Cross-Linking of Corneal Collagen with UVA and Riboflavin for the Treatment of Corneal Disease

Farhad Hafezi, MD, PhD[1,2]

Introduction

In recent years, reduced corneal biomechanics have been identified as an important element in the pathogenesis of various corneal diseases. The biomechanical characteristics of a connective tissue such as strength and resistance against mechanical stress are indispensable prerequisites to maintain regular shape and function of that tissue. Intra- and intermolecular cross-links between collagen molecules are essential elements of these biomechanical properties.[1-3] Accordingly, collagen cross-links occur physiologically in all organs and tissues with certain biomechanical characteristics.

In other surgical fields, cross-linking has been used for decades to increase the biomechanical properties of connective tissue structures: in cardiac surgery, porcine aortic valve bioprosthesises are treated with glutaraldehyde prior to implantation to ensure increased cross-linking, which increases biomechanical resistance against biodegradation. Additionally, in otolaryngology, polymers inducing cross-linking are used in the treatment of destabilized vocal cords and for nasal reconstruction.[4-9]

In the cornea, a variety of conditions such as primary acquired (keratoconus and pellucid marginal corneal degeneration) and secondary induced (iatrogenic keratectasia after refractive laser surgery) ectatic disorders lead to a reduced biomechanical resistance. Corneal collagen cross-linking with riboflavin/UVA (CXL) represents a new approach to these diseases. To assist researchers and clinicians interested in the field, this article attempts to provide a structured overview on the current state of the method, basic principles, technique, and application of CXL in primary and iatrogenic keratectasia. Furthermore, it addresses safety issues and potential complications of the method.

Keywords: Collagen, Cross-linking, Ultraviolet A, Riboflavin, Keratoconus, Pellucid Marginal Degeneration, Iatrogenic Keratectasia

Iranian Journal of Ophthalmology 2009;21(2): 3-12 © 2009 by the Iranian Society of Ophthalmology

The principle of cross-linking and CXL in the cornea

Comparable to other connective tissues of the human body, the number of collagen cross-links in the cornea does not remain stable throughout the life, but increases physiologically with age. This increase provides a potential explanation for the fact that human ectatic corneal disorders such as keratoconus usually occur in young individuals and show an arrest with age progression.[2,3,10,11]

1. Associate Professor of Ophthalmology, University of Zurich, Switzerland
2. Institute for Refractive and Ophthalmic Surgery (IROC), Zurich, Switzerland

Received: April 4, 2009
Accepted: May 7, 2009

Correspondence to: *Farhad Hafezi, MD*
Institute for Refractive and Ophthalmic Surgery (IROC), Zurich, Switzerland, Tel: +41 43 4883800, Email: farhad@hafezi.ch

A pathological condition associated with increased cross-linking is diabetes mellitus (DM).[12-14] Seiler and colleagues have shown the protective effect of DM type II against keratoconus in a retrospective case-control study.[15] Recently, Koop and coworkers have shown that DM decreases the odds of severe keratoconus in a retrospective study.[16]

Inter- and intrafibrillar collagen cross-links are a major factor ensuring the mechanical stiffness of connective tissues. A number of techniques can be used to induce additional cross-links: exposure to aldehydes (glutaraldehyde or aldehyde sugars), enzymatic treatment (lysyloxydase), and photo-polymerization using UV light.[17] For the transparent cornea, some approaches were either too toxic (exposure to glutaraldehyde or UVA alone) or too time-consuming (ribose 0.5 molar solution for 14 days).[18,19] The most promising technique was photo-polymerization by generation of free radicals using a nontoxic and soluble photomediator. This photomediator should have the following characteristics: it should absorb ultraviolet A (UVA) light strong enough to generate free radicals that induce cross-links and should protect deeper ocular structures from the potential hazards of free radical formation. These parameters were achieved by using a 0.1% aqueous solution of riboflavin-phosphate (vitamin B_2). It provides an adequate shielding effect at a wavelength of 370 nm, and generates sufficient free radicals to induce formation of additional collagen cross-links. These cross-links are intramolecular rather than intermolecular as suggested by an increase in collagen fiber diameter by approximately 10% following CXL.[20] This increase was more pronounced in the anterior stroma.[20] Whether interfibrillar cross-links are also induced is still unclear.

To reduce uncontrolled stromal swelling, riboflavin solution was diluted in a carrier isoosmolar to the corneal stroma (dextrane 20%). Since riboflavin is a macromolecule (molecular weight of 376.37 g/mol), the corneal epithelium represents a barrier that decreases the absorption rate.[21] The corneal epithelium should therefore be removed prior to instillation of riboflavin solution. The intensity of UVA irradiation was set to 3 mW/cm^2, corresponding to a surface dose of 5.4 J/cm^2. These parameters induce corneal cross-links to a depth of 310 µm. A stromal thickness of at least 400 µm should be respected in CXL. At this depth, the irradiation intensity is two times lower than the damage threshold level.[22-24]

CXL leads to a marked increase of corneal biomechanical stability. To determine the stiffening effect, the key experiment is the stress-strain measurement performed in a micro-material tester using corneal strips.[25-28] In human cadaver corneas, Young's modulus increased by a factor of 4.5 using the standard parameters.[26]

Besides the biomechanical effect, a biochemical effect also contributes to the increased resistance of cross-linked corneas. Spoerl and colleagues have experimentally investigated the effect of enzymatic digestion on porcine corneas in a solution containing pepsin, trypsin and collagenase with and without precedent CXL. The cross-linked corneas were markedly more resistant to the proteolytic process. Changes in the tertiary structure of the collagen molecule may explain the stabilizing biochemical effect of cross-linking. These changes prevent proteolytic enzymes to access their specific cleavage sites by steric hindrance.[29]

Wollensak and coworkers studied the cytotoxicity of the riboflavin/UVA standard treatment (for parameters see below) on keratocytes and endothelial cells.[22,23,30] In rabbit corneas, keratocyte apoptosis was detected up to 300 µm depth at 24 hours following standard CXL treatment. Smaller irradiances led to shallower cell depth following Lambert-Beer's law.[30] In cell cultures established from porcine keratocytes, the damage threshold of the irradiance of UVA in combination with 0.025% riboflavin solution was determined at 0.45 mW/cm², which is 10 times lower than for UVA irradiation alone.[23] A similar experimental setup was used to measure the damage threshold for porcine endothelial cells.[22] At an irradiance of 0.3 mW/cm,² no signs of cell damage were detected; whereas, at 0.35 mW/cm², 98% of the cells stained positively for both Trypan Blue and Yopro in their nuclei. The authors concluded that when using the standard riboflavin/UVA technique, a preoperative minimal corneal thickness of 400 µm after removal of the epithelium is mandatory to avoid damage to the corneal endothelium.

The treatment protocol

Treating Corneas with more than 400 µm preoperatively (Standard Technique)

These treatment parameters are currently widely used to treat corneas thicker than 400 µm after abrasion of the epithelium.

After an abrasion of the corneal epithelium of 9 mm, isoosmolar 0.1% riboflavin solution with Dextrane T500 is applied on the cornea every three minutes for 30 minutes. Successful penetration of riboflavin through the cornea ("riboflavin shielding") is assured by visualization of riboflavin in the anterior chamber by slit lamp biomicroscopy (using blue light).

Prior to treatment, ultrasound pachymetry (five repetitive measurements) is performed on the deepithelialized cornea at the thinnest point to ensure a minimal corneal thickness of 400 µm. Thereafter, the eye is irradiated for 30 minutes with UVA at a working distance of 5 cm with an irradiance of 3 mW/cm^2 corresponding to a surface dose of 5.4 J/cm^2 (UV-X^{TM}, Peschke Meditrade, Cham, Switzerland).

During treatment, isoosmolar 0.1% riboflavin solution and topical anesthetic (oxybuprocaine 0.4%) are administered every five minutes to saturate the cornea with riboflavin. After treatment, antibiotic ointment (ofloxacine) and a bandage contact lens soaked with preservative free antibiotic (Ofloxacin 0.3%) are applied until complete healing of the corneal epithelium and followed by application of fluorometholon eye drops twice daily for 6 weeks.[31]

A slight haze, comparable to the healing reaction following corneal abrasion in photorefractive keratectomy, can be seen in the first six to eight weeks following surgery. Postoperative controls are performed daily until complete healing of the epithelium, at one, three, six and 12 months followed by yearly controls (Figure 1).

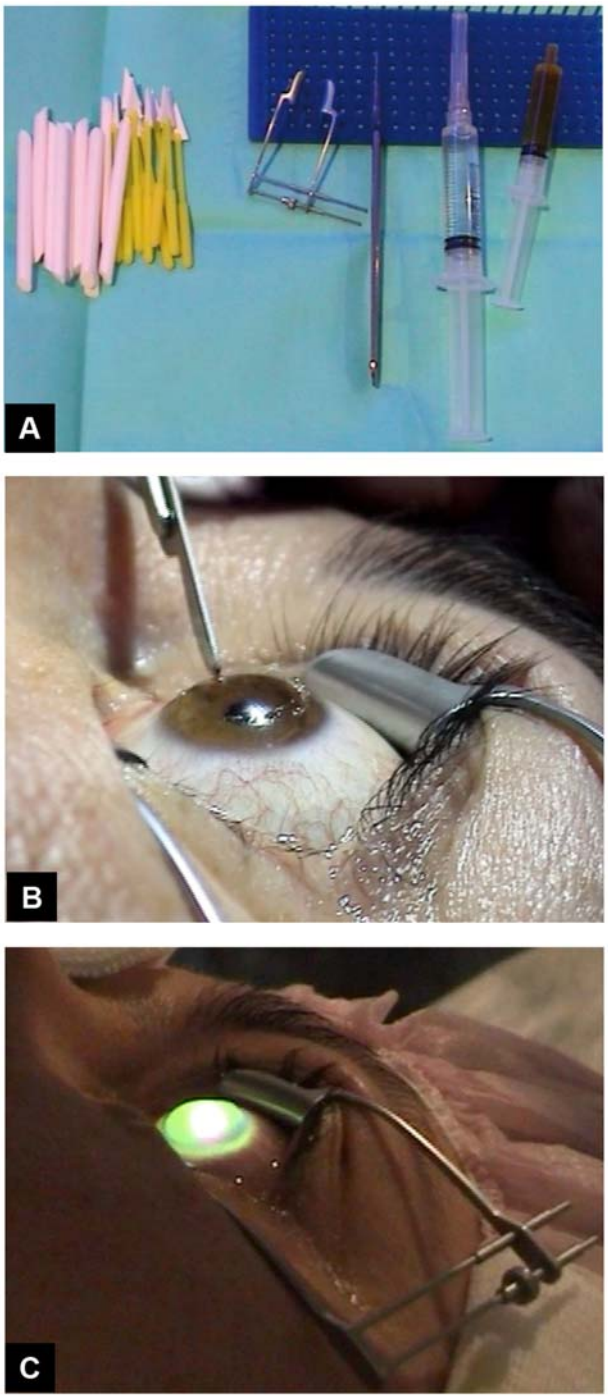

Figure 1. The CXL procedure
A: Only few surgical instruments are needed for CCL of the cornea. B: Epithelial removal over an area of 9 mm in diameter. C:, Fluorescence of the riboflavin-saturated cornea during illumination with UVA.

Treating Thin Corneas with less than 400 µm preoperatively

In many cases of advanced progressive keratectasia, patients still achieve a satisfying visual acuity with contact lenses. A low minimal stromal thickness of less than 400 µm after abrasion of the epithelium is the only parameter prohibiting safe CXL. We have therefore modified the technique using hypoosmolar riboflavin solution to induce stromal swelling, thus increasing the stromal thickness prior to CXL in cases with preoperatively thin corneas.[32]

The standard CXL technique was modified as follows: hypoosmolar 0.1% riboflavin solution is generated by diluting Vitamin B2-riboflavin-5-phosphate 0.5% (G. Streuli & Co. AG, Uznach, Switzerland) with physiological salt solution (Sodium chloride 0.9% solution, B. Braun Medical AG, Sempach, Switzerland) (310 mOsmol/l). Hypoosmolar riboflavin solution does not contain Dextrane T500. The solution is protected from light and used within two hours. After removal of the corneal epithelium and 30 minutes of instillation of isoosmolar riboflavin solution, the corneal stromal thickness was measured using ultrasound pachymetry. In cases where the remaining stromal bed was thinner than 400 µm, hypoosmolar riboflavin was applied every 20 seconds for five more minutes, and the corneal thickness was checked again. Hypoosmolar riboflavin solution was administered repeatedly until the minimal corneal thickness reached 400 µm, which usually occurred within five to 15 minutes.[32]

The absolute increase in corneal thickness that can be achieved using this modified protocol ranges between 36 to 110 µm. The technique has been used successfully with primary progressive keratoconus and iatrogenic keratectasia after refractive laser surgery; results are similar to those in patients where the standard protocol (ie, an isoosmolar solution) was used.

To remove or not to remove the epithelium

Recently, Pinelli and coworkers suggested a modification of the technique where no epithelium is removed. They claim that this modification is an enhancement of the technique for several reasons: first, the procedure is painless for the patient and second, complications of epithelial healing are avoided. However, no peer reviewed data on this suggested modification is available and several findings raise serious concerns about the "epithelium-on"-modification: Firstly, riboflavin is a macromolecule (molecular weight 376.37 g/mol), and the corneal epithelium represents a barrier that drastically decreases the absorption rate of riboflavin into the corneal stroma.[21] Secondly, Baiocchi et al have recently investigated the concentration of riboflavin in the corneal stroma using high-pressure liquid chromatography (HPLC) in ex vivo human corneas. In "epithelium-on" corneas, a limited stromal riboflavin concentration could only be measured; it was 40-fold lower than in corneas with removed epithelium.[33] Lastly, Wollensak et al have shown recently in rabbit corneas that the increase in biomechanical strength in corneas where the epithelium had not been removed is only one-fifth of that of corneas in which the epithelium had been removed prior to riboflavin instillation.[34]

Monitoring the cornea after CXL

The techniques used to monitor successful CXL are corneal topography, corneal pachymetry (ultrasound or optical) and corneal confocal microscopy. In the latter, the depth of effective treatment can be monitored due to a change in the reflectivity/refractive index of treated vs. untreated cornea. Furthermore, we have recently shown that the depth of effective treatment might even be detected biomicroscopically in slit lamp examination.[35] Figure 2 shows the stromal demarcation line at a depth of approximately 300 µm. At three and six months after surgery, corneal topography may reveal an arrest of progression of the ectatic process. Wollensak and coworkers have shown even a regression of maximal K-values by an average of 2 diopters (D) in 70% of all patients treated. Regression took up to 30 months to stabilize.[27]

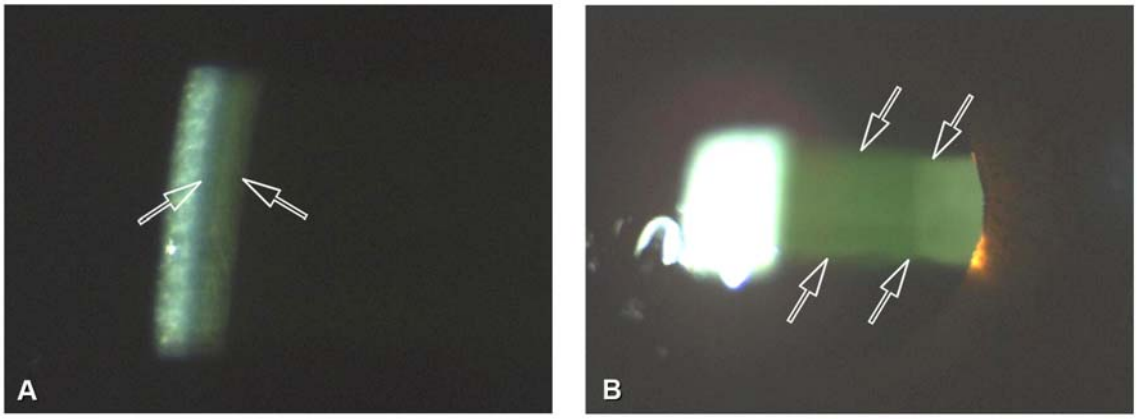

Figure 2. Riboflavin application to the cornea
A: After 30 minutes of instillation of riboflavin 0.1% drops in 20% dextrane, slit lamp inspection of the cornea reveals complete penetration of the corneal stroma by riboflavin (greenish aspect). B: Slit lamp inspection of the anterior chamber reveals a "green Tyndall" phenomenon. Riboflavin has penetrated the anterior chamber, which is an indicator of "riboflavin shielding".

Although corneal topography only indirectly measures the biomechanical changes related to CXL, it currently is the technique of choice to monitor the CXL effect on the cornea. Future detection methods may include devices that measure corneal biomechanics directly. Noguera and colleagues have recently shown a significant increase in corneal hysteresis in human cadaver eyes after CXL when compared to untreated controls.[36]

CXL in keratoconus and pellucid marginal degeneration

Between 1999 and 2002, 22 patients with progressive keratectasia were treated in a clinical phase one study and were followed for an average of two years (range three months to four years).[27] A distinction of clinical subentities such as keratoconus and pellucid marginal degeneration was not performed. The progression halted in every case, and no side effects were observed except for slight corneal edema, photophobia, and minimal intrastromal scarring in the early postoperative phase. Sixteen eyes showed a regression of the keratectasia with a reduction of the maximal K-readings by 2 D.[27] Endothelial cell counts were unaffected by the treatment. In the follow-up five-year study, 48 patients were included and again, no patient showed further progression of keratoconus. Regression was observed in 31 patients by an average of 2.87D.[37] In a follow-up long-term study, Raiskup-Wolf and colleagues analyzed 241 eyes of 272 patients with progressive keratectasia with a maximum follow-up of six years. The maximal K-readings decreased significantly by 2.68 D in the first year, 2.21 D in the second year, and 4.84 D in the third year (Figure 3). The best corrected visual acuity (BCVA) improved significantly (> or = 1 line) in 53% of 142 eyes in the first year, 57% of 66 eyes in the second year, and 58% of 33 eyes in the first year or remained stable (no lines lost) in 20%, 24%, and 29%, respectively.[38]

Mazzotta and coworkers presented a six-month follow-up after CXL for keratoconus including in vivo-confocal microscopy in 10 eyes of 10 patients.[39] Confocal microscopic analysis at one month after CXL using the standard parameters showed that the outer 270 to 350 µm of the stroma were free of keratocytes. This confirms the experimental results of Wollensak et al who detected keratocyte apoptosis up to 300 µm depth following CXL.[30] At six months after treatment, repopulation by activated keratocytes led to an even higher density than preoperative state. An increase of approximately 20% of corneal thickness was attributed to corneal edema, which gradually returned to preoperative levels at six months after treatment. Although not numerically documented, the authors did not observe a change in endothelial cell counts or appearance (morphometry) at any time after treatment.[39]

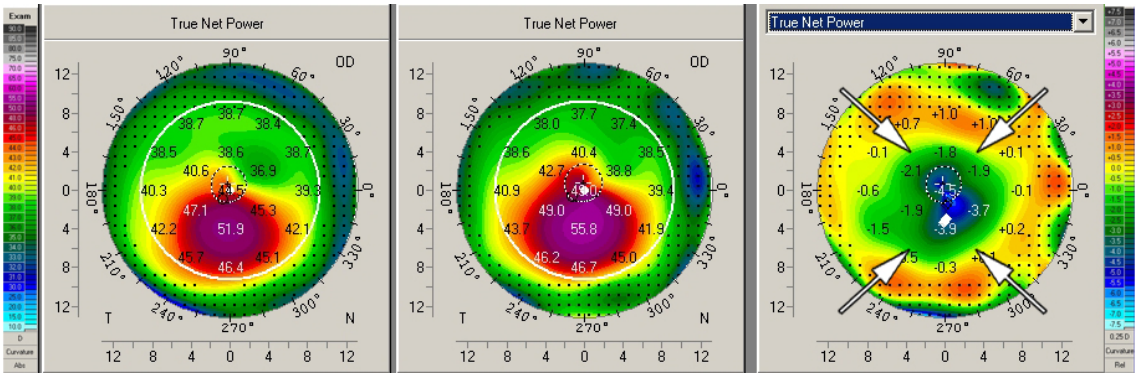

Figure 3. Regression of keratoconus after CCL. Scheimpflug true net power analysis of the anterior corneal surface preoperatively and at 6 months after CXL. The difference map shows the decrease in the maximal K-readings (arrows).

Iseli and colleagues have further investigated the regularization process at 12 months after CXL by Scheimpflug analysis. They showed that regularization is not completed at 12 months after surgery.[40] Additionally, they observed a shift in refraction towards myopia but no statistically significant increase in best spectacle-corrected visual acuity (BSCVA). This finding is in contrast to results published previously.[27,41]

CXL in iatrogenic keratectasia after refractive laser surgery

Kohlhaas and coworkers reported the first CXL in a case of iatrogenic keratectasia after LASIK.[42] The keratectasia occurred one month after LASIK, and the progression was documented for the following ten months. Within a follow-up period of 18 months after CXL, corneal topography, K-readings, and refraction were stable. No side-effects regarding corneal endothelium were reported. Hafezi and coworkers presented ten cases of CXL after iatrogenic keratectasia with a follow-up time of up to 25 months.[31] Their results showed that CXL can distinctly reverse otherwise progressive iatrogenic keratectasia after LASIK. The observed reduction of maximal K-values is probably due to the increased biomechanical stability of the cornea after cross-linking and is in line with similar findings in keratoconus patients that were treated similarly.[27] After CXL in keratoconus, Caporossi et al found a trend for a more regular cornea concomitant with an increase in BSCVA.[41] This effect, to a larger degree, was also found in CXL after iatrogenic keratectasia: four of the ten eyes gained more than two lines in BSCVA. The cause of this optical regularization process is still unknown.

CXL in corneal melting processes

The first report on clinical use of CXL in the cornea was when Schnitzler et al reported four cases of corneal melting ulcers that were resistant to other conventional therapies in 2000.[43] In three of the four cases, the melting process could be stopped, and a keratoplasty was at least temporarily avoided. The arrest of the melting process was probably due to a combination of the known biomechanical and biochemical effects of CXL as well as to the UVA irradiation that may have eliminated any potential microorganisms actively involved in the melting process.[29] The aim of CXL in these cases is to arrest the melting process and to avoid a keratoplasty à chaud. In the past two years, our group has successfully used CXL in five patients with infectious keratitis associated with corneal melting. CXL was performed when the infection did not respond to systemic and topical antibiotic therapies. Follow-up after cross-linking ranged from one to nine months. In all cases, the progression of corneal melting was halted after CXL treatment.[44]

Potential future applications of CXL

CXL of collagen may not only improve the aforementioned conditions. Additionally, a number of potential applications can be envisioned.

In 1994, McBrien and Norton have demonstrated that prevention of collagen

cross-linking increases form-deprivation myopia in an animal model of progressive myopia.[45] Wollensak et al have demonstrated successful cross-linking of scleral collagen in the rabbit by UVA irradiation and, alternatively, by using glutaraldehyde.[46,47] Once all issues regarding potential cytotoxicity to the underlying choroid and outer retina is overcome, this technique may open new experimental approaches in the treatment of progressive myopia.

In penetrating keratoplasty for keratoconus, the rate of keratoconus recurrence is increasing with time after surgery (7.1% at 20 years and 11.7% at 25 years after surgery).[48] A common hypothesis suggests that recurrence of keratoconus is the manifestation in donor tissue of the same mechanisms that caused ectasia in the host cornea. CXL prior to penetrating keratoplasty for keratoconus might be a means to prevent the recurrence of keratoconus.

A combination of Intacs and CXL might be beneficial for patients. Chan and coworkers have reported a significant reduction in postoperative cylinder in patients that were treated with Intacs and subsequent CXL when compared to Intacs implantation alone.[49]

Another potential approach using CXL is the extension of the current limits in refractive laser surgery. The lower limit of 250 µm residual corneal thickness might be overcome once ablation profiles are adjusted to the new biomechanical properties of cross-linked cornea. However, careful consideration should be taken because surface ablation can potentially weaken corneal biomechanics in exchange for better optical homogeneity. Thus, the biomechanical stability induced by CXL may be at risk again.

Quite the contrary, Kanellopoulos et al have performed a topography-guided surface ablation following CXL for keratoconus. The aim was to homogenize the irregular corneal surface. With a follow-up of 18 months, the patient showed no signs of keratoconus recurrence and corneal topography, K-readings, and refraction were stable.[50]

Ultimately, in cataract surgery, continuous curvilinear capsulorhexis (CCC) might be facilitated in selected cases. A combination of Trypan Blue as a chromophore and illumination with diffuse white light of 6000 lux intensity increases the stiffness of the anterior capsule, thus making CCC easier-to-perform.[51]

CXL safety issues and potential complications

Cross-linking of the cornea implies irradiation with UVA light and generation of free radicals. The prevention of damage to the corneal endothelium and deeper ocular structures such as the iris, the lens and the retina is mandatory. Spoerl et al have unambiguously demonstrated that the UVA intensity used during CXL is far below the damage threshold for the corneal endothelium, the iris, lens and retina (for review see[24]). The structures being at greatest risk of damage from the induced radicals are the keratocytes and the corneal endothelium. Keratocytes show apoptosis after CXL to a stromal depth of 320 µm.[39] As long as the corneal stroma shows a thickness of 400 µm and the irradiance is 3 mW/cm^2 or less, the endothelium is protected by the riboflavin concentration in the stroma (riboflavin shielding).[24]

Nevertheless, various cases with complications after CXL were reported by several groups and at the 4th CXL congress in Dresden in 2008.[52,53] Complications were either related to epithelial healing after abrasion (ie, infectious keratitis) or to variable degrees of stromal scarring-the latter dissolving after several weeks and even months of topical steroid treatment. Interestingly, only one case of endothelial damage was reported. Here, a cornea too thin to be eligible for the standard treatment protocol was nevertheless treated.

Conclusion

Cross-linking of corneal collagen is a promising approach for the treatment of various corneal disorders. In progressive ectatic corneal diseases, it reduces the need for penetrating keratoplasty. The ease-of-use and inexpensiveness make it particularly interesting for countries where penetrating keratoplasty is difficult due to donor availability and/or financial reasons. However, CXL remains an operative procedure with serious potential side effects and complications. Therefore, only surgeons with sufficient experience in the management of corneal wound healing should perform this procedure.

The question of the durability of the treatment remains an open issue. To date, no retreatments have become necessary although an estimated several thousands of patients have undergone CXL worldwide in the past six years. Additionally, the turnover of corneal collagen is very slow.[1] Therefore, to investigate on potential long term side effects and complications, prospective studies with a follow-up of at least eight to ten years will be necessary.

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