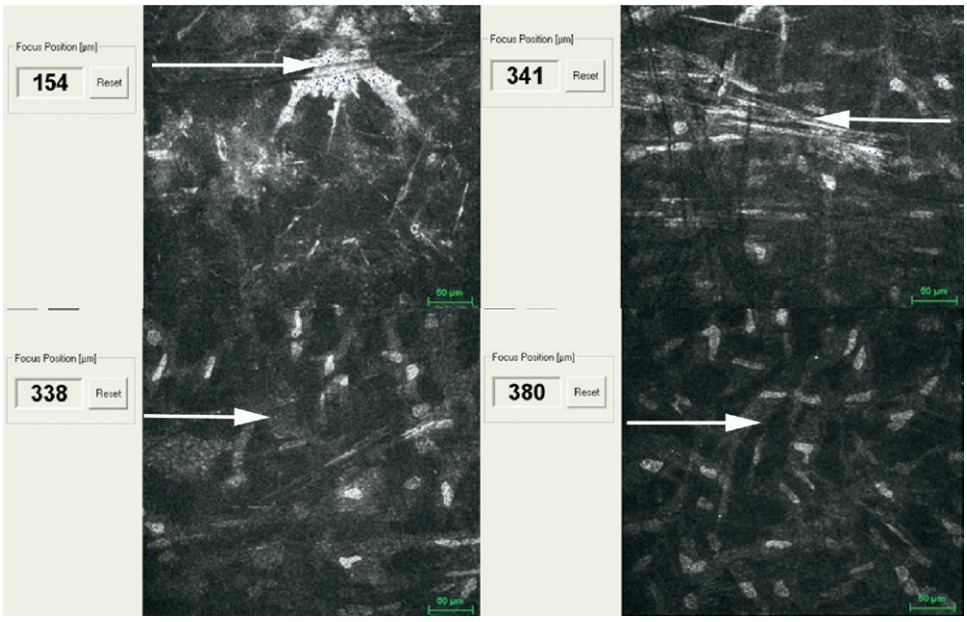

FIGURE 5. Riboflavin UV-A collagen CXL. Late "needle-shaped" hyperreflective bands or bridges in the anterior midstroma (Top left and Top right, white arrows) suggest new structured collagen and lamellar interconnection. Increased reflectivity of extracellular matrix (Bottom left, white arrow); normal reflectivity of deep stroma beyond depth of 340 µm (Bottom right, white arrow).

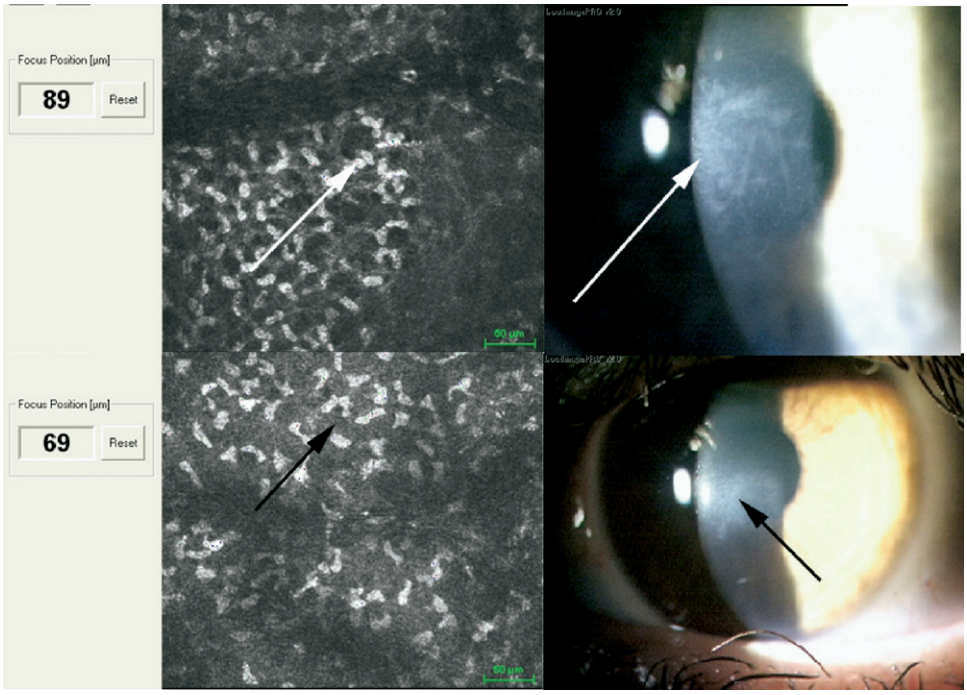

FIGURE 6. Riboflavin UV-A collagen CXL. Preoperative scans of anterior stroma to a depth of 80 µm (Top left, white arrow, and Bottom left, black arrow) showing hyperactivated hyperdense keratocyte nuclei and early post-CXL haze (Top right, white arrow, and Bottom right, black arrow).

cellular matrix combined with activated keratocyte nuclei. After six months the reflectivity (hyper) of the anterior midstroma was inverted with respect to the initial postoperative reflectivity (hypo). This feature expressed as a late demarcation line, different from the early edematous demarcation demonstrated in our[6] and other studies.[16] This aspect was maintained up to three years' follow-up as shown by confocal examination (Figure 4). Some scans two years after the operation showed "bridge-like" and "needle-shaped" hyperreflective bands (Figure 5).

Transitory corneal opacity similar to haze was detected in five of 44 cases, usually with onset in the first three months (four cases) and in one case with late onset after six months. It was characterized confocally by higher reflectivity of the stroma with undistinguished keratocyte nuclei and biomicroscopically by the presence of a central corneal opacity. Reexamination of preoperative confocal scans in this small group of patients showed that in two cases there were hyperactivated hyperdense keratocyte nuclei in the anterior stroma (Figure 6). This aspect was also noted "a posteriori" in the patient with late-onset haze. These two patients were under 20 years old. In the other three cases, confocal evidence of dark, reticular patterned microstriae and slit-lamp evidence of strong preoperative Vogt striae were established as possible causes of corneal opacity.[9]

DISCUSSION

RIBOFLAVIN UV-A CORNEAL COLLAGEN CXL IS THE ONLY "pathogenetic approach" to progressive keratoconus and post-LASIK corneal ectasia that can delay or block their progression, reducing demand for donor keratoplasty. In vivo confocal study in humans demonstrated early and late modification of corneal microstructure after corneal collagen CXL treatment. The limbal region, where the corneal epithelium joins the conjunctival epithelium, contains a radial arrangement of trabecular conjunctival processes known as palisades of Vogt. These are thought to be the site of origin of corneal stem cells.[17] In the corneal collagen CXL procedure, irradiation of the limbal region should be carefully avoided to protect the proliferative compartment of the cornea. Limbal protection is possible if the peripheral epithelium is left in situ beyond a central scraped area, 9 mm in diameter, and the whole corneal surface is covered in riboflavin 0.1% solution 10 minutes before and throughout the treatment. The epithelial ring beyond 9 mm, associated with the riboflavin solution, provides protection by absorbing 95% of the UV-A energy in a cornea 400 μm thick.[7] The lateral diffusion of UV-A irradiation during corneal collagen CXL has been found less than 20 μm.[6] The possibility of direct visual control of the UV-A spot by the micro-camera available in the CBM XL UV-A source is a good method to ensure patient fixation and avoid tilting and defocusing of radiation on the limbus.[18] In our experience, use of poly-methyl methacrylate rings of different diameters (Janach, Como, Italy) ensures absolute limbal protection in low-compliance patients who do not maintain the fixation adequately during the 30 minutes of corneal CXL treatment.

After corneal collagen CXL and four days of soft contact lens bandage, the corneal epithelium showed rapid and normal regrowth, good compensatory function, smoothing of corneal surface irregularities during progressive stratification, and visual improvement, especially appreciable after the third month postoperative. Oral amino acid supplements for a week before and after the operation improved epithelial healing as confirmed in some studies.[19,20]

In all cases, disappearance of nerve fibers, attributable to epithelial scraping in the case of the subepithelial plexus and UV-A irradiation in the case of anterior-midstromal nerves, was observed after corneal collagen CXL. Confocal analysis showed that nerve regeneration after corneal collagen CXL was more rapid than after other corneal surgical procedures, such as photorefractive keratectomy, LASIK, or penetrating keratoplasty.[7] The presence of disconnected nerves in the first 6 months and the appearance of interconnected fibers after six months suggested primary regeneration leading to partially restored corneal sensitivity and lacrimal reflex, followed by gradual interconnection of secondary nerves that improves corneal sensitivity. Follow-up confocal analysis excluded the possibility of late neurodystrophic lesions.

Regarding stromal modifications, after six months the reflectivity of the anterior midstroma was inverted (hyper) compared with initial postoperative reflectivity (hypo), demonstrated in our previous studies.[6] This characteristic was expressed as a late demarcation line different from early edematous demarcation.[6,16] Changes in stromal reflectivity recorded in all patients at follow-up after the sixth month are in our opinion an important indirect (confocal) sign of corneal CXL (Mazzotta C. I, II, III International Cross-linking Meeting, Zurich, Switzerland, 2005 to 2007). Slit-lamp evidence of changes in stromal density at postoperative follow-up was not strictly haze, but a new condition of compacted collagen lamellae of different etiopathogenetic origin expressed by stromal hyperdensity. This important finding may be attributable to new well-structured collagen produced by repopulating keratocytes. "Stiffening" of the cross-linked cornea demonstrated ex vivo by Wollensak and associates[11] is reasonably explained and sustained in vivo in humans by this confocal aspect. As well, the "needle-shaped" hyperreflective bands or bridges detected in the stroma during the healing process could be a micromorphological qualitative expression of newly replaced collagen and different lamellar interconnections created by CXL and repopulating keratocytes, as suggested by experimental biochemical, biomechanical,[4,11] and recent experimental evaluation by Wollensak and Redl[12] that reports in a gel electrophoretic analysis of cross-linked cornea samples, the presence of an additional intense polymer band in the stacking gel, compared with control corneas not cross-linked with the typical electrophoretic collagen pattern of normal cornea, that was resistant to mercaptoethanol, heat, and pepsin treatment with high molecular size. The new polymer band complies well with the collagen morphologic changes after CXL treatment. The different collagen structure observed in our in vivo confocal analysis supports the results of a major chemical and structural stability and could be a

possible explanation of the long-term effect of corneal collagen CXL.

According to the data recorded in our series of cases, the clinical stability of CXL could be related not only to CXL itself but also to biosynthesis of new well-structured collagen and more compact lamellar interconnections demonstrated by late postoperative in vivo confocal scans. The process of keratocyte-induced apoptosis, only possible if epithelium is removed before riboflavin soaking and UV-A treatment, seems to be essential for replacement of cells and collagen in the anterior midstroma, which in various studies is reported to be the main site of origin of metabolic disorders affecting keratoconic corneas, especially the anterior stroma and Bowman lamina.[21–26]

Transient corneal opacity similar to haze was the only complication in our series of cases (five of 44 patients) with early onset (first three months; four cases) and late onset (after six months; one case). The opacity only lasted 30 to 40 days and was successfully managed with topical preservative-free dexamethasone drops for four weeks. Preoperative confocal analysis in three of these patients who were under 20 years old showed hyperactivated keratocyte nuclei in the anterior stroma to a depth of 80 μm, and dark, reticular-patterned microstriae in the other two patients (over 20 years of age). The latter also had preoperative slit-lamp evidence of strong Vogt striae. These could be risk factors for corneal opacity after corneal collagen CXL.[9] Confocal microscopy can significantly contribute to the optimization of postoperative medical therapy.

Steroids are recommended in these cases but should not, in our opinion, be prolonged beyond a month to avoid inhibiting collagen synthesis.[27,28] There is experimental evidence of the impact of prednisone on collagen in diaphragm muscle of mdx mice and of steroid efficacy in preventing collagen accumulation. Steroids promote hydroxyproline CXL, presumably by decreasing collagen turnover.[29] They can therefore be considered a valid alternative therapy in the first three to four weeks after corneal collagen CXL and obviously for the management of complications.

In our opinion, keratoconus therapy based on diagnostic staging; patient age; clinical, topographic, and pachymetric progression; general condition; and compliance could benefit from corneal collagen CXL treatment to delay or block progression of ectasia. Corneal collagen CXL is primarily indicated in early stages of keratoconus and in younger patients where progression is faster; it can prevent corneal shape modifications and irregularities. Age-induced corneal CXL[30] reduces the need for this treatment in patients over 35 years of age, because of a generally spontaneous biochemical and biomechanical stability of the disease in the third to fourth decades of life, except in a few cases of contact lens intolerance or patients with low compliance, because of its positive effects in improving corneal symmetry and visual outcome.[8] Obviously, this treatment can also be applied in patients over 35 years of age if progression of ectasia is detected during follow-up. More long-term studies and clinical data are necessary to establish the efficacy and the duration of riboflavin UV-A collagen CXL, though preliminary results[3,8] and in vivo confocal evidence[6–9] speak eloquently in favor of this new approach to progressive corneal ectasia.

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