UVA-light and Riboflavin-mediated Corneal Collagen Cross-linking

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Background

Keratoconus and other corneal conditions associated with progressive stromal thinning including keratectasia after laser-assisted in situ keratomileusis (LASIK) and pellucid marginal degeneration represent therapeutic challenges. Until recently, available therapies including contact lenses, epikeratoplasty, intrastromal corneal rings, and corneal transplantation targeted, in principle, the abnormal shape of the cornea by mechanical means. The underlying molecular pathogenic mechanisms of corneal ectasia were not addressed with any of these therapies until the introduction of corneal collagen cross-linking to clinical practice in 1999.[1] This breakthrough clinical application was the result of multiple laboratory observations and discoveries made over preceding decades.

Early experiments of Cannon and Foster[2] showed that degraded normal collagen or synthesis of abnormal collagen might play a role in the pathogenesis of keratoconus. This concept was later confirmed by subsequent clinical and experimental studies. Keratoconus was characterized by progressive stromal thinning and ectasia[3] as a result of increased expression of lysosomal and proteolytic enzymes[4–7] and decreased concentration of protease inhibitors,[5,8] which result in corneal thinning and altered configuration of corneal collagen lamellae.[9,10]

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Owing to the young age of onset, typically in the second decade of life, and progressive nature, keratoconus has a negative impact on quality of life.[11] The Collaborative Longitudinal Evaluation of Keratoconus Study[12,13] found a significant decline in visual acuity within 8 years, an increase in astigmatism, subepithelial scarring, and corneal thinning in untreated keratoconus. In fact, keratoconus was the most common indication for corneal transplant in the past decades.[14]

The idea of therapeutically targeting the underlying pathogenic mechanisms of keratoconus was explored in the 1990s by Khadem et al[15] who pursued the identification of biological glues that could be activated by heat or light to increase the resistance of stromal collagen. It was discovered that the gluing effect was mediated by oxidative mechanisms associated with hydroxyl radical release. A similar mechanism related to active glycosylation of age-dependent tropocollagen was shown in aging corneas.[16] The phenomenon of collagen cross-linking after ultraviolet-A (UVA) light exposure was reported by Klingman and Gebre[17] in 1991 when the investigators observed biochemical changes in the skin of hairless mice after chronic exposure to UVA radiation. Their experiments rendered collagen highly resistant to pepsin digestion indicating increased collagen cross-linking induced by UVA.

Subsequent studies using corneal tissue showed similar effects on corneal collagen after exposure to riboflavin and UVA light.[18] In experimental studies using rabbit and porcine eyes an increase in corneal rigidity by approximately 70% in untreated versus treated corneas[19] after collagen cross-linking by combined riboflavin/UVA treatment was shown. After developing collagen cross-linking using the photosensitizer riboflavin and UVA similar to photopolymerization in polymers,[20] Wollensak et al[1] were the first to clinically induce UVA-light and riboflavin-mediated corneal collagen cross-linking (CXL) initially in a patient with non-seeing eyes in 1998 and later in a series of patients with keratoconus.

UVA Light and Riboflavin

The solar spectrum represents a band of radiation including UVC (220 to 290 nm), UVB (290 to 320 nm), and UVA (320 to 340 nm), infrared radiation, and visible light. UVC is blocked by the ozone layer in the stratosphere of the earth. UVA and UVB penetrate to the surface of the earth and are known to cause damage to ribonucleic acids. UVA radiation alone can induce corneal endothelial cell damage after a relatively high surface dose of 42.5 J/cm^2.[21,22] The typical UVA surface dose clinically used for CXL is only 5.4 J/cm^2, which, based on one study,[23] would be an estimated dose received by the cornea in 15 to 20 minutes of sun exposure during a summer day.

Animal studies showed that the threshold endothelial cytotoxic dose is 0.65 J/cm^2.[24] The currently used treatment parameters are set so that

the anterior 250 to 350 μ of corneal stroma are treated[25]; thus, preventing damage to corneal endothelium. With current treatment parameters the endothelial cytotoxic dose could only be delivered in human corneas thinner than 400 μm.[24] For patients with corneas thinner than 400 μm, a hypoosmolar riboflavin solution has traditionally been used to increase the corneal thickness.[26] The conventional hypoosmolar 0.1% riboflavin solution is prepared by diluting vitamin B_2 (riboflavin-5-phosphate, 0.5%) with physiological salt solution (sodium chloride, 0.9% solution) to achieve osmolarity of 310 mOsmol/L.[26] In the conventional isoosmolar solution vitamin B_2 (riboflavin-5-phosphate, 0.5%) is diluted in dextran T500 (20%) to achieve osmolality of 402.7 mOsmol/L.

Riboflavin plays a critical role during exposure of the cornea to UVA. Riboflavin saturated cornea increases UVA absorption in the cornea to 95%[19] compared with 32% without riboflavin[27]; therefore, enhancing the collagen cross-linking effect in the corneal stroma on one hand and reducing the exposure of the endothelium and intraocular tissues to UVA on the other. The CXL results in an increase in biomechanical rigidity (stiffening) of human corneas. It has been postulated in the literature that this could possibly be caused by an increase in the collagen fiber diameter because of intrafibrillar cross-links.[28] The cross-linking effect is strongest in the anterior 300 microns of the corneal stroma,[29] which has been previously found to play a significant role in maintaining the corneal curvature.[30] Consequently, collagen cross-linking results in corneal flattening and reduction of spherical error (SE).

The mechanism of CXL is not completely understood. Riboflavin is known to generate active oxygen species [singlet oxygen ($^1O_2$) and superoxide anion radicals (O_2^-)], which have been shown to contribute to enzyme inactivation.[31,32] However, Kato et al[33] showed that the active oxygen species do not seem to contribute to collagen cross-linking. Instead, riboflavin-sensitized photodynamic modification of collagen is responsible for collagen aggregation, which is accompanied by the loss of tyrosine and the formation of dityrosine.[33] Another study suggested that photooxidized histidine and lysine might contribute to collagen cross-linking as well.[34] Much speculation exists with regard to these mechanisms, and controversy and lacunae in understanding the science in this area remain. Further experiments are needed to better understand the mechanism of CXL.

■ Clinical Application of CXL

Keratoconus

In 2003, Wollensak et al[1] reported on the first series of patients with progressive keratoconus treated with CXL and showed that the procedure

was not only able to halt progression in all eyes, but regression was noted in 70% of the eyes. Their results showed the mean decrease in maximum K was 2.01 D and the mean decrease in refractive error was 1.14 D over a mean follow-up period of 23 months. These clinical observations were later confirmed by multiple other reports.[35–51]

Caporosi et al[38] showed mean K reduction of 2.1 D in the central corneal zone of 3.0 mm in diameter, which was associated with increase of uncorrected visual acuity (UCVA) by 3.6 lines, sphere spectacle-corrected visual acuity by 1.85 lines, and best spectacle-corrected visual acuity (BSCVA) by 1.66 lines at 6 months after the treatment and a reduction of 2.5 D in the mean spherical equivalent.

Raiskup-Wolf et al[37] reported on a large cohort of 480 eyes of 272 patients with keratoconus treated with CXL and followed for up to 6 years (mean, 26.7 mo). Their results showed a mean decrease in maximum K by 2.68 D in the first year, 2.21 D in the second year, and 4.84 D in the third year, suggesting that there is a trend toward continued improvement for at least 3 years after the treatment. One or more line of improvement in BSCVA was noted in 53%, 57%, and 58% during the first, second, and third postoperative year, respectively. The investigators also reported on 2 of their patients whose keratoconus progressed despite cross-linking and who required a repeated application of UVA/riboflavin.

Wittig-Silva et al[36] conducted a randomized controlled trial of collagen cross-linking in progressive keratoconus. Their preliminary results showed a decrease in maximum K by an average 0.74 D at 3 months, 0.92 D at 6 months, and 1.45 D at 12 months in treated eyes. In contrary, the control eyes showed gradual increase in maximum K by 1.28 D at 12 months.

Grewal et al[35] reported on 1 year follow-up data of 102 patients with progressive keratoconus. None of the patients showed progression after cross-linking. Their results showed a mean reduction of 1.43 D in spherical equivalent, reduction in keratometry readings, and improvement in BCVA. However, none of the parameters followed showed a statistical significance compared with preoperative data. The investigators also examined central corneal thickness, corneal volume, lens density, foveal thickness, and retinal nerve fiber layer, none of which showed statistically significant difference during the follow-up for up to 1 year after surgery. The investigators pointed to a tendency toward decrease in foveal thickness and cautioned that this observation should be studied further.

Coskunseven et al[40] compared the results of CXL in patients with keratoconus to their fellow untreated eyes and found a significant improvement in UCVA and BSCVA, and significant decrease in spherical equivalent and astigmatism in treated eyes during a mean follow-up period of 9 months. Similar results were reported by Vinciguerra et al[41,42] and Agrawal.[43] More convincing than Grewal

et al data, these studies showed a statistically significant improvement in UCVA, BSCVA, and keratometry readings. Corneal wavefront measurements in some studies did not seem to change significantly within the first 6 to 9 months after the procedure,[43,44] but a significant change was noted at 12 months.[42] In contrast, a significant reduction in coma can be seen earlier, at 6 months after cross-linking according to 1 report.[43]

Combining CXL with other surgical procedures to correct corneal ectasia has been explored in several clinical studies. Chan et al[45] compared clinical outcomes of 13 eyes with keratoconus treated with intrastromal corneal rings combined with transepithelial CXL (also referred to as C3-R) with 12 eyes treated with Intacs without CXL. They found that the patients treated with Intacs and C3-R had a significantly greater reduction in cylinder than the Intacs-only group. Another study made similar observations.[46] Coskunseven et al[47] compared 2 groups of patients with reversed sequence of intrastromal corneal ring implantation and CXL with the mean interval between the 2 treatments of 7 months. Their results showed a greater improvement in corrected distance visual acuity (CDVA), SE, and mean K in the group treated with intrastromal corneal ring implantation first and with subsequent CXL later. Photorefractive keratectomy (PRK) is another surgical procedure that has been combined with CXL to treat keratoconus. Kanellopoulos[48] compared a group of 127 eyes treated with topography-guided PRK followed by CXL 6 months later in a group of 198 eyes treated with both procedures on the same day and found that the same-day group had statistically significantly better improvement in vision and K readings. Kymionis et al[49] also showed some benefit of combining PRK and CXL in a smaller cohort of patients who received both procedures on the same day. An improvement in vision was reported in 1 eye with keratoconus treated with CXL and followed by topography-guided PRK 12 months later.[50] Unlike the combination of PRK or intrastromal corneal rings implantation with CXL, the effect of same-day conductive keratoplasty combined with CXL according to one study resulted in regression 3 months after the procedures.[51]

Post-LASIK Ectasia

Halting the progression of keratectasia in a patient after LASIK using CXL was initially reported by Kohlhaas et al.[52] Hafezi et al[53] reported later on a case series of 10 patients with post-LASIK ectasia treated with cross-linking. The investigators observed that progression of keratectasia was arrested in 5 patients and in the remaining 5 patients keratectasia regressed during the postoperative follow-up of up to 25 months. According to one case report, the initial improvement in SE and K values in a patient with post-LASIK keratectasia treated with Intacs, was followed by regression, which was subsequently successfully reversed using CXL.[54]

Infectious Keratitis

Laboratory studies have shown that the exposure of UVA light combined with riboflavin application kills a large variety of microorganisms.[55–60] Infact, this technology is used clinically to sterilize blood products and kill bacteria, viruses, and protozoa. Neither the UVA light nor the riboflavin alone was shown to have bactericidal effect. Several published reports examined the effect of UVA-light and riboflavin application in patients with infectious keratitis and found that the progression of ulcers was stopped and corneal re-epithelialization and resolution of the infectious process was observed.[61–63] There has been a debate about the mechanism of action of UVA-light and riboflavin application in infectious keratitis and several unifying hypotheses were proposed: (1) inhibition of proteolytic collagenase enzymes by the photochemical process; (2) strengthening of the corneal collagen by the cross-linking, which increases resistance to the infectious process; and (3) direct sterilization effect of the UVA light and the riboflavin.

Other Indications

Persistent corneal edema is another previously studied indication for CXL.[64–69] Improvement in pain, corneal transparency, and a decrease in central corneal thickness were typically seen initially, but return to preoperative levels within months suggested that the procedure might not have a long lasting effect in patients with corneal edema. Despite this limitation, it might be a suitable alternative for patients with pain symptoms and poor visual prognosis.[69] Kymionis et al[70] treated a patient with pellucid marginal degeneration with simultaneous photorefractive keratectomy and collagen cross-linking. The investigators noted a remarkable improvement of UCVA from counting fingers to 20/40 in both eyes and BCVA from 20/50 and 20/63 to 20/25 and 20/32, right and left eye, respectively. A summary of the reported indications for CXL is in Table 1.

■ Complications

Although riboflavin and UVA application is considered to be a safe and well-tolerated procedure, complications can occur (Table 2). Endothelial cytotoxicity is always a concern with any corneal surgery. Animal studies showed when a cornea less than 400-μ thick is irradiated with a standard dose of 5.4 J/cm^2 (3 mW/cm^2), the endothelial dose can reach cytotoxic levels ($\geq$0.65 J/cm^2 = 0.36 mW/cm^2) and cause significant necrosis and apoptosis of endothelial cells within 24 hours of application.[24] Therefore, caution must be exercised in patients with central corneal thickness of less than 400 μ. In these patients, application of hypotonic riboflavin to cause corneal swelling to levels above 400 μ or reduction of UVA dose has been used. One study showed that 2 mW/cm^2

Table 1. *Reported Indications for Ultraviolet-A Light and Riboflavin-Mediated Corneal Collagen Cross-linking*

Diagnosis	References
Keratoconus	35–51
Post-LASIK ectasia	52–54
Infectious keratitis	61–63
Corneal edema	64–69
Pellucid marginal degeneration	70

LASIK indicates laser-assisted in situ keratomileusis.

was the lowest dose that produced a significant mechanical stiffening effect[83] and an increase in resistance to enzymatic digestion.[18] With this low irradiance level the endothelial UVA dose is only 0.54 J/cm^2 (0.3 mW/cm^2), well below the threshold of the endothelial cytotoxic dose.

According to a study involving 117 eyes of 99 patients, the complication rate defined as loss of 2 or more Snellen lines was 2.9% and the failure rate defined as continued progression of keratectasia was 7.6%.[39] Age older than 35 years and preoperative CDVA better than 20/25 were identified as significant risk factors for complications. A preoperative maximum keratometry reading of 58.0 D or more was a significant risk factor for failure. Interestingly, the investigators also found that the procedure was more likely to fail in female patients when compared with their male counterparts. This study used the traditional protocol in which epithelial removal was performed. Sterile infiltrates were seen in 7.6% eyes and central stromal scarring was noted in 2.8% eyes. The investigators found that a preoperative maximum K reading of <58.0 D may reduce the rate of failure to less than 3%, and restricting patient age to younger than 35 years may reduce the complication rate to 1%. During UVA/riboflavin application approximately 7% of the UVA passes

Table 2. *Reported Complications of Ultraviolet-A Light and Riboflavin-mediated Corneal Collagen Cross-linking*

Complication	References
Vision significant stromal haze	71,72
Infection	
Bacterial keratitis	63,73–76
Acanthamoeba keratitis	77
Herpes simplex keratitis and uveitis	78
Sterile inflammation	
Keratitis and corneal scaring	79
Stromal infiltrates and melt	80,81
Diffuse lamellar keratitis	82

HSV indicates *Herpes simplex* virus.

the cornea.[1] In the eye UVA is absorbed primarily by the crystalline lens, which also contains riboflavin and, hence, lenticular cross-linking can potentially occur. However, to the best of our knowledge, no case of CXL-induced cataract formation has been reported in the literature over more than a decade since the first use of CXL. Systemic conditions such as pregnancy[84] and neurodermatitis[37] have been proposed to be risk factors for progression of keratectasia despite the application of CXL.

Postoperative corneal haze that does not limit vision can be seen after UVA/riboflavin cross-linking.[71] However, visually significant corneal haze, with or without stromal infiltration, has been also reported. According to one study in a series of 163 eyes, clinically significant stromal haze persisting for 1 year after cross-linking developed in 8.6% of eyes (n = 14) of 13 patients.[72] The investigators proposed that, based on their data, preoperative increased K value at corneal apex and decreased corneal thickness may possibly represent risk factors for persistent clinically significant stromal haze. Koppen et al[79] reported on 4 patients who developed multiple white stromal infiltrates and ciliary injection after cross-linking, which responded to topical and subconjunctival steroids. The infiltrates resolved with stromal scars and in some patients caused reduction of the visual acuity. Sterile infiltrates that resulted in stromal melt were observed after CXL by some investigators.[80,81] In addition, in 1 patient with post-LASIK keratectasia a diffuse lamellar keratitis developed after CXL.[82]

Infectious keratitis is a known complication associated with epithelial corneal defects and/or bandage contact lens wear, both of which are present after the traditional CXL with epithelial debridement, which is typically performed to facilitate penetration of the riboflavin into the corneal stroma. A spectrum of microorganisms causing infectious keratitis after CXL including bacteria,[63,73–76] *Acanthamoeba*[77] and *Herpes simplex* virus[78] have been reported.

■ Summary

Despite the lack of large multicenter prospective randomized trials, CXL has gradually become a favorite treatment tool and first line treatment for keratoconus and related corneal conditions. Although available data suggest that CXL administered with the currently widely adopted treatment parameters is safe, effective, and well tolerated, further improvements are likely to come in the future. One of the improvements is development of protocols that do not require epithelial removal, which will likely lead to reduction of risk for infectious keratitis and stromal scarring, and increase in patient comfort. Delivery of riboflavin into the cornea through intact epithelium[45,46,85] or through a femtosecond laser-created pocket[86] could become an alternative to currently widely accepted administration of riboflavin that requires removal of the corneal epithelium.

Reduction of procedure time will further enhance the patient comfort. A method named "flash-linking" was recently studied in porcine eyes as an alternative to traditional administration of CXL.[87] Flash-linking used a customized photoactive cross-linking agent and only 30 seconds of UVA-light exposure time, at the same power and wavelength as currently accepted treatment parameters. Flash-linking showed a corneal stiffening effect comparable to traditional cross-linking in porcine eyes. Another improvement might come from changing the riboflavin solution. Recent experiments by McCall et al[88] suggested that if riboflavin is dissolved in deuterium oxide the concentration of riboflavin could be decreased or the effect could be significantly enhanced, as deuterium oxide is known to increase the half-life of singlet oxygen species.

Ultimately, development of topical medications capable of inducing CXL that could be self-administered would further revolutionize treatment of keratoconus and related conditions. Several drugs including genipin,[89] glutaraldehyde,[90] glyceraldehydes,[91] and aliphatic β-nitro alcohols[92] were identified, but further investigations are needed before any of these compounds could be introduced to clinical practice.

In refractive surgery, which is considered elective and required to have the highest standards for safety, a complication is defined as a loss in CDVA of 2 or more Snellen lines[93] within 6 to 12 months postoperatively. A refractive surgical procedure is deemed to be safe if a complication rate is lower than 5%.[94] Although the exact rate of complications after CXL has not been established in larger multicenter clinical trials, the reported success rates suggest that the procedure should invariably be considered at the very least for patients with progressive keratectasia elective, as, at present, it is the only treatment shown to have the ability of halting or reversing the natural course of this condition.[39] A good argument could also be made that, considering the risk of no treatment and subsequent progression of keratoconus, CXL should be considered in any patient diagnosed with forme fruste or clinically significant keratoconus or similar corneal ectatic condition.

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