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INVITED REVIEW

# Corneal cross-linking – a review

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## Abstract

*Purpose:* To review cross-linking the cornea using riboflavin and ultraviolet A light, which has been widely adopted, refined and applied in a range of corneal surgeries and pathologies where the strength of the cornea might be compromised.
*Recent findings:* A large number of clinical trials have been carried out, most of which have demonstrated that standard cross-linking is a successful method to halt the progression of keratoconus or even aid regression.
*Summary:* This review describes our current understanding of the technique, focussing on how cross-linking works, how the treatment is being optimised, the clinical results that have been reported to date and the potential use of the therapy in the treatment of other corneal disorders.

## Introduction

The potential of ultraviolet-A light (UVA) to cross-link tissues in the presence of the non-toxic photosensitising agent riboflavin had been known for some time, but it was not until 1998 that a group from Dresden suggested it as a potential therapeutic treatment to strengthen the corneal stroma. The concept was based on the observation that naturally occurring protein cross-linking, which accelerates with age, strengthens and stiffens the cornea. This suggested that artificial cross-linking may have a similar effect, particularly in conditions such as keratoconus, where the constituent collagen is prone to enzymatic degradation and fibrillar slippage. This review discusses the development of corneal cross-linking (commonly referred to as CXL) with riboflavin and UVA, the basic scientific principles behind the technique and its success as a treatment option for keratoconus and other corneal disorders. It also explores issues of safety, side-effects and long-term prognosis to provide Ophthalmologists and Optometrists with the necessary information to advise patients on possible treatment options and eligibility for cross-linking.

## Mechanism of cross-linking

There is considerable experimental evidence supporting the creation of cross-link formation following CXL: increased stiffness,[1] increased resistance to proteolytic enzymes such as collagenase,[2] reduced corneal permeability[3] and formation of large collagen molecular aggregates when examined by SDS electrophoresis.[4] The chemical process is believed to start with the excitation of riboflavin into its excited singlet and triplet states. Two mechanisms are then possible, one of which (Type I) is favoured at low oxygen concentrations producing radicals or radical ions, and the second (Type II) in which excited riboflavin reacts with oxygen to produce singlet molecular oxygen ($^1O_2$).[5] Under aerobic conditions, which occur during the initial 15 seconds exposure to UVA, sensitised photo-oxidation of stromal proteins occurs mainly by its reaction with reactive oxygen species such as ($^1O_2$) – a Type II reaction.[6] After this brief phase, oxygen is depleted and the reaction between riboflavin and proteins is predominantly Type I. The reactive species can then, in principle, induce covalent cross-linking of many different molecules including, in the corneal stroma, collagens, proteoglycans (extracellular matrix molecules consisting of a protein core to which are attached sulphated glycosaminoglycans), DNA and RNA. Lesions in nucleic acids are cytotoxic and lead to apoptosis of keratocytes and, unless precautions are taken, also to endothelial cells. The riboflavin is crucial to the process – applied to the anterior stroma it induces the cross-links, while at the same time absorbing the ultraviolet radiation and thus preventing damage to the posterior layers of the cornea.[5,7]

At present, it is still not known exactly what the nature of the cross-links is, and precisely where they occur within

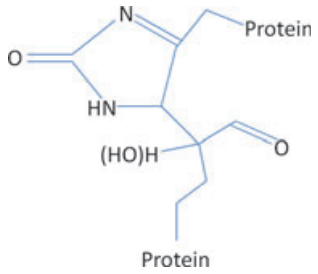


**Figure 1.** Example of a likely Advanced Glycation Endproduct (AGE) cross-link formed following CXL.[8] The size of the bond limits the inter-protein distances that can be cross-linked.

the extracellular matrix. Carbonyl and free amine groups are commonly involved in cross-linking processes. A careful study by McCall *et al.*[8] showed that, following CXL, carbonyl-based cross-links dominate in the cornea, with relatively little cross-linking of free amine groups. It appears that the carbonyl-dependent cross-linking involves the formation of advanced glycation endproducts, similar to those that result from non-enzymatic glycosylation.[9] *Figure 1* shows one such cross-link that may occur. This type of cross-link could involve amino acids such as histidine, hydroxyproline, hydroxylysine, tyrosine, and threonine,[8] but the exact amino acids involved in cross-linking, and their molecular locations, remain to be determined.

The constituents of the cornea involved in cross-linking are also unknown. Theoretically cross-linking could occur not just between collagen molecules but also between collagens and proteoglycan core proteins. Zhang *et al.*[10] have studied interactions between the various constituents of the extracellular matrix, both in isolation and within the tissue. Their results are summarised in *Table 1*. It was evident that collagen could not only cross-link with itself but also with two proteoglycan core proteins – mimecan and decorin. The core proteins could cross-link to themselves but the attached sulphated glycosaminoglycans (keratan sulphate and chondroitin sulphate) were not involved in cross-linking. Interestingly, after cross-linking, decorin appeared to form distinct dimers rather than large aggregates like the other proteoglycan core proteins.

Swelling studies have also shed light on the location of cross-links. In an *in vitro* study, Wollensak *et al.*[11] demonstrated that cross-linked pig corneas placed in a humidity chamber swell less than untreated corneas. However, the deturgescent agent dextran is normally included to limit swelling caused by the riboflavin, and it was not clear whether the altered swelling properties were caused by the presence of dextran within the cross-linked tissue or whether it was due to the cross-links themselves. In a more recent study in which corneal buttons were allowed to swell freely in saline solution (and consequently leach proteoglycans and riboflavin solution from the tissue), we found no difference (*Figure 2*) in the swelling rate of CXL treated, riboflavin-only treated, or untreated corneas,[12] suggesting the absence of significant collagen-proteoglycan cross-linking.

Hayes *et al.*[12] also showed that CXL does not increase the bulk separation between adjacent collagen molecules within fibrils, as would be expected if cross-links such as the one shown in *Figure 1* were to occur throughout the fibril. This, together with their swelling results, led the authors to conclude that cross-linking predominates within and between molecules on the fibril surfaces, and within proteoglycan core proteins in the interfibrillar space.[12] If the latter is the case, it may be that the term "collagen cross-linking", so often used to describe CXL, is in fact an incomplete description of the mechanism.

## Effect of treatment on the cornea: biomechanics

CXL significantly increases corneal rigidity immediately after treatment, with an 80% increase of Young's modulus in pigs and a 450% increase in the thinner human cornea at 6% strain.[13] Longer term *in vitro* studies in rabbits have confirmed that the stiffening effect persists at eight months after treatment.[1] Later reports have demonstrated that the stiffening is depth-dependent, being confined mostly to the anterior 200 μm or so of the cornea.[14–16] In fact, 70% of the incident UVA is absorbed within the anterior 200 μm and 90% within the anterior 400 μm.[14]

The ocular response analyser provides two *in vivo* measures of corneal biomechanical properties, corneal hysteresis

**Table 1.** Cross-linking that occurs (Y) and does not occur (N) between corneal stromal macromolecules (based on the results of Zhang *et al.*[10])

| Molecule | Collagen | Keratocan | Lumican | Mimecan | Decorin | Keratan Sulphate | Chondroitin Sulphate |
|---|---|---|---|---|---|---|---|
| Collagen | Y | N | N | Y | Y | N | N |
| Keratocan | N | Y | – | – | – | – | – |
| Lumican | N | – | Y | – | – | – | – |
| Mimecan | Y | – | – | Y | | – | – |
| Decorin | Y | – | – | – | Y – dimer | | |
| Keratan Sulphate | N | – | – | – | – | N | N |
| Chondroitin Sulphate | N | – | – | – | | N | N |

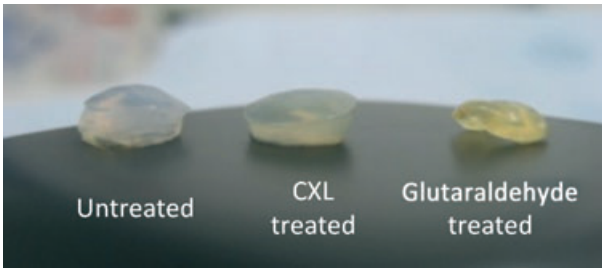


**Figure 2.** Untreated (left), standard CXL treated (middle) and 2.5% glutaraldehyde treated (right) corneal buttons shown following immersion in saline solution for 24 h. The hydration of the untreated and CXL treated corneas increased from H = 5.5 to H = 14 whilst the glutaraldehyde -treated cornea increased from H = 5 to H = 6. The cross-links formed by glutaraldehyde fixation restrict tissue swelling *in vitro* whereas those formed by CXL do not; this is likely due to difference in the nature and location of the cross-links.

(a measure of viscous damping) and corneal resistance factor (related to the viscoelastic resistance of the cornea to deformation). These parameters have lower values in keratoconus patients and appear to be unaltered after CXL.[17] However, corneal hysteresis is not correlated with Young's modulus and the ocular response analyser only measures the viscoelastic properties in a sagittal direction using an air-puff system whereas stress/strain measurements are made in the tangential direction. In fact, this has been confirmed by Wollensak *et al.*[18] who showed that collagen cross-linking does not change the interlamellar cohesion force thus allowing an interlamellar sliding movement[19] that is not affected by cross-linking. This study also shed light on the mechanism of cross-linking, showing that it probably does not halt keratoconus progression by preventing lamellar slippage.

## Effect of treatment on the cornea: structure

The effects of CXL on the various structures within the corneal stroma have been studied by a number of imaging techniques, both *in vivo* and *ex vivo*. Immunofluorescence confocal microscopy revealed a highly organised anterior fluorescence zone with a compaction of the collagen bundles following CXL.[20] Transmission electron microscope studies showed that there was a 12% increase in the constituent collagen fibril diameters within this anterior region, providing direct evidence that the collagen fibrils themselves were involved in the cross-linking process.[21] This was supported by enzyme digestion experiments that showed that CXL confers the collagen with markedly increased resistance to pepsin, trypsin and collagenase.[2] However, x-ray scattering studies failed to support the finding of increased fibril diameters in cross-linked corneas.[12] It is hypothesised that cross-linked corneas may appear to have relatively larger fibril diameters than untreated tissue when viewed by electron microscopy as the newly formed cross-links may provide greater resistance to the tissue shrinkage that is known to occur during tissue processing for electron microscopy.

Cross-linking has also been imaged using non-linear microscopy.[22,23] In an *in vivo* rabbit study, two-photon microscopy was employed to visualise and quantify the collagen cross-linking following CXL by means of collagen's intrinsic autofluorescence; a strong autofluorescence signal was generated from cross-linked collagen that allowed the cross-linked region to be clearly demarcated from the uncross-linked region.[23] It has since been shown in human corneas that the boundary between cross-linked and uncross-linked tissue occurs at a stromal depth of about 300 μm from the anterior surface in epithelium-debrided cross-linked corneas.[24] In the case of epithelium-intact treated corneas the cross-linked region is limited to the anterior 90–110 μm of the tissue.[25]

UVA treatment is known to be associated with cytotoxicity. The original studies on the effects that irradiation has on stromal keratocytes used cell cultures treated with 0.025% riboflavin solution and a range of UVA irradiances. An abrupt cytotoxic level occurred at 0.5mW $cm^{-2}$, which was 10-fold lower than when riboflavin was omitted.[26] Using the standard irradiance methods, this cytotoxic level was expected to be reached down to a stromal depth of 300 μm. This was confirmed by examination of enucleated rabbit corneas, removed 24 h after standard CXL, which revealed complete depletion of keratocytes down to a depth of 300 μm.[27] This leads to several questions – when CXL is carried out in humans is the cornea repopulated by activated keratocytes and if so, how long does the re-population take, and is fibrotic connective tissue laid down by the keratocytes during the process? To address some of these questions, a second phase prospective non-randomised study was carried out in 10 keratoconus patients treated with CXL. *In vivo* confocal microscopy showed a loss of keratocytes in the anterior and mid-stroma immediately after treatment. After 3 months, keratocytes had repopulated the exposed area and the initial oedema disappeared. At 6 months, keratocyte repopulation was complete, accompanied by an increased density of collagen fibres.[28] However, it is known that the collagen, proteoglycans and keratocytes in keratoconus are abnormal[29–31] and there still remains no ultrastructural study of precisely what these (presumably keratoconic) migrating cells are doing in terms of collagen and proteoglycan deposition when they repopulate the stroma, and therefore to what extent a "normal" stromal ultrastructure is being attained, if at all.

## Safety

A major concern when irradiating the cornea with UVA is the safety aspects associated with endothelial cell damage and corneal sensitivity if nerves are injured. This aspect has been

comprehensively covered in a separate review[32] so will only be briefly discussed here. *In vitro* cell culture studies have been carried out by the Dresden group on rabbits[33] and pigs.[34] Apoptosis was detected histologically using either TUNEL[33] or trypan blue/Yopro staining.[34] In both cases an abrupt endothelial cytoxicity occurred for 370 nm wavelength at an irradiance level close to 0.36 $mWcm^{-2}$. To protect the endothelial cells therefore requires precise knowledge of how much radiation penetrates the stroma, and that in turn requires careful measurement of the absorption coefficient and the effects of riboflavin. This parameter has been measured in human donor corneas with and without riboflavin. The riboflavin led to a 50% increase in absorbance after 30 minutes of riboflavin treatment,[35] with an absorbance coefficient of $56.36 \pm 4.80\ cm^{-1}$ although other workers have found a significantly lower value[36] which may be a cause for concern. This level of absorbance has been calculated to yield a UVA irradiance at a depth of 400 μm of 0.18 $mWcm^{-2,}$ which is less than half the toxic level[32] and for this reason, the maximum thickness of the cornea that can be treated by the standard method was set at 400 μm. The very small amount of riboflavin and UVA that penetrate the cornea is thought not to affect the aqueous, which in any case contains high levels of ascorbate, a free radical scavenger.[32]

Another cause for concern is the possibility that the corneal limbus, in which the epithelial stem cells are located, may be damaged during CXL. A prospective non-randomised clinical trial found no damage to the limbus[34] but an *in vitro* study showed cytotoxicity and reduced cell expansion of human limbal epithelial cells[37] following riboflavin/UVA exposure. Therefore, as an added protection it is advised that polymethacrylate rings or other forms of masking should be used to ensure absolute limbal protection, particularly in low-compliance patients who cannot maintain fixation adequately during the 30 min CXL procedure.[34]

Corneal nerves are damaged during CXL mostly as a consequence of the epithelial removal process. Immediately after CXL the subepithelial plexus and anterior/mid-stromal nerve fibres disappear. In humans and rabbits, regeneration of nerve fibres is complete after about 6 months[34,38] and plexus structure after 1 year.[34] Corneal sensitivity recovers quickly and is completely normal six months[39] to one year after treatment.[38,40]

## Patient selection for CXL

Although CXL is not recommended in patients with a corneal thickness of less than <400 μm (due to the risk of endothelial damage), in some cases, a hypotonic riboflavin solution may be used to increase the pre-operative stromal thickness of thin keratoconus corneas to 400 μm and allow CXL to be performed.[41] Another postulated contraindication for CXL is a history of incisional refractive surgery (such as radial keratotomy or astigmatic keratotomy), as it has been suggested that post-CXL alterations within the corneal stroma might cause the keratotomy incisions to rupture.[42] However, evidence for this complication is uncertain and indeed recent reports have indicated that CXL may have a role in actually halting ectasia induced by radial keratotomy.[43] It has been suggested that CXL is unlikely to benefit patients with central corneal opacities and associated poor best-corrected visual acuity and so other treatment options (e.g. graft surgery) should be considered for such cases.[42] Severe dry eye is a further contraindication as it may hinder re-epithelialisation and thereby increase the risk of post-surgery infection[32,42]; such conditions should be managed with punctual plugging and lubricants before considering CXL.[32,42] This is also true of patients with conjunctival vernal disease, where cases of sterile keratitis have been reported.[44] In such eyes, pre-operative management with topical steroids and even systemic immune-suppressives should be instigated to ensure any conjunctival atopic disease is in full remission before considering CXL. Similarly, corneal melting in eyes after CXL with herpes simplex keratitis have been reported and therefore caution must be advised in such eyes, where systemic prophylaxis with anti-herpetic medication is probably a sensible precaution.[45] It has also been noted that CXL treatment of patients with a preoperative keratometry reading of >58 D presents a greater risk of continued keratoconus progression[46] and permanent postoperative stromal haze.[47] Additionally, patients over the age of 35 years old with a preoperative corrected distance visual acuity of better than 20/25 have a higher risk of complications (loss of two or more Snellen lines) than younger patients.[46] On the basis of these findings it has been predicted that by restricting treatment eligibility criteria to include only those under the age of 35 years with a maximum keratometry reading of less than 58 D the frequency of complications and failures may be reduced to less than 1%.[46] As keratoconus progression is more frequent and faster in patients under the age of 18 years than in older patients and has a higher probability of culminating in the need for corneal transplantation,[48,49] Caporossi *et al.*[50] have recommend that standard CXL be the first choice therapy for progressive keratoconus in patients under 26 years of age, provided they meet with all other safety requirements for the treatment. At present it is felt that the treatment of pregnant and nursing mothers and patients with systemic collagen diseases should be delayed until sufficient investigations into the safety of the treatment in these populations has been carried out.[42]

## Standard procedure

The standard procedure suggested for clinical use involves anaesthetising the eye (for example with proxymetacainhy-

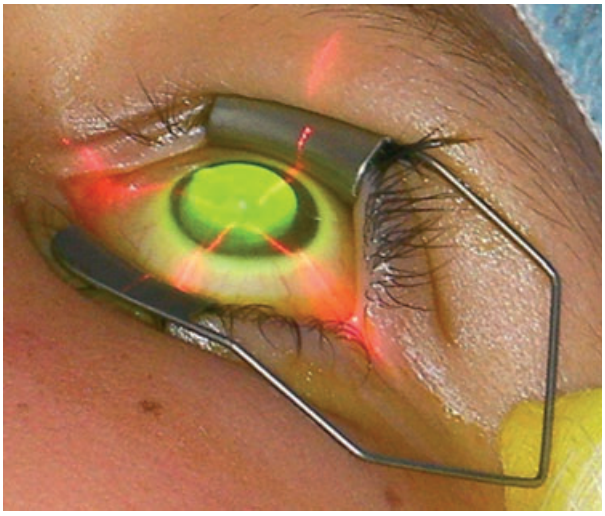

**Figure 3.** Standard CXL involves exposing the epithelium-free central cornea (pre-soaked with riboflavin) with UVA light for 30 minutes, with the addition of more riboflavin every 5 minutes. Image courtesy of Dr. Peter Hersh, Hersh Vision Group.

drochloride 0.5% drops) under sterile conditions and then removing the central 7–9 mm of the epithelium. A riboflavin solution (0.01% riboflavin-5-phosphate and 20% dextran T-500) is then applied to the corneal surface every 5 min for 30 min before irradiation and at 5 min intervals during the course of a 30 min exposure to 370 nm UVA radiation, calibrated prior to surgery with a UV light meter at 3 $mWcm^{-2}$ (*Figure 3*). A wavelength of 370 nm was chosen as this corresponds to the absorption peak for riboflavin and an irradiance of 3 $mWcm^{-2}$ was selected to avoid potential UV overdose.[5,51] The purpose of removing the epithelium is to allow penetration of riboflavin (MW 456) which would otherwise be prohibited by the epithelial cells' tight junctions.[52] After treatment, antibiotic eye drops are applied and a therapeutic soft contact lens with good oxygen transmissibility may be placed upon the eye to decrease pain without decreasing the quality of the regrowing epithelium.[42] Application of topical antibiotics is required for 1 week after the operation and mild steroids may also be prescribed. Patients are usually pain-free within 5–7 days when the contact lens is removed.[42] Patients are typically reviewed at day 1 and 5 and again at months 1, 6 and 12 post-surgery.[53]

## Clinical trials

A large number of clinical trials have been carried out, nearly all of which have demonstrated that standard CXL is a successful method to halt the progression of keratoconus or even aid regression. The results of several of these investigations are summarised in *Table 2*. Widely accepted parameters for evaluating the clinical outcome of refractive corrections and CXL include uncorrected visual acuity and corrected visual acuity. Uncorrected visual acuity is usually measured from a distance chart without the use of contact lenses or spectacles, representing the habitual vision status of the eye. Corrected visual acuity is also measured on a distance chart, referring to the best available vision, and depending upon the context, may represent the use of contact lenses, spectacles, or both. In the last case this is described as best corrected visual acuity. However, it should be noted that visual acuity of any kind is a highly subjective measure in a keratoconic subject.[54] Classically, keratoconus induces significantly large magnitudes of irregular astigmatism, higher-order aberrations and some forward light-scattering (even for keratoconic eyes without apical stromal scarring, such as those undergoing CXL)[55] which are each partly responsible for the poor and more often than not variable best corrected visual acuity achieved for patients with this disease. Consequently, keratoconic patients suffer from substantial glare in addition to refractive error. Keratoconic patients also demonstrate increased irregular cylinder[56] and increased higher order aberrations[57] compared to normal eyes. Variations in visual acuity results measured in keratoconic eyes is likely to be due to the large variability in the measurement of high cylinder powers[58] and to the variability in higher order aberrations (for example, due to changes in fixational saccadic eye movements[59] and variations in the pre-corneal tear film between blinks or changes with increasing accommodation, as demonstrated by Radhakrishnan *et al.*[57]).

Although a lesser measure of visual function, topographical information may be viewed as a more objective way of assessing the outcome of treatment. Keratometry measures the power of the principle meridians of the cornea in dioptres (D). This provides two figures in an astigmatic cornea, Kmax which represents the steeper meridian and Kmin the flattest. Kmax is used as a measure to assess the severity of keratoconus and a decrease or absence of change in Kmax demonstrates cone flattening or stability, respectively. This parameter may be measured manually using a keratometer, automatically using an autokeratometer that may also measure refraction, or 'simulated K's' may be derived from topographical (corneal topography) information of the whole cornea. As the purpose of CXL is to halt the ectasia associated with keratoconus, Kmax is the parameter consistently measured to assess the effectiveness of the treatment. Stability or reduction in Kmax has therefore been the measure used to assess the percentage of patients for whom CXL had been an effective treatment (*Table 2*).

The outcome parameters chosen for inclusion in *Table 2* are therefore uncorrected and corrected visual acuity, improvement in keratometry (Kmax) (although it should be noted that different techniques have been used to

**Table 2.** Results from published clinical trials using the standard CXL procedure

| Name | Maximum follow-up time | No. of treated eyes at start/end of study | Mean age (yr) | % Halted or (improved) assessed by Kmax change | Mean post-operative reduction in Kmax value at end of study | Mean increase in uncorrected visual acuity at end of study | Mean increase in corrected visual acuity at end of study |
|---|---|---|---|---|---|---|---|
| Wollensak *et al.*[51] | 4 years | 23/2 | 31.7 | 95.5 (70) | 2.01 D | ? | 1.26 lines |
| Caporossi *et al.*[61] | 3 months | 10/10 | 31.4 | ? | 1.9 D | 3.6 lines | 1.66 lines |
| Raiskup-Wolf *et al.*[60] | 6 years | 241/5 | 30.04 | 81 (57) | 2.44 D | ? | −0.18 LogMAR |
| Jankov *et al.*[139] | 6 months | 25/25 | 28 | 100 (52) | 2.14 D | -0.11 LogMAR | 0 |
| Wittig-Silva *et al.*[140] | 12 months | 33/9 | 26.9 | (>50) | 1.45 D | ? | −0.12 LogMAR |
| Vinciguerra *et al.*[141] | 2 years | 28/28 | Age range 24–52 | ? | 1.35 D | -0.24 LogMAR | -0.15 LogMAR |
| Agrawal[142] | 1 year | 37/37 | 16.9 | 92 (54) | 2.47 D | ? | >1line |
| Coskunseven *et al.*[62] | 1 year | 19/19 | 22 | ? | 1.57 D | -0.06 LogMAR | −0.10 LogMAR |
| Koller *et al.*[46] | 1 year | 117/105 | ? | 92.4 (37.1) | ? | ? | ? |
| El-Raggal[143] | 6 months | 15/15 | 26.4 | ? | 1.63 D | −0.04 LogMAR | −0.02 LogMAR |
| Koller *et al.*[144] | 1 year | 192/155 | 29.3 | 98 (37.7) | 0.89 D | ? | −0.55 LogMAR |
| Derakhshan *et al.*[63] | 6 months | 31/31 | 22.3 | 90.3 (77) | 0.65 D | 2.0 lines | 1.7 lines |
| Asri[145] | 1 year | 142/64 | 24.12 | 90.2 (21.3) | 0.49 D | 0 | −0.01 LogMAR |
| Hersh *et al.*[146] | 1 year | 49/49 | ? | 89.8 (51.0) | 2.0 D | −0.05 LogMAR | −0.14 LogMAR |
| O'Brart *et al.*[147] | 18 months | 24/22 | 29.6 | 100 (23) | ? | 0.07 Snellen decimal equivalent | −0.1 Snellen decimal equivalent |
| Guber *et al.*[66] | 1 year | 33/33 | 26.36 | ? | 0.16 D | ? | −0.042 LogMAR |
| Viswanathan and Males[148] | 4 years | 51/? | 24.25 | ? | 0.96 D | ? | −0.05 LogMAR |

measure this parameter), and the percentage of patients seeing stability or regression. For consistency, *Table 2* shows the maximum duration of each trial and the number of eyes examined at the start and end of the trial – it should be noted that in most trials, fewer eyes were examined towards the latter stages due to drop out of subjects. Due to variation in the literature, changes in uncorrected visual acuity and best corrected visual acuity are sometimes reported in lines and sometimes in LogMAR.

The first published clinical trial was carried out by Wollensak *et al.*[51] and showed that CXL was effective in halting the progression of keratoconus. Further trials have confirmed that Kmax may be reduced by 2 D or more, with modest increases in visual acuity (*Table 2*). Raiskup-Wolf *et al.*,[60] used a much larger cohort and confirmed the general conclusions of the earlier work regarding the efficacy of the technique in halting progression, and subsequent trials continue to support these findings. All trials have indicated a time-dependence of the effects of CXL, both in terms of transient haze and oedema in the early stages, as well as in refractive outcome, which seems to improve over the first year or more following treatment. Long-term comparative analysis showed that functional results after CXL among paediatric and young patients (up to 26 years) were better than in patients over 27 years.[50]

The majority of the studies listed in *Table 2* showed no significant changes in intraocular pressure, where this was measured, or in endothelial cell density. There is some disagreement as to the effects of the treatment on corneal thickness. Some authors reported no long-term change,[46,61,62] whereas Raiskup-Wolf *et al.*,[60] showed a small reduction of $21 \pm 31$ μm. On the other hand, Derakhshan *et al.*[63] reported a small but significant average increase of 9.1 μm. A careful evaluation of corneal thickness by Greenstein *et al.*[64] showed that there is an initial thinning of the cornea which then recovers towards baseline.

The reduction in Kmax noted in most studies, indicated that in many patients CXL leads to regression of the symptoms of keratoconus by flattening the cornea. The causes of

this flattening are as yet unknown. Vinciguerra *et al.*[65] concluded that the refractive outcomes were achieved by a simultaneous flattening of the cone apex and a steepening of the part of the cornea symmetrically opposite the cone. It has been suggested that flattening results from the contractive properties of the keratocytes as they migrate to repopulate the wound.[66] There may also be some rearrangement of the collagen and the surrounding matrix brought about by cross-linking.[67] Tu *et al.*[68] explained the effect by considering the stiffening/shortening effect of collagen fibrils on a non-central cone, claiming that CXL would tend to pull the cone towards the corneal centre, thus leading to a flattening effect. This raises the interesting question of whether or not the effects of CXL would depend on the position of the cone. Finite element modelling does indeed suggest that this is the case and that the topographic effects of CXL may be greatest if treatment is centred on the cone.[69]

Recently, initial results have been presented indicating that CXL is also effective in treating recurrent keratoconus.[70] Three cases were examined and in all cases, Kmax and best corrected visual acuity were stabilised, suggesting that CXL can arrest the progression of recurrent keratoconus after penetrating keratoplasty.

## Side effects

In addition to the pain and potential visual loss caused by epithelial removal in the first few post-operative days,[71] several other potential complications of CXL have been reported, some temporary and some not. It is estimated that re-epithelialisation requires at least four days for completion and up to three months for qualitative improvement of the epithelial cell mosaic compared with the pre-operative state.[34] Stromal haze typically develops during the first few weeks or months after surgery which can result in transient deterioration of an already compromised visual performance.[34] Haze has been reported to be greatest at one month, to plateau at 3 months, then to significantly decrease between 3 and 12 months.[72] This haze has a distinctive spatial profile; at one month is was noted to be more pronounced in the superficial stroma, gradually diminishing to zero at 240 μm, and more pronounced in the centre than 1–3 mm from the centre.[73] This is in accordance with confocal microscope observations of keratocyte apoptosis and repopulation. At 6 months a second region of light scatter appeared between 240 μm and 340 μm corresponding to the "demarcation line" which Seiler and Hafezi[24] have suggested results from some difference in refractive index or reflectivity between the cross-linked and the deeper uncross-linked regions. Permanent corneal haze (leading to a loss of two or more lines of corrected visual acuity) has been shown to occur in approximately 8.6% of all treated eyes.[47]

Because CXL involves de-epithelialisation followed usually by the application of a bandage contact lens, there is always the risk of infection. There have been several case studies reporting the development of keratitis.[74–77] Another case report described sterile keratitis as a result of pre-existing vernal keratoconjunctivitis,[44] emphasising the importance of careful selection of patients with other pre-existing conditions, whether these are being treated or not. Corneal melting has also been reported, often associated with infections.[78,79] However, there are other reports of melting and perforation that do not appear to have a clear explanation[80,81] and this suggests that there may still be unresolved issues regarding the safety of the technique or the way it is performed.

Reports of other side effects of the treatment are sporadic. There have been accounts of irreversible endothelial damage, even when CXL was apparently carried out appropriately, which have resulted in the need for penetrating keratoplasty.[82] Corneal permeability was measured *in vivo* by monitoring the time course of pilocarpine on pupil diameter, and *ex vivo* by measuring fluorescein diffusion. In both cases, permeability was significantly reduced following CXL.[3] This reduced permeability may have consequences for the diffusion of nutrients through the cornea as well as for the intraocular penetration of topically applied medications, so long-term studies are required. Similarly, there is some debate as to the effects of CXL on intraocular pressure. While most reports indicate no significant changes,[60,61,83] Kymionis *et al.*[84], in a study of 55 eyes from 55 patients, showed that intraocular pressure remained elevated by 14% one year after cross-linking. These elevated levels were not correlated with patient age, pachymetry or preoperative keratometry. Coskunseven *et al.*[62] also found that intraocular pressure increased significantly by up to 6 mmHg. At present it is not clear if elevated intraocular pressure in some patients persists in the longer term and what, if any, would be the long term effects on vision of this elevated pressure.

## Modifications to the standard procedure

1. To reduce patient discomfort

One clinical drawback of the standard CXL procedure is the postoperative discomfort associated with the removal of the corneal epithelium, which can be mild to severe and last for several days. In addition to this, epithelial debridement can lead to complications such as wound infection and other problems related to the activation of the wound healing responses in the stroma. Consequently, some authors have suggested modifications in which the procedure is carried out without epithelial removal.[85,86] However, *in vitro* studies in pig corneas have shown that riboflavin penetration through the intact epithelium is minimal,[87] and is patchy if the epithelium is partially disrupted.[88] Follow-on *in vivo* human studies have confirmed

the need for complete removal of the epithelium[52,89] when standard CXL is performed, although not all clinicians agree and the debate continues.[89,90]

Recently, several methods of trans-epithelial cross-linking have been proposed in which the anti-swelling agent dextran is typically omitted on the basis that its high molecular weight may inhibit the penetration of riboflavin solution across the epithelium. In these procedures, chemical agents, such as benzalkonium chloride (BAC),[91] EDTA[25] or gentamycin[54] are added to the riboflavin solution (individually or in combination) to loosen the tight junctions of the epithelial cells and thereby facilitate passage of riboflavin into the stroma without the need for epithelial removal. Although transepithelial cross-linking by these methods undoubtedly offers patients a faster and less invasive treatment than can be provided by the standard technique and facilitates the treatment of paediatric and uncooperative patients as well as those with thinner corneas (nearing 380 μm), its effectiveness remains uncertain. Experimental comparative studies in rabbit corneas have shown that cross-linking of corneas with an intact epithelium using BAC 0.0005% results in an increase in biomechanical rigidity (Young's modulus) of about one-fifth of that induced by standard CXL with epithelial debridement (21.30% vs 102.45%)[92]; this is presumably due to limited riboflavin absorption, since increasing the concentration of BAC to 0.02% produces an increase in the absorption co-efficient and an increase in Young's modulus.[93] It is not yet known whether the full stiffening effect of the standard CXL treatment is actually needed to stop keratoconus progression or whether the effects produced by trans-epithelial cross-linking may be sufficient. The latter is supported by two prospective cohort studies[25,54] and one non-randomised retrospective study,[94] with follow-up times of up to 12[54,94] and 18[25] months which have independently found significant improvements in visual and topographic outcome measures after trans-epithelial CXL. The long term efficacy and side effects of each procedure need to be ascertained by longer follow-up, randomized, controlled studies.

Several other new approaches to cross-linking are also being investigated. Daxer *et al.*[95] have proposed a technique to treat keratoconus whereby a flexible full-ring implant is placed into a "closed" corneal pocket into which the riboflavin is instilled, thus avoiding the need to remove the epithelium[91] or use other drugs. Iontophoretic delivery of riboflavin (using a mild electrical current) also holds promise as a useful modification to the standard protocol as it could greatly reduce the time required for administering riboflavin, and possibly also eliminate the need for epithelial removal[96] (*Figure 4*). According to Dr George O Waring IV, riboflavin is especially suitable for delivery by this method since it has a low molecular weight, is negatively charged at physiological pH levels and is highly water soluble.[96]

2. To reduce treatment time

With the aim of reducing treatment time and increasing the throughput of patients, investigators are now considering the use of higher illumination intensities in the CXL

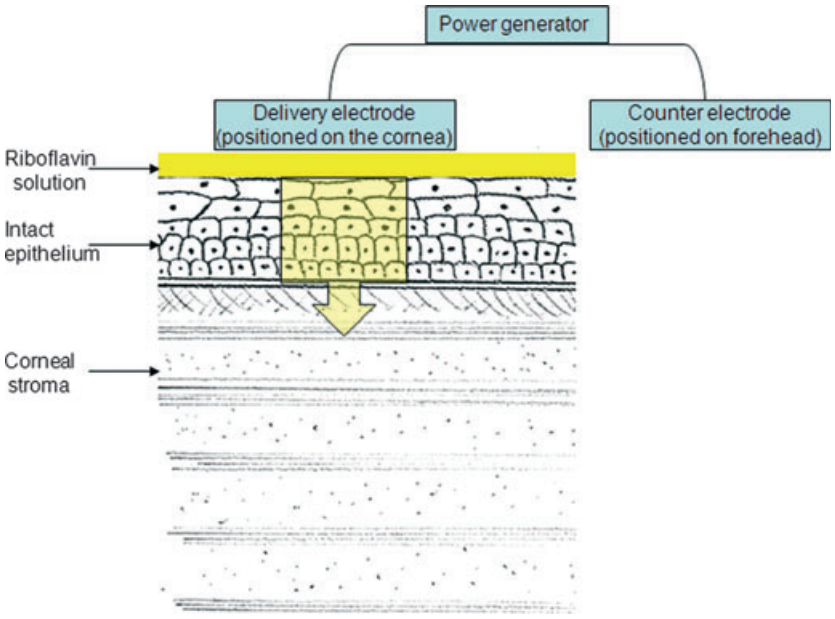


**Figure 4.** Schematic showing iontophoretic delivery of riboflavin into the corneal stroma. A negatively charged delivery electrode is placed on the cornea and a counter electrode (small plaster patch) is placed on the patient's forehead. A low intensity electrical current flows between the two electrodes to drive riboflavin solution across the intact epithelium and into the corneal stroma.

procedure. In the standard CXL procedure 3 $mWcm^{-2}$ is applied to a 9 mm treatment zone for 30 min, resulting in a total energy dose of 3.4 J or a radiant exposure of 5.4 $Jcm^{-2}$. However, this same level of radiant exposure can be achieved by applying a higher intensity for a shorter time, and studies conducted on pig corneas have shown that increasing the illuminance intensity to 10 $mWcm^{-2}$ and reducing the exposure time to 9 min produces a similar increase in corneal stiffness to that gained using the standard procedure.[97] The safety of using higher intensities *in vivo* has not yet been examined.

3. To facilitate the treatment of very thin keratoconic corneas

In order to overcome the contra-indication of treating corneas with a thickness bordering on 400 μm, Kymionis *et al.*[98] developed the use of pachymetry-guided epithelial debridement- a treatment modification in which the epithelium is only removed from regions of the cornea with a thickness in excess of 400 μm. Although the safety and efficacy of the treatment has yet to be fully validated, a study of 2 patients revealed that no adverse events had occurred during the treatment and after 9 months, both the corneal topography and endothelial cell density remained unchanged. An alternative solution for the treatment of very thin corneas was proposed by Hafezi *et al.*[41] They suggested replacing the standard iso-osmolar riboflavin solution (containing dextran) with a hypo-osmolar riboflavin solution (without dextran) to swell the cornea to an acceptable thickness prior to cross-linking.[41] X-ray scattering studies have shown that this phenomenon of increasing corneal thickness in cross-linked corneas is caused not by an increase in the diameter of the collagen fibrils but by an increase in the spacing between individual fibrils.[99] Using the modified technique, Hafezi *et al.*[41] treated 20 patients with thin corneas (minimum preoperative stromal thickness of 323 μm) and reported a cessation of keratoconus progression in all cases. However, the technique is not without limitations and CXL failure has been reported following the treatment of an extremely thin cornea (preoperative minimal thickness after abrasion of 268 μm).[100] The outcome of this case led the authors to suggest that a minimal preoperative stromal thickness of 330 μm is required for successful CXL using the modified protocol.[100]

## Other uses of CXL

1. Non-keratoconus ectasia

In recent years, several authors have reported the successful use of CXL to treat other forms of non-keratoconus ectasia, such as pellucid marginal degeneration[101–104] or keratectasia following LASIK[105–108] and radial keratotomy.[43] In all cases, an arrest and even a partial reversal in the ectasia was seen after cross-linking. In fact Hafezi and Iseli[109] have so far been the only ones to report an exacerbation of keratectasia despite CXL. They described a case in which a pregnant woman developed bilateral iatrogenic keratectasia 26 months after LASIK surgery. CXL was performed on both eyes and a regression in ectasia was observed at 22 months follow-up. However, the patient's subsequent pregnancy led to an exacerbation of the keratectasia, possibly as a result of hormonal changes during pregnancy altering the biomechanical properties of the cornea.

2. Stabilisation of corneoplastic procedures

Although the corneoplastic effects of intra-corneal ring segment implantation generally remain stable for many years,[110] CXL is being considered as a useful adjunct to the procedure to further stabilise the altered corneal shape. The development of this combination treatment is in its early stages and the optimal time to perform each stage of the treatment has yet to be ascertained.[111–113] Combining LASIK with CXL may result in improved corneal integrity and thereby reduce instances of post-LASIK keratectasia. Indeed, a recent study investigating this found that patients treated with combined LASIK and CXL had a similar or slightly better clinical outcome than those treated with LASIK alone.[114]

The use of CXL with topography-guided photorefractive keratectomy was first described by Kanellopoulos and Binder.[115] Since then, Kymionis *et al.*[116] have shown that the simultaneous treatment of topography-guided photorefractive keratectomy followed by CXL for keratoconus results in reduced refractive error and keratometry readings and improvements in visual acuity that remain stable at a mean follow-up of nearly 20 months. Similar results have been obtained by Stojanovic *et al.*[103] However, it is worth noting that *in vitro* studies of untreated and CXL treated pig corneas have shown that the efficacy of laser ablation is lower in CXL treated corneas[117] and so it may be necessary to modify existing ablation algorithms for the treatment of cross-linked corneas.[117]

Further investigations into the use of CXL as a means of stabilising corneal moulding have produced mixed results. Early studies of accelerated CXL in combination with microwave keratoplasty (a novel technique used to induce axial shrinkage of collagen and thereby flatten the keratoconus cornea), found it to be only minimally effective as an adjunct to the procedure as it failed to maintain the flattening effect and regression occurred.[118] When used in conjunction with orthokeratology it was found that CXL failed to stabilise the moulding effect (corneal topography and wave front error returned to baseline levels within 1 month of orthokeratology interruption) but nevertheless resulted in improved visual acuity, which remained above baseline levels 1 year after the combined treatment was performed.[119]

3. Infectious keratitis

The antimicrobial properties of CXL against common bacterial and fungal pathogens were demonstrated *in vitro* by Martins *et al.* in 2008.[120] Due to its ability to inhibit pathogen growth CXL is seen as a promising treatment option for the management of cases of infectious keratitis which are unresponsive to antibiotic therapy, and the clinical studies support this.[121,122] In a study involving 40 patients with infectious keratitis, the use of CXL and continued antibiotic treatment resulted in 85% of the cases being resolved without the need for emergency penetrating keratoplasty.[122] It was noted however that the success rate was higher for bacterial infections than fungal infections and that the treatment should be avoided in eyes with prior herpes simplex. The encouraging results of another study involving 16 patients, in which CXL was used as a primary treatment for bacterial keratitis[123] indicate that larger randomized trials are warranted to compare the benefits of CXL treatment with customary antibiotic therapy in terms of the healing time and complication frequency.

4. Oedema

On the basis of Wollensak *et al.*[11] demonstrating that cross-linked pig corneas placed in a humidity chamber swell less than untreated corneas, CXL was proposed as a therapeutic option for the treatment of conditions involving corneal oedema. In a study of 25 eyes of 25 patients in which CXL was used to treat oedema related to Fuchs endothelial dystrophy, corneal graft failure, and postoperative bullous keratopathy, the mean corneal thickness was found to be significantly reduced following treatment.[124] However, at 3 months follow-up 56% of the patients had developed epithelial bullae and only 44% of the 25 patients remained asymptomatic at 6 months follow-up.[124] Two other studies describing CXL treatment of bullous keratopathy reported significant reductions in pain, irritation and discomfort but no change in corneal thickness and visual acuity.[125, 126] Another showed short term improvements in pain, corneal thickness and transparency but found no lasting effects.[127] With the aim of producing more favourable and longer lasting results, others have tried modified CXL techniques in which the oedematous cornea is dehydrated to a normal thickness prior to treatment by means of a 1 day pre-treatment of 40% glucose[128] or a 30 minute pre-treatment of 70% glycerol.[129] Using these methods, distinct reductions in corneal thickness and patient discomfort have been reported immediately after treatment[129] and at 8 months follow-up.[128] Although CXL may not prevent the need for corneal transplantation in conditions involving corneal oedema it has the potential to improve the patient's visual comfort and extend the time interval for an upcoming corneal transplantation.[128]

## Frequently asked questions

Corneal cross-linking with riboflavin and UVA has to date been carried out on tens of thousands of patients with a very high success rate. Nevertheless, from the discussion above it is clear that there are still a number of questions that need to be answered. We conclude by seeking opinions from some leading experts in this field about some of the most common questions.

**1. At what point should a patient be referred for cross-linking?**

Mr D. O'Brart, Guy's and St Thomas' NHS Foundation Trust, UK

My indication at present for CXL is to perform it in any suitable patient (adequate corneal thickness, K max less than 58D, no central scarring, age typically less than 40) with reported or documented evidence of progression, although that is changing to any such suitable patient with keratoconus or ectasia, as it not only halts progression but also improves corneal shape.

**2. Can the patient return to wearing soft contact lenses after cross-linking?**

Prof. Dr. F. Hafezi, University of Geneva, Switzerland

Contact lens wear can be started again, once the exam at 4 weeks after CXL shows that the corneal epithelium is well closed and without irregularities. Between month 1 and 6, reduced sensitivity might be an issue, and we advise to not excessively wear contact lenses during that period and have the cornea checked regularly. I do not think that contact lens wear should be an issue after these 6 months. Please note that at 6 months after CXL, an assessment of the anterior corneal curvature will be made. To properly assess the cornea, the patients should refrain from wearing contact lenses for 2 weeks to avoid misinterpretation due to corneal warpage.

**3. How long is the treatment expected to last? Will re-treatments be needed?**

Prof. Dr. E. Spoerl, Augenklinik Universitätsklinikum, Dresden, Germany

The half-life-time of the cornea is about 7 years and with cross-linking this half-life time will be increased thus we can expect that the CXL effect should last more than 10 years. However, under certain situations such as pregnancy,[109,130–134] neurodermatitis,[60] stress and hormonal changes[135,136] and application of prostaglandins,[137,138] a new progression of keratoconus can occur in spite of CXL. In our series of 730 eyes which we cross-linked since 1998 the rate of

re-CXL is about 2.5%. For that reason a yearly control of the cornea by topography (until another measurement device for the corneal biomechanical parameters is available) is also necessary after CXL to detect slight changes before worsening of the vision and if necessary a re-CXL should be performed immediately.

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