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Corneal collagen crosslinking in keratoconus and other eye disease

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Abstract

• **Keratoconus is a condition characterized by biomechanical instability of the cornea, presenting in a progressive, asymmetric and bilateral way. Corneal collagen crosslinking (CXL) with riboflavin and Ultraviolet-A (UVA) is a new technique of corneal tissue strengthening that combines the use of riboflavin as a photo sensitizer and UVA irradiation. Studies showed that CXL was effective in halting the progression of keratoconus over a period of up to four years. The published studies also revealed a reduction of max K readings by more than 2 D, while the postoperative spherical equivalent (SEQ) was reduced by an average of more than 1 D and refractive cylinder decreased by about 1 D. The major indication for the use of CXL is to inhibit the progression of corneal ecstasies, such as keratoconus and pellucid marginal degeneration. CXL may also be effective in the treatment and prophylaxis of iatrogenic keratectasia, resulting from excessively aggressive photo ablation. This treatment has been used to treat infectious corneal ulcers with apparent favorable results. Most recent studies demonstrate the beneficial impact of CXL for iatrogenic ecstasies, pellucid marginal degeneration, infectious keratitis, bullous keratopathy and ulcerative keratitis. Several long-term and short-term complications of CXL have been studied and documented. The possibility of a secondary infection after the procedure exists because the patient is subject to epithelial debridement and the application of a soft contact lens. Formation of temporary corneal haze, permanent scars, endothelial damage, treatment failure, sterile infiltrates, bullous keratopathy and herpes reactivation are the other reported complications of this procedure.**

• **KEYWORDS:** keratoconus; collagen; corneal cross-linking; ultraviolet radiation and riboflavin

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INTRODUCTION

Tiny fibers of protein in the eye called collagen help hold the cornea in place and keep it from bulging. When these fibers become weak, they cannot hold the shape and the cornea becomes progressively more cone shaped [1]. Keratoconus (from Greek: *kerato*-horn, cornea; and *konos* cone) is a degeneration disorder of the eye in which structural changes within the cornea cause it to thin and change to a more conical shape than the more normal gradual curve. Keratoconus can cause substantial distortion of vision, with multiple images, streaking and sensitivity to light all often reported by the patient. It is typically diagnosed in the patient's adolescent years. If afflicting both eyes, the deterioration in vision can affect the patient's ability to drive a car or read normal print. The prevalence in general population is 50-200 per 100 000 [1]. A 20% of keratoconic patients will suffer of severe visual deterioration due to irregular astigmatism, myopia, corneal scarring and optical means such as spectacles and rigid gas permeable contact lenses do not offer any visual rehabilitation [2].

The genetic basis of keratoconus has been studied through linkage mapping and mutation analysis to reveal its molecular basis and pathogenesis [3]. Several studies have reported a strong association between eye rubbing and the development of keratoconus [4]. This association may be due to the activation of wound healing processes and signaling pathways secondary to mechanical epithelial trauma direct rubbing-related mechanical trauma to the keratocytes and increased hydrostatic pressure in the eye [5]. Contact lens wear is another form of corneal microtrauma associated with keratoconus [6]. Keratoconus usually starts in the teenage years. It can though begin in childhood or in people up to the age of 30. It's possible it can occur in people at the age of 40 and older, but that is less common [2]. The changes in the shape of the cornea can happen quickly or may occur over several years.

Keratoconus can involve each layer of the cornea. The corneal epithelial cells will be enlarged and elongated [7]. Early degeneration of basal epithelial cells can follow by

disruption of the basement membrane. This disruption results in the growth of epithelium posterior to the Bowman's layer and collagen anterior to the epithelium, forming typical Z-shaped interruptions or breaks in the Bowman's layer [8]. Scarring of the Bowman's layer and the anterior stroma are common and present histopathologically with collagen fragmentation, fibrillation and fibroblastic activity. The stroma has normal-sized collagen fibers but low numbers of collagen lamellae, which results in stromal thinning. Endothelial cell pleomorphism and polymegathism may also be manifested. With increasing severity and duration increase, greater change and damage occurs at the base of the cone than at the apex [9].

Corneal topography is a valuable diagnostic tool for diagnosing subclinical keratoconus and for tracking the progression of the disease. Rabinowitz [10] has suggested four quantitative videokeratographic indices for screening keratoconic patients. These indices include central corneal power >47.2 D, inferior-superior dioptric asymmetry over 1.2 D, simulated keratometric (Sim K) astigmatism >1.5 D and skewed radial axes >21° [10].

HISTORY OF CORNEAL COLLAGEN CROSSLINKING AND STRUCTURE OF COLLAGEN

Collagen molecules are secreted from connective tissue cells as procollagen, a biosynthetic precursor of collagen that comprises of three α-chains with additional N- and C-terminal extensions. Fibrils are formed from the aggregation of collagen molecules from which the procollagen peptides have been cleaved. Collagen undergoes a number of post-translational modifications, including the formation of cross-links by enzymatic oxidation of lysine and hydroxyl sine residues to their respective aldehydes. Keratoconus is a central noninflammatory ectasia of the cornea, with histopathological studies showing decreased numbers of collagen fibrils, keratocytes with membrane anomalies, fragmentation of the epithelial basement membrane, fibrillation and disintegrations of Bowman's membrane, and electron microscopic appearance of degenerative changes of the basal epithelial cells. Early experiments by Cannon and Foster implicated the role of degraded normal collagen or synthesis of abnormal collagen in the pathogenesis of keratoconus. Increased lysosomal and proteolytic enzymes expression and reduced protease inhibitors are seen in keratoconus. This is responsible for the corneal thinning and collagen lamellae configuration alterations seen in keratoconus [11]. In the early to mid-1990s, Khadem *et al* [12] worked on the identification of biological glues and their activation by heat or light to affect an increase the resistance of stromal collagen. This work perhaps marked the beginning of the search for therapeutic targeting of the underlying pathogenic mechanisms of keratoconus. The gluing effect was found to be mediated by oxidative mechanisms associated with hydroxyl radical release. In 1991 Klingman and Gebre evaluated the dermatological biochemical changes that occur following chronic exposure to UVA radiation and demonstrated that the treated collagen was highly resistant to pepsin digestion, indicating increased corneal collagen crosslinking (CXL) induced by UVA. The biomechanical strength of the keratoconic cornea is reduced with the central and inferior regions commonly affected, resulting in cone formation. The reduced interlamellar cohesive strength has been attributed as the causative factor of the cone in these ectatic areas and can be strengthened by CXL [13]. CXL of the cornea using UVA and riboflavin has been found to be effective in halting the progression of keratoconus [14]. Based on the use of UVA light in dentistry for hardening filling materials and the work carried out by Milne and Zika, elaborating a wide variety of CXL techniques, the riboflavin/UVA therapy was adapted to the cornea by Seiler *et al* [15] in the mid-1990s, who also tested other CXL systems. In 2003, a clinical and experimental breakthrough was achieved by Wollensak *et al* [16] when they reported on the clinical efficacy and biocompatibility of the riboflavin/UVA system in human corneas, which paved the way for clinical trials. During CXL new chemical bonds are induced and there is an increased corneal resistance to enzymatic degradation after CXL treatment.

PHYSICOCHEMICAL CHANGES IN THE CORNEAL STROMA INDUCED BY COLLAGEN CROSSLINKING

Spoerl *et al* [17] were the first to investigate the use of riboflavin and UVA irradiation to achieve crosslinking of corneal collagen. Using this method, Wollensak *et al* [18] and Kohlhaas *et al* [19] were able to demonstrate a marked positive effect of CXL on the biomechanical properties of both porcine and human corneal tissue. CXL was shown to increase the rigidity of human corneal tissue (as expressed by Young's modulus at 6% strain) [18]. In a similar experiment, the increase in Young's modulus (67% at 5% strain) was shown to be confined to the anterior 200 microns of stroma where most of the UVA radiation is absorbed [19]. Within 1h of UVA-riboflavin treatment of eye bank cornea, changes can already be observed in stromal keratocytes with cell shrinkage, chromatin condensation and apoptotic bodies [20]. Keratocyte toxicity studies have shown that (at least in the rabbit model) saturation of the corneal stroma using 0.1% riboflavin in a dextran solution and 30min of UVA irradiation with a wavelength of 370 nm and a surface irradiance of 3 mW/cm^2 may limit keratocyte death to a depth of approximately 300 μm [21]. In this study, there was no observable toxic effect of CXL at a depth beyond this level. CXL has been shown to increase stromal shrinking

temperatures [22] and to increase resistance to enzymatic digestion [16]. CXL of corneal collagen may also increase the impedance of the corneal stroma to the diffusion of some solutes, as has been demonstrated for fluorescein [23].

CORNEAL COLLAGEN CROSSLINKING

CXL with riboflavin and UVA is a new technique of corneal tissue strengthening that combines the use of riboflavin and UVA irradiation. Riboflavin works as a photo sensitizer for the induction of crosslinks between collagen fibrils and at the same time act as a shield from the penetration of UVA in the underlying tissues [24]. CXL is the only available treatment directed at the underlying pathology in keratoconic cornea, which stromal biomechanical and structural instability is leading to progressive ectasia. CXL induces covalent inter- and intrafibrillar collagen cross-links creating an increase in biomechanical rigidity of human cornea by about 300%. The crosslinking effect is maximal only in the anterior stroma [25]. CXL may also be effective in the treatment and prophylaxis of iatrogenic keratectasia, resulting from laser *in situ* keratomileusis [26, 27]. This treatment has also been used to treat infectious corneal ulcers with apparently favorable results [24, 28]. CXL has been used in combination with other treatments, such as intra corneal ring segment implantation [29, 30] and limited topography-guided photo ablation.

The major indication for the use of CXL is to inhibit the progression of corneal ecstasies, such as keratoconus and pellucid marginal degeneration [25,29]. Some studies have detected a high rate of progression of keratoconus in younger patients, so it has suggested to treat with CXL at the diagnosis, and not to wait for progression [31]. With some success *in vitro* studies [32,33] have shown that the cornea absorbs approximately 30% of UVA light while an additional 50% of UVA absorption occurs in the lens [4]. Corneal UVA absorption can be considerably increased with a photo sensitizer such as riboflavin. With an irradiance of 3 mW/cm^2 of UVA and 0.1% riboflavin, as much as 95% of UVA light will be absorbed within the cornea. This results in a 20-fold reduction of the original irradiance of 3 mW/cm^2 of UVA (at the corneal surface) down to 0.15 mW/cm^2 (at the endothelial level), which is well below 0.36 mW/cm^2, the threshold considered cytotoxic for the endothelium [34, 35].

Using a wavelength of 360-370 nm with an accumulated irradiance of 5.4 J/cm^2 ensures that the exposure of all structures is below harmful levels [25]. The riboflavin in the cornea itself also serves as a further protective layer, which has reported to reach more than 400 μm after 30min of application, penetrating the anterior chamber, where it is visible with the slit lamp as a yellow flare. Therefore, there is an ultraviolet absorption coefficient that shields the more posterior structures such as the endothelium, the crystalline lens and the retina [25].

Kymionis *et al* [32] described the indirect effect of CXL through the change in corneal thickness during and after the treatment due to a more compact and rigid cornea. They found a statistically significant decrease (mean, 75 microns) in central corneal thickness at the interval of the epithelial removal (415.7±20.6 microns) and at the end of riboflavin solution instillation (340.7 ±22.9 μm; $P<0.001$) and no statistically significant change during irradiation ($P>0.05$). Pre-operative and 1-month post-operative endothelial cell count were not statistically different (pre-operative, 2780±197 to 1mo post-operative, 2713±116; $P=0.14$).

CROSSLINKING TECHNIQUE AND PROCEDURE

CXL treatment helps to stabilize progressive keratoconus. It can be performed as treatment after epithelial debridement or transepithelial treatment. CXL treatment with and without epithelial debridement also leads to changes in corneal sensitivity and nerve morphology changes. Corneal sensitivity is decreased in the treatment areas for 3d and then steadily recovers to normal levels for up to 3mo [35].

Treatment after Epithelial Debridement Topical anesthesia of proparacaine hydrochloride 0.5% is administered for 2min. With an 8 mm diameter trephine blade, the central mark is placed over the epithelium. Mechanical epithelial debridement of the previously marked central 8 mm of the cornea is carried out gently using an iris repositor or merocel sponge or with a rotating soft brush without disturbing the subepithelial components. This is to ensure that the riboflavin penetrates the stroma in order to achieve a high level of UVA absorption. As a photosensitizer, 0.1% riboflavin solution was instilled onto the cornea every 5min for 30min before irradiation to allow sufficient saturation of the stroma. Instillation of riboflavin drops for 10-15min has only recommended by some surgeons to prevent maximum thinning of the cornea by the dextran before irradiation to lessen the risk of damage to the endothelium. To ensure that the cornea and the anterior chamber are saturated by riboflavin the eye is examined at the slit lamp just prior to the application of the UV light. Following this, an 8.0 mm diameter of central cornea is irradiated with UVA light of 370 nm wavelength and an irradiance of 3 mW/cm^2 for 30min. During the 30min of irradiation, riboflavin 0.1% drops may be applied to the cornea at 5min intervals to sustain the necessary concentration of the riboflavin. At the end of the procedure, a combination of topical steroid and antibiotic drops (moxifloxacin eye drops 0.5%, prednisolone acetate 1% eye drops) are administered followed by a bandage contact lens application. In most cases, the contact lens is removed within 5d after treatment [36].

Transepithelial Technique (Crosslinking Without Removal of the Epithelium) Alternatively, CXL can be

performed transepithelially [37]. The ability to achieve predictable CXL without epithelial removal is a desirable modification to lessen discomfort and shorten recovery time. With the aid of 20% Dextran T500, topically instilled riboflavin can penetrate the cornea without removal of the epithelium ("Epi-on" procedure). UVA light then focused on the cornea for 30min to activate the riboflavin. The Epi-on procedure does not cause the patient any pain during or after the procedure. Femtosecond laser-created pocket collagen CXL in early keratoconus with riboflavin has recently been described [38]. Following the procedure, there may be some foreign body/gritty sensations, and haziness during the day of the procedure that clears up in 1d. This is a minimally invasive method and prevents the progression of keratoconus by increasing the corneal tensile strength, with no medium-term adverse effect on its normal architecture. Several substances had used to loosen the tight junctions of the epithelial layer and thus increase the penetration of riboflavin. One is a riboflavin solution containing benzalkonium chloride (BAK), the most commonly used preservative in ophthalmic medications. BAK is also a tensioactive substance, surfactant or an active surface agent that changes the surface tension value, and hence would facilitate the penetration of substances through the epithelium. However, in a comparative *in vitro* study, Samaras *et al*[39] compared 20% alcohol, partial or complete epithelial removal by analyzing light transmission properties of porcine corneas after CXL and concluded that complete removal of the corneal epithelium appears to be necessary to allow sufficient riboflavin absorption into the stroma to alter the normal light transmission properties of the porcine cornea.

ACCELERATED CROSSLINKING

Accelerated crosslinking of cornea is a new advanced procedure with faster recovery which takes the advantage of the combined action of riboflavin (vitamin B) and UVA light exposure. In the accelerated crosslinking procedure, riboflavin (vitamin B_2) is dripped onto the cornea and then exposed to ultra violet light. The light causes the riboflavin to fluoresce, which leads to the formation of bonds between collagen molecules or collagen crosslinking. In a matter of minutes, the procedure is complete. It has revolutionized standard crosslinking by reducing treatment time from one hour to 3min or less. The reduction in speed is made possible by increasing the UVA power and reducing the exposure time, thereby maintaining the same energy on the eye as standard crosslinking while reducing crosslinking time by an order of magnitude. In the acceleration crosslinking we can use two systems: The UV-X illumination system, a 30min riboflavin soaks time and then typically 30min of UVA exposure, this takes about 1h in the theatre time. The avedro KXL accelerated crosslinking system a 3-5min soak time and then typically 3min of UVA exposure.

PATIENT SELECTION

The primary purpose of crosslinking is to halt the progression of ectasia. Likewise, the best candidate for this therapy is an individual with keratoconus or post-refractive surgery ectasia who has documented progression of the disease. There currently are no definitive criteria for progression, but parameters to consider are change in refraction (including astigmatism), uncorrected visual acuity, best corrected visual acuity, and corneal shape (topography and tomography).

Contraindications 1) Corneal thickness of less than 400 microns is a contraindication to the standard treatment protocol; 2) prior herpetic infection is a contraindication because it may result in viral reactivation; 3) concurrent infection; 4) severe corneal scarring or opacification; 5) history of poor epithelial wound healing; 6) severe ocular surface disease (*e.g.* dry eye); 7) autoimmune disorder.

CLINICAL RESULTS

The initial clinical experience in Dresden was reported by Wollensak *et al* [14] in 2003 and a report of a smaller Italian study of 10 patients followed in 2006 [36]. In 2008, Raiskup-Wolf *et al* [40] described what remains the largest published series comprising 241 eyes followed in Dresden for up to 6y after CXL. This uncontrolled, retrospective study confirmed earlier findings with statistically significant improvements in astigmatism, best-corrected visual acuity (BCVA) and maximum simulated keratometry values (Kmax) at 12mo. Flattening was observed in 54% of eyes with a mean change in Kmax of -1.91 D. The effects of CXL were maintained over the duration of follow up with progression of the disease documented in only two patients. Wittig-Silva *et al* [41], found similar results regarding BCVA and K reading, with no difference in SEQ and endothelial cell density between treated and control eyes after 12mo follow-up. In another study, conducted by Jankov *et al* [42], it was found an arrest in the progression of keratoconus in a group of patients after CXL treatment. In a period of six months prior to the treatment all patients of this group presented deterioration in terms of astigmatism and corneal stability. Kmax readings decreased by more than 2 D (from 53.02±8.42 to 50.88±6.05 D), SEQ in less than 1 D (from -3.27 ±4.08 to -2.68 ±3.02 D), while refractive cylinder decreased by less 0.5 D (from -2.29±1.77 to -1.86±0.92 D). No eyes lost any line of BCVA, 12 maintained the preoperative BCVA, 7 gained one line, 5 gained two lines, and 1 patient gained three lines of BCVA [42,43]. Many reports from several other centers have described similar results as what we can see in Table 1.

Table 1 Results of different studies

Authors	Country	Design	Follow up (mo)	Finding
Wollensak *et al* (2003) [14]	Germany	Case series (23)	3-47	Kmax decreased by 2.01; SEQ reduced by 1.14D; BCVA improved by 1.26 lines.
Caporossi *et al* (2006) [36]	Italy	Case series (10)	3	Kmax decreased by 1.9 D; BSCVA improved 1.66 lines.
Raiskup-wolf *et al* (2008) [40]	Germany	Retrospective case series (241)	6-72	Kmax decreased by 2.57D at 3 years; BCVA improved in 58%.
Witting-silva *et al* (2008) [41]	Australia	Randomized, controlled trial (24/23)	3-12	Kmax decreased by 1.45D; BSCVA improved by 0.12 l.
Jankov *et al* (2008) [42]	Serbia	Case series (25)	6	Kmax decreased by 2.14 D; UCVA improved by 0.11.
Hoyer *et al* (2009) [44]	Germany	Retrospective series (153)	12-36	Kmax decreased by 4.34 D at 3 days; BCVA improved by 1 line or more in 60.6 % at 3 years.
Fournie *et al* (2009) [45]	France	Uncontrolled, prospective trial (20)	3-18	Kmax decreased by 1.68 D; BCVA improved by 0.14.
Grewal *et al* (2009) [46]	India	Uncontrolled, prospective trial (102)	12	Stable BCVA, spherical equivalent and corneal curvature.
Coskunseven *et al* (2009) [47]	Turkey	Controlled (fellow eye) non-randomized (19/19)	5-12	Kmax decreased by 1.57 D; BSCVA improved by 0.10.
Agrawal (2009) [48]	India	Retrospective series (37)	12	Kmax decreased by mean of 2.73 D in 66%; BCVA improved ≥1 line in 54%.
Vinciguerra *et al* (2009) [49]	Italy	Controlled (fellow eye), non-randomized (28/29)	12-24	Kmax decreased by 6.16 D at 12 months; BSCVA improved by 0.14 at 12 months.
Koller *et al* (2009) [50]	Switzerland	Controlled (fellow eye), non-randomized (21/21)	12	Kmax increased minimum radius of curvature by 0.62D, reduction in 4 of 7 keratoconus indices.

Published clinical studies of corneal collagen cross linking in the treatment of keratoconus. B[S] CVA=Best [Spectacle]-corrected visual acuity; Kmax: Maximum curvature or steepest simulated keratometry value derived from computerized video keratography; *n*: Eyes subject to analysis; NS: Not statistically significant; SEQ: Spherical equivalent; UCVA: Uncorrected visual acuity.

CORNEAL CROSSLINKING AND SAFETY

From the beginning of the CXL research the safety of the treatment stood in the center of attention because this procedure was not applied in tissue engineering of isolated collagen structures. The aim was to develop a clinically applicable method for eyes *in vivo* to create additional chemical bonds inside the corneal stroma by means of a highly localized photopolymerization while minimizing exposure to the surrounding structures of the eye [51].

There were some points to taken under consideration: 1) the duration of the treatment should not be too long; 2) the transparency of the cornea should not be changed; 3) the crosslinking effect should only include the cornea.

The treatment parameters should fulfil two requirements: the biomechanical effect and the safety. Many investigations were necessary for the fine-tuning of these treatment parameters [52].

The Choosing of the Photochemical Crosslinking There are two different collagen cross-linking methods: the chemical crosslinking uses solutions like glutaraldehyde, transglutaminase, genepin and nitroalcohol, and the photooxidative (also called physical) crosslinking uses light especially UV-light [14]. The application of liquid cross linker to the curved cornea is not easy and the diffusion of the liquid cannot be controlled during application. Thus, we recommend using the photooxitative crosslinking for a safer application.

The Choosing of the Treatment Parameters with Respect to Safety Singlet oxygen is necessary for the photooxidative crosslinking. Riboflavin is one of the most potent producers of these oxygen radicals. Riboflavin (vitamin B_2) is not toxic and it is used as a food dye. However, riboflavin is not only a photosensitizer-it acts also as a UV absorber. Since the UV light is effective only in the absorbed areas, it is desirable that the irradiation is absorbed in about 400 μm thick corneal stroma tissue [53, 54]. For the absorption of ultraviolet light in the cornea the concentration of the superficially applied riboflavin solution of 0.1% and the time course of the diffusion process is relevant. Applied riboflavin must diffuse into the corneal stroma and this process requires certain time [53]. The intact epithelium acts as a barrier that inhibits the diffusion of riboflavin into the cornea [55, 56]. For that reason the epithelium must be debrided from the intended treatment area because this simple procedure removes a diffusion barrier for the riboflavin molecule and speeds saturation of the corneal stromal tissue, then riboflavin diffuses through the cornea and a concentration gradient is formed. Though the highest

concentration of riboflavin is reached in the anterior stroma [57], however after 20-30min a sufficient concentration is reached also in the posterior stroma. After the riboflavin has traversed the cornea, it enters into the anterior chamber. The aqueous humour without riboflavin does not have any relevant absorption at 370 nm but clinically it starts to stain after about 5min of surface exposure to riboflavin. By means of slit lamp inspection using blue light the surgeon has to assure that riboflavin has appeared in the anterior chamber before the UV-irradiation has started [58]. The yellow staining of the anterior chamber serves as a safety feature, indicating that the riboflavin has penetrated the cornea and the cornea is thoroughly saturated. Only a circular area of 8 mm in diameter is then exposed to UV-light with a wavelength of 370±5 nm and an irradiance of 3 mW/cm^2 for a total time of 30min. This corresponds to a total dose of 3.4 Joule (J) or a total dose density of 5.4 J/cm^2 to the cornea. Therefore, due to the additional riboflavin shielding all structures behind the corneal stroma including the cornea endothelium, anterior chamber, iris, lens, and retina are theoretically exposed to a residual UV-dose density that is less than 1 J/cm^2 as recommended by the UV-guidelines [59]. The aim in the biomechanical effect is to reach a stiffness of the keratoconic cornea similar to the normal one. The stiffness of the keratoconic cornea is about 70% of a healthy cornea [60]. It was not our aim to harden the cornea extremely and to convert it in a non-physiological range. For that reason a low irradiance of 3 mW/cm^2 for 30min is choose. Thus, the stability of the cornea was increased and the crosslinked collagen network allowing keratocyte repopulation [61, 62].

Design of a Light Source with Respect to Safety All the above safety considerations are based on an irradiance that is homogenous within the field of UVA-application. If optical inhomogenities such as hot spots are present the damage thresholds may be exceeded locally leading to localized endothelial damage although the average irradiance may be less than 3 mW/cm^2. Therefore, clinically used light sources must guarantee a perfect homogeneity of the irradiance across the beamed area. A beam path according to Koehler is focused through a variable diaphragm onto the corneal surface with a special beam homogenizing micro structure [63] in order to ensure homogeneity of illumination on the cornea. In the optical design according to Koehler the UV light diaphragm is imaged onto the corneal front surface and as a consequence is the UV light that is focused on the front surface of the cornea strongly scattered in the ocular media behind the cornea. Thus the safety considerations are fully applied and the real radiant exposures in the eye are probably even lower.

Prevention of the Corneal Endothelium Damage The photopolymerization process inducing additional crosslinks in the corneal stroma is carried out by free radicals mediated by the riboflavin irradiated with UV-light. Such radicals can create cell damages that may be tolerable in keratocytes population but not in the corneal endothelium. The cytotoxicity of the riboflavin/UVA treatment on keratocytes and endothelium cells was studied by Wollensak *et al* [64, 65] and the cytotoxic threshold of the UVA/Riboflavin for endothelial cells and keratocytes was determined [64, 65]. The cell population that is the most critical to suffer from damages either from UV-light directly or from the free radicals is the corneal endothelium because it is immediately adjacent to the corneal stroma and these cells in the normal human cornea have low regenerative capacity.

Complications of Corneal Collagen Crosslinking Several long-term and short-term complications of CXL have been studied and documented [40,66] which may be direct or primary due to incorrect technique application or incorrect patient's inclusion or indirect or secondary complications related to therapeutic soft contact lens, patient's poor hygiene, and undiagnosed concomitant ocular surface diseases (dry eye, blepharitis, *etc*).

Postoperative Infection/Ulcer Debriding the corneal epithelium theoretically exposes the cornea to microbial infection. Bacterial keratitis has been reported 3d following treatment in which scraping revealed an E. coli infection [67]. Acanthamoeba keratitis due to eye washing under tap water as the patient was unaware of a bandage contact lens being inserted has been reported [68]. Poor contact lens hygiene resulting in polymicrobial keratitis caused by streptococcus salivarius, streptococcus oralis, and coagulase-negative staphylococcus sp. has been reported recently [69]. A patient with no history of herpetic keratitis developed herpes simplex keratitis geographical ulcer and iritis five days after treatment [70]. Staphylococcus epidermidis keratitis has been reported 2d after treatment [71]. Diffuse lamellar keratitis (stage 3) has been reported following treatment in a case of post-LASIK ectasia [72]. Severe keratitis with patient's contact lens and cornea scrapings positive for pseudomonas aeruginosa has also been reported recently [73]. Reactivated herpetic keratitis and neurodermatitis have also been reported following CXL [47, 70]. One study reported four cases of severe keratitis in a group of 117 keratoconic eyes treated with standard CXL [74]. Keratitis can occur following CXL because of presence of an epithelial defect, use of soft bandage contact lens, and topical corticosteroids in the immediate postoperative period. In cases of corneal infection after CXL, contact with the infectious agent likely occurred during the early postoperative period rather than during surgery because CXL not only damages keratocytes, but it also kills bacteria and fungi. This effect is used to advantage when CXL is performed for infectious keratitis.

Corneal Haze In a recently published retrospective study of 163 eyes with grade I-III keratoconus, approximately 9% of the 127 patients developed clinically significant hazes after 1-year follow up. The subset of patients developing steroid resistant haze appeared to have more advanced keratoconus, as reflected in a lower mean corneal thickness and higher keratometry value of the apex compared with the control group [74]. Advanced keratoconus should be considered at higher risk of haze development after CXL due to low corneal thickness and high corneal curvature [75].

After collagen crosslinking using riboflavin and UV-A, a lacunar honeycomb-like hydration pattern can be found in the anterior stroma with the maximum crosslinking effect, which is because of the prevention of interfibrillar crosslinking bonds in the positions of the apoptotic keratocytes [76]. The polygonal crosslinking network might contribute favorably to the biomechanical elasticity of the cross-linked cornea and to the demarcation of the anterior stroma after CXL on biomicroscopy, thus making lacunar edema a positive sign of efficient crosslinking [76]. Another study documented stromal haze in 5 of 44 patients within 6mo of undergoing CXL. There has been a debate as to whether stromal haze is a normal finding after CXL because of its frequency [77]. Previous confocal microscopy studies [77] report that a dense extracellular matrix compatible with clinical haze forms between 2mo and 3mo postoperatively. Koller *et al* [78] evaluated anterior stromal haze, which was graded on a scale used in cases after PRK [79]; the mean grade was 0.78, 0.18, and 0.06 at 1, 6, and 12mo, respectively. The haze after CXL differs from the haze after PRK in stromal depth. Whereas haze after PRK is strictly subepithelial, haze after CXL extends into the anterior stroma to approximately 60% depth which is on average equal to an absolute depth of 300 μm.

Haze formation after CXL may be a result of back-scattered and reflected light, which decreases corneal transparency [33]. Greenstein *et al* [80] studied the natural course after CXL and found a significant postoperative increase in haze measured by both Scheimpflug densitometry and slit lamp assessment. The increase peaked at 1mo and plateaued between 1mo and 3mo. Between 3mo and 6mo, the cornea began to clear and there was a significant decrease in CXL-associated corneal haze which usually does not require treatment except for some low dose steroid medication in some cases. From 6mo to 1y postoperatively, there continued to be a decrease in haze measurements.

In vitro and *ex vivo* studies [20,21] show that CXL leads to an immediate loss of keratocytes in the corneal stroma. In a confocal microscopy study, Mazzotta *et al* [61] found that in eyes with keratoconus, activated keratocytes repopulated the corneal stroma starting at 2mo and that the repopulation was almost complete at 6mo. It is possible that these activated keratocytes contribute to the development of CXL-associated corneal haze. Other factors that may contribute to CXL-associated corneal haze include stromal swelling pressure changes [81], proteoglycan-collagen interactions [30], and glycosaminoglycan hydration [82].

Endothelial Damage The endothelial damage threshold was shown to be at an irradiance of 0.35 mW/cm^2, which is approximately twice compared with the 0.18 mW/cm^2 that reaches the corneal endothelium when using the currently recommend protocol [34]. It may be due to a stromal thickness less than 400 μm or incorrect focusing. If the procedure is done on a thinner cornea, it may lead to perforation. The recommended safety criteria must be observed because UV irradiation has potential to damage various intraocular structures.

Peripheral Sterile Infiltrates Sterile corneal stromal infiltrates occur as a result of enhanced cell-mediated immunity to staphylococcal antigens deposited at high concentrations in areas of static tear pooling. Sterile infiltration after CXL may be related to staphylococcal antigen deposition in areas of static tear pooling beneath the bandage contact lens [83].

Herpes Reactivation Exposure to UV light can also induce oral and genital herpes in humans and ocular herpes in animal models. Development of herpes keratitis and iritis after riboflavin-UVA treatment has been reported. It seems that UVA light could be a potent stimulus to trigger/induce reactivation of latent HSV infections even in patients with no history of clinical herpes virus ocular infections. Significant corneal epithelial/stromal trauma or actual damage of the corneal nerves could be the mechanism of HSV reactivation. The use of topical corticosteroids and mechanical trauma caused by epithelial debridement may be additional risk factors [70].

Treatment Failure CXL failure is largely defined as keratoconic progression following treatment. One study of 117 eyes from 99 patients who underwent CXL documented a failure rate of 7.6% at one-year follow up. The results also indicated that 2.9% of eyes lost two or more lines of Snellen visual acuity. Age older than 35y, cornea thickness <400 μm, and a preoperative spectacle-corrected visual acuity better than 20/25 were identified as significant risk factors for complication. A high preoperative maximum keratometry reading was a significant risk factor for failure. Risk factors for CXL failure included a preoperative patient age of 35y or older, spectacle-corrected visual acuity better than 20/25, and a maximum keratometry reading greater than 58.00 D [77].

ADDITIONAL APPLICATIONS OF CORNEAL COLLAGEN CROSSLINKING

Corneal Collagen Crosslinking After Refractive Surgery It is known that both LASIK and photorefractive keratectomy (PRK) modify corneal stability and overall tissue strength. Prophylactic CXL has been advocated in such patents prior to (or at the time of) PRK in an attempt to prevent this complication [84]. This treatment appears to be particularly applicable to patients who are suspected of having form frust keratoconus or those with a correction that exceeds -8 D. These patients may benefit from stiffening of the cornea because keratectasia might not develop. Early treatments of iatrogenic keratectasia after LASIK performed at the University of Dresden seem to be effective in preventing further progression of the post-refractive surgery ectasia. Hafezi *et al* [26] reported that CXL arrested and/or partially reversed keratectasia of 10 patients over a postoperative follow-up of up to 25mo as demonstrated by preoperative and postoperative corneal topography showing a reduction in maximum keratometry reading. CXL could also conceivably alter both the stromal ablation rate and the biomechanical response of the cornea to the ablation and further reduce the refractive predictability of PRK. The use of CXL as means of extending the range of patients eligible for laser vision correction would be difficult to justify on the basis of current evidence.

Corneal Collagen Crosslinking in Pellucid Marginal Degeneration Other ectatic conditions of the peripheral cornea, such as pellucid marginal degeneration, may also benefit from CXL provided that any risk to the limbal stem cells is adequately addressed. Kymionis *et al* [85] performed simultaneous PRK and CXL in a 34-year-old woman with progressive pellucid marginal corneal degeneration in both eyes. 12mo postoperatively, BCVA improved from 20/50 and 20/63 to 20/25 and 20/32 in the right and left eye, respectively. Corneal topography revealed significant improvement in both eyes.

Corneal Collagen Crosslinking in Bullous Keratopathy CXL has also been suggested as a treatment for corneal edema. This concept is supported by changes in the hydration behavior of the porcine cornea after CXL and the observation that stromal compaction follows. Ehlers and Hjortdal[86] reported a reduction in corneal thickness in 10 of 11 eyes treated with CXL with the majority experiencing some improvement in vision. Wollensak *et al* [87] examined if this effect can be used for the treatment of bullous keratopathy. This clinical interventional case series included 3 patients with bullous keratopathy due to pseudophakia, corneal transplant rejection and Fuchs' endothelial dystrophy. The bullous changes of the epithelium were markedly improved, resulting in loss of pain and discomfort. After dehydration for 1d using 40% glucose, the standard CXL technique was used by Wollensak *et al* [87] for treatment. Corneal thickness was reduced by 90.33±17.04 microns 3d after crosslinking and by 93.67±14.22 microns 8mo after CXL. Visual acuity was significantly improved in the case that did not present prior stromal scarring. In similar results concluded Krueger *et al* [88] who performed staged intrastromal delivery of riboflavin with UVA crosslinking in a case of advanced bullous keratopathy. Despite the encouraging results, longer follow-up is necessary to confirm the long-term impact of CXL in bullous keratopathy. However, this putative application for CXL is attractive in that it offers the potential to reduce the need for corneal transplantation in a condition other than keratoconus. It may also offer another means of controlling pain in patients with bullous keratopathy who are either unsuitable for or awaiting keratoplasty.

Corneal Collagen Crosslinking in Infectious Keratitis The antimicrobial effects of the photoactivation of riboflavin may also be harnessed to treat infections of the cornea. Riboflavin has a modest affinity for nucleic acid and its absorption of UVA leads to the oxidation of guanine bases, thus preventing the replication of the viral and bacterial genome [89]. This effect is synergistic with any direct antimicrobial effect of UVA irradiation itself and with any damage to microbial cell membranes and DNA caused by oxygen radicals. In 2008, Martins *et al* [90] demonstrated the antimicrobial properties of CXL against common pathogens. A group of bacteria include pseudomonas aeruginosa (PA), staphylococcus aureus (SA), staphylococcus epidermidis (SE), methicillin-resistant S. aureus (MRSA), multidrug-resistant P. aeruginosa (MDRPA), drug-resistant Streptococcus pneumoniae (DRSP), and candida albicans (CA) was tested. Riboflavin/UVA was effective against SA, SE, PA, MRSA, MDRPA, and DRSP, but was ineffective on CA.

Iseli *et al* [24] evaluated the efficacy of CXL for treating infectious melting keratitis. Five patients with infectious keratitis associated with corneal melting were treated with CXL. CXL was performed when the infection did not respond to systemic and topical antibiotic therapy. Follow-up after crosslinking ranged from 1 to 9mo. In all cases, the progression of corneal melting was halted after CXL treatment. Emergency keratoplasty was not necessary in any of the 5 cases presented. CXL should be proven capable of sterilizing the cornea to a diameter of at least 8 mm and to a depth that includes the anterior and mid-stroma, it follows that many corneal infections may be controlled with a single treatment.

Corneal Collagen Crosslinking in Complicated Bullous Keratopathy With Ulcerative Keratitis Experimental evidence that CXL increases the resistance of porcine

corneal stroma to digestion by collagenase and other proteolytic enzymes has led to the suggestion that CXL may have a role in slowing or preventing stromal ulceration resulting from infective, traumatic or immune-mediated corneal disease [28]. CXL's antimicrobial and anti-edematous properties were demonstrated by Kozobolis *et al* [91]. In their report of two patients with combined bullous keratopathy and ulcerative keratitis, resistant to conventional treatments. Both patients presented significant improvement of their vision-threatening corneal ulcer, corneal edema and BCVA for a follow-up period of two months.

Donor Cornea Modification As CXL is capable of depleting the stroma of keratocytes and other antigen-presenting cells, it has been suggested that pretreatment of donor corneal tissue may prevent or reduce the severity of allograft reactions following penetrating keratoplasty and thereby prolong graft survival [92].

Other Potential Applications Although CXL resulted in a decrease of SEQ, astigmatism and max K, uncorrected visual acuity (UCVA) and BSCVA increased only modestly in the majority of studies of CXL for keratoconus. Other studies with alternative treatment methods for keratoconus, such as implantation of intracorneal rings, have reported more than a two-line increase in BSCVA [93,94]. These observations lead us to the following hypothesis: If the treatment with CXL stops or slows the progression of keratoconus, while other methods can reshape the cornea, a logical solution would be to combine the two treatment methods in order to synergize their effects. In this combined method, a pre-treatment with an alternative method would significantly reshape the cornea by flattening and regularizing corneal shape, which would be followed by CXL to stabilize the cornea. Alternatively, the CXL procedure could be performed first, followed by a reshaping procedure.

CXL Combined With Intracorneal Rings Kamburoglu *et al* [95] reported a case of post-operative LASIK ectasia that underwent Intacs SK implantation and CXL treatment in both eyes. The pre-operative BSCVA was 20/60 in the right eye and 20/80 in the left eye. The pre-operative SEQ was -14.50 D in the right eye and −10.50 D in the left eye. Mean keratometry pre-operatively was 56.20 D in the right eye and 50.70 in the left eye. Following bilateral Intacs SK implantation, CXL was performed the following day in the left eye and after 1mo in the right eye. Eight months after combined treatment, BSCVA was 20/25 and 20/25, manifest refractions were -1.50 ×170 and -1.25 ×50 and mean keratometric values were 47.20 and 44.20 D in the right and left eyes, respectively. In 2007, Chan *et al* [29] performed a retrospective, non-randomized, comparative case series of 12 eyes of nine patients who had inferior-segment INTACS placement without CXL and 13 eyes of 12 patients who had inferior-segment INTACS placement followed by CXL. The INTACS with CXL group had a significantly greater reduction in cylinder than the INTACS-only group and there was a significantly greater reduction in Kmax in the INTACS with CXL group Chan *et al* [29] concluded that the addition of CXL to the INTACS procedure resulted in greater improvements than INTACS insertion alone for keratoconus cases.

Corneal Collagen Crosslinking Combined With Limited Topoguided Photorefractive Keratectomy One of the most promising uses of the CXL procedure is in combination with a modified version of PRK. In a prospective study, Kanellpoulos and Binder [84] included a total of 325 eyes with keratoconus. The first group (n=127 eyes) underwent CXL with subsequent topography-guided PRK performed 6mo later (sequential group) and the second group (n =198 eyes) underwent CXL and PRK in a combined procedure on the same day (simultaneous group) using the Allegretto (WaveLight, Erlangen, Germany) topography- guided laser platform to normalize the shape of the cornea. Statistically, the simultaneous group performed better in all parameters evaluated, including UCVA and BSCVA, SEQ refraction and keratometry, and less corneal haze [84]. It is important to emphasize that combined treatment of CXL/PRK is a specialized inter vention with the goal of normalizing the cornea as much as possible to increase BSCVA rather than treating the refractive error itself. Therefore, the primary treatment target is cylinder in order to improve the irregular astigmatism and the secondary target is correcting some of the sphere. Most importantly, the eye may not require a corneal transplant.

Corneal Collagen Crosslinking Combined With Conductive Keratoplasty Kymionis *et al* [96], recently showed that corneal remodeling with conductive keratoplasty in patients with keratoconus seems to have a temporary effect despite the subsequent application of CXL in two patients with keratoconus. Conductive keratoplasty spots were applied on the flatter side of the cornea followed by CXL. Immediately after conductive keratoplasty, a significant corneal topographic improvement was observed. However, the effect of conductive keratoplasty regressed 3mo post-operatively and remained unchanged until the sixth post-operative month in both patients.

CONCLUSION

Keratoconus is a progressive ectatic disorder leading to visual deterioration due to irregular astigmatism and in advanced cases corneal scarring. CXL with riboflavin and UVA irradiation is a minimal invasive technique that modifies corneal stromal structures and increases corneal stability. Collagen crosslinking is recommended for patients with early keratoconus who cannot be optically corrected

and those who demonstrate recent progression. It may be preferable to delay such treatment for patients that are adequately corrected and show very slowly progressive or non-progressive disease. Some studies have detected a high rate of progression of keratoconus in younger patients, so it has suggested to treat with CXL at the diagnosis, and not to wait for progression. It is also proved the efficacy of the procedure in reducing the corneal curvature, SEQ refraction and refractive cylinder in keratoconic eyes after the application of CXL. A reduction in corneal curvature, SEQ, and cylinder has detected in some eyes and cannot be "promised" to all patients. The safety of the method is also demonstrated from the fact that there was no discrepancy in terms of endothelial cell density between treated and no treated eyes. As long as the corneal stroma treated has a minimal thickness of 400 microns (as recommended), neither corneal endothelium nor deeper structures such as lens and retina will suffer any damages. The CXL technique is promising in treating corneal melting conditions or infectious keratitis because cross-linking would strengthen a collagenolytic cornea while UVA irradiation eliminates the infectious agent. CXL anti-edematous and antimicrobial properties were demonstrated in a series of studies suggesting its therapeutic indications in bullous keratopathy and in infectious keratitis as an adjuvant treatment to conventional therapeutic modalities. CXL appears to be a promising tool whose indications and results should be more investigated. Apart from haze and stromal hyper density after CXL with early or late onset as direct complication of the treatment, no other direct or primary complications of the procedure have been reported. Complications described in the literature are in the major part of indirect origin [infections, therapeutic contact lens, previous surgery (LASIK), coexisting disorders of ocular surface, incorrect patient inclusion in the treatment, technical problems with UVA solid state emitter, wrong technique application, bad focusing, tilting, defocus, *etc*.]. Therefore, only surgeons with sufficient experience in the management of corneal wound healing should perform this procedure. Repeat crosslinking treatments may become necessary in the long term. Considering that the turnover rate of stromal collagen fibers is several years, prospective studies with a follow up of at least eight to ten years will be necessary.

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