

## Corneal Crosslinking with Riboflavin and Ultraviolet A. I. Principles

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**ABSTRACT** Changes in the biomechanical properties of the human cornea play an important role in the pathogenesis of corneal ectatic diseases. Biomechanical investigation shows significant differences between human ectatic corneas and normal corneas, including decreased stiffness and reduction of collagen crosslinks in the ectatic cornea. Induction of crosslinks is a well-established procedure in polymer chemistry to increase the elastic modulus of materials. Crosslinking (CXL) in connective tissue can occur during aging and as a side effect of diabetes mellitus. CXL has been used medically to increase stability and reduce the biodegradation of collagen-based biomaterials for bio-prostheses. CXL of the cornea using riboflavin and UVA light with a wavelength of 370 nm and a dosage of 5.4 J/cm<sup>2</sup> is a new approach that increases the mechanical and biochemical stability of stromal tissue. This technique combines the principles of CXL (chemical and nonenzymatic) and the biochemical mechanisms of photo-oxidative CXL with riboflavin as a photosensitizer. In this review, the enrichment of riboflavin in the stroma by standard (epi-off) and transepithelial (epi-on) CXL is discussed. The theoretical and experimental measurements of the absorption of UV light explain the stronger CXL effect in the anterior stroma and its importance for the prevention of damage to the

endothelial cells. UV devices are described. Changes of the physical properties after CXL, as well as the cellular changes, are discussed. From these basic investigations, treatment parameters for effective and safe CXL are identified.

**KEY WORDS** cornea, crosslinking, keratectasia, keratoconus, riboflavin, ultraviolet radiation

### I. INTRODUCTION

The term crosslinking (CXL) is used in the biological sciences to express the formation of chemical bridges after chemical reactions between proteins or other molecules. The crosslinks can be formed by chemical reactions that are initiated by heat, pressure, or radiation. The result of such reactions is change to the physical properties of the crosslinked material. During the natural aging process of the human body, both enzymatic and nonenzymatic CXL reactions occur in various parts of the organism. One of the observations that led to the concept of CXL for the treatment of corneal ectatic diseases is that patients suffering from diabetes mellitus tend not to show progression of corneal ectasia because of naturally occurring nonenzymatic CXL.<sup>1-4</sup> Corneal collagen CXL with the use of riboflavin and ultraviolet A irradiation (UVA) is a novel treatment for corneal ectatic diseases.

Since the publication of the first paper on this topic 15 years ago, almost 400 papers dealing with corneal CXL and its new modalities have been published and can be found on PubMed. Until recently – in the “pre-CXL era” – all of the treatment options for corneal ectatic diseases addressed only the refractive consequences of the disorder and not the underlying pathology, namely a stromal instability stemming from collagen abnormalities. CXL directly targets these stromal imbalances by using UVA and a photosensitizer to create new covalent crosslinks between collagen fibrils to increase corneal stiffness.<sup>5</sup> The most common use of CXL is to manage ectatic corneal disorders by halting their progression. Nevertheless, a continuous line of new applications is under clinical investigation with promising results.

In the case of keratoconus, visual acuity worsens due to an increasing irregularity in the corneal surface, which causes deterioration of the optical imaging properties.

Accepted for publication December 2012.

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This work received no sponsored support.

The authors have no proprietary or commercial interest in any concept or product discussed in this article.

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Part II of this review (Clinical Indications and Results) is published in the Clinical Practice section of this issue of *The Ocular Surface*.

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© 2013 Elsevier Inc. All rights reserved. *The Ocular Surface* ISSN: 1542-0124. Raiskup F, Spoerl E. Corneal crosslinking with riboflavin and ultraviolet A. I. Principles. 2013;11(2):65-74.

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Although in keratoconus, the total collagen content of the cornea does not differ significantly from that of the normal cornea, corneal stiffness is decreased by a factor of approximately 0.7.<sup>6</sup> This suggests that disruption of the CXL within (eg, within tertiary and quaternary structure) and/or between the collagen molecules must be present. Reduction of the CXL has been discussed as a cause of keratoconus, among other things,<sup>7</sup> but no experiments to correct this pathological condition have been undertaken.

A similar reduction of corneal stiffness also occurs after laser in situ keratomileusis (**LASIK**), where in very rare cases, owing to complex causes (eg, pre-existing keratoconus, excessive tissue ablation, connective tissue diseases, etc.), keratectasia can develop. LASIK weakens the biomechanical stiffness of the cornea because the effective corneal thickness is reduced as a result of the thickness of the flap plus the depth of the ablation. If the remaining corneal stroma can no longer entirely compensate for the higher mechanical tension (force per cross-sectional area), then there will also be a bulging of the cornea with a deterioration of the optical imaging properties.<sup>8</sup>

The goal of collagen CXL with riboflavin/UVA light is to artificially increase the degree of crosslinks in the corneal

stroma and thus to reestablish sufficient mechanical stability. In this review (Part I), the fundamentals of photo-oxidative collagen CXL with riboflavin and UV light are described. Clinical applications of CXL are discussed in Part II, which is published in the *Clinical Practice* section of this journal.

**II. PRINCIPLES OF CXL****A. Mechanical Stability of Tissues and Crosslinks Containing Collagen**

The mechanical stability of the cornea is primarily determined by the structure of the collagen molecules and their spatial arrangement, that is, by the long collagen fibrils, which branch but nevertheless extend from limbus to limbus. Crosslinkages stabilize this mechanical state and also prevent the collagen fibrils in the curved cornea from sliding apart. Pathological changes to the tissue occur because of either an increase in the degree of crosslinkage (eg, in diabetes mellitus or scars) or a decrease (eg, in Ehlers-Danlos syndrome).

The maintenance of the physiological function of the degree of crosslinkage must be very well regulated. With age alone, the number of crosslinks, along with the rigidity of the structures, increases.<sup>9,10</sup> This can be observed in the cornea, the skin, the ocular lens, the blood vessels, and the cartilage of the joints. Sunlight and smoking<sup>11,12</sup> cause analogous changes. The biochemical process of CXL is exploited to stabilize the tissues that contain collagen,<sup>13</sup> for collagen-based implants and tissue constructions used in tissue engineering,<sup>14</sup> and in the treatment of keratoconus<sup>15</sup> or for keratectasia following LASIK.<sup>16,17</sup>

**B. CXL Production****1. Physiological Basis**

Under physiological conditions, collagen molecules are enzymatically crosslinked in the extracellular space by the enzyme *lysyl oxidase* after they have left the cell (post-translationally).<sup>18</sup> The collagen thereby attains its natural firmness, stability, and tissue-specific elastic properties. Lysyl oxidase transforms the amino groups of certain amino acids into aldehyde groups, and these groups can either spontaneously react with neighboring aldehyde groups in an aldol condensation reaction or react with  $\epsilon$ -amino groups of amino acids to create covalent, aldimine crosslinkages. In Ehlers-Danlos syndrome, for example, there is a lysyl oxidase deficiency; in keratoconus, it is assumed that a lysyl oxidase gene defect is present<sup>19,20</sup> or that an increased pH level in the tear liquid, seen with keratoconus, interferes with the activity of the lysyl oxidase<sup>21</sup>; and with keloids and scars, the activity of this enzyme is heightened.<sup>22</sup>

In addition, nonenzymatic CXL can be produced by<sup>23</sup>:

- 1) *Chemical CXL agents* (glutaraldehyde, formaldehyde, diphenylphosphoryl, nitroalcohols,<sup>24,25</sup> genipin<sup>26,27</sup>).

The preferred use of these agents is for the modification of the properties of tissues containing collagen in the process of tissue engineering.

- 2) *Sugar aldehydes (advanced glycation endproducts [AGEs])*. With age, and particularly in diabetes mellitus, these sugar crosslinkages increase. These AGEs-linkages have a protective effect against the development of keratoconus (prevention or a decrease in the severity of the keratoconus).<sup>28,29</sup>
- 3) *Photo-oxidative CXL (UV, ionizing radiation)*.

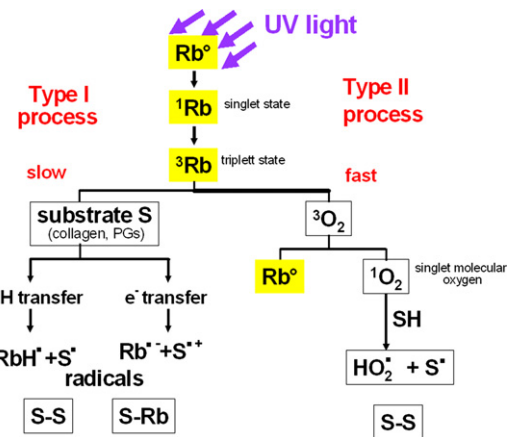
## 2. Photo-Oxidative CXL with Riboflavin and UVA Light

The photo-oxidative CXL method with riboflavin and UVA light was chosen for stiffening of the cornea because it has a localized effect, a short period of therapy is sufficient, and it leaves the transparency of the cornea unaltered. Riboflavin (vitamin B<sub>2</sub>) is also used as a coloring agent in food processing (eg, vanilla pudding). It is nontoxic and is bioavailable as medication. In this photochemical reaction, radicals are created by UV light. To increase the effectiveness of this process in the presence of UV radiation, riboflavin, a special photosensitizer (transfer molecule), is used. If riboflavin absorbs energy from UV light, it excites (excited singlet riboflavin <sup>1</sup>Rf\*, lifetime 10<sup>-8</sup>s). In an exchange mechanism, the excited singlet riboflavin is transformed into a triplet-excited riboflavin (<sup>3</sup>Rf\*, lifetime 10<sup>-2</sup>s).<sup>30</sup> Thus, type I and type II reactions can be differentiated (Figure 1). For type II reactions, oxygen is necessary to form singlet oxygen. Singlet oxygen is the physically excited form of the oxygen molecule<sup>31</sup>; the number of electrons does not change, but, rather, the spin, ie, the direction in which an electron is turning, changes. Through interaction with triplet oxygen (<sup>3</sup>O<sub>2</sub>), very reactive singlet oxygen (<sup>1</sup>O<sub>2</sub>), an oxygen radical that continues to interact with the carbonyl group of collagens, is created. However, if the necessary oxygen is depleted by UV, then type I reactions dominate.<sup>32</sup> In CXL, both reactions take place.

In this photochemical process, active locations along the molecule chain react with each other, intermolecularly or intramolecularly, and create covalent connections between the amino acids (especially histidine, hydroxyproline, hydroxylysine, tyrosine and threonine); thus, the so-called crosslinkages are created. The formation of dityrosine from tyrosine, which can create the intermolecular and intramolecular linkages between collagen molecules, has also been observed.<sup>33-35</sup> The amine groups do not play a major role in riboflavin-UV CXL.<sup>36</sup> However, this CXL is carbonyl-dependent and involves the formation of advanced glycation end-product crosslinks,<sup>37</sup> such as in age-related CXL. Recent work indicates that CXL generates crosslinks not only between collagen molecules but also between proteoglycan core proteins.<sup>38</sup> Thus, interfibrillar bonds appear to also be possible, whereas the CXL does not increase interlamellar crosslinks.<sup>39</sup>

The prerequisite for the initiation of a chemical reaction by light is the absorption of this light by the reactive system. The photochemical CXL effect (photopolymerization) occurs only where riboflavin is activated by UV light.

Riboflavin is effective not only as a generator of singlet oxygen but also as a radical scavenger at high



**Figure 1.** Excitation of riboflavin and the two possible reaction mechanisms.

concentrations; thus, there is a balance between the formation and the destruction of singlet oxygen.<sup>30</sup> Hence, the increase in the concentration of riboflavin does not necessarily increase the rate of creation of singlet oxygen; rather, a state of saturation is reached.

## C. Enrichment of Riboflavin in the Stroma

A sufficient concentration of riboflavin is prerequisite for a strong biomechanical CXL effect in the stroma. This concentration in the stroma can be achieved by several methods: 1) diffusion in the de-epithelialized stroma (standard method); 2) diffusion through the epithelium into the stroma (transepithelial method); or 3) direct introduction of riboflavin into the stroma (pocket technique, ring technique, needle technique).

### 1. Standard or “Epithelium-Off” Method

The intact epithelium constitutes a diffusion barrier for riboflavin (molecular weight 376 g/mol) and must therefore be mechanically removed prior to the application of the riboflavin drops.<sup>40,41</sup> A certain amount of time is required for the diffusion of riboflavin into the deeper levels of the stroma, in accordance with the law of diffusion.<sup>42,43</sup> For this reason, it is very important to instill drops of riboflavin solution onto the de-epithelialized cornea approximately 20-30 minutes prior to radiation to achieve a high total concentration and a high total absorption, thus guaranteeing the protection of the corneal endothelium, the lens, and the retina. In the anterior stroma, the concentration is the highest, and 30 minutes after riboflavin application, a dynamic equilibrium has been established and the concentration will show a linear decrease deeper in the stroma, as has been demonstrated by calculations and measurements.<sup>42,44-47</sup>

### 2. Transepithelial or “Epithelium-On” Method

The penetration of riboflavin through the epithelium can be increased by several methods: 1) increasing contact time (viscous solution, ring application); 2) changing the permeability of the epithelium (benzalkonium chloride ([BAC],

ethylendiaminetetraacetic acid [EDTA], channel forming peptides, mechanical changes); or 3) altering physicochemical properties of the riboflavin solution (osmolarity, iontophoresis, concentration).

BAC increases epithelial permeability by loosening the tight junctions. This pharmacological modification of corneal epithelial permeability represents a novel method to avoid epithelial debridement in CXL.<sup>48,49</sup> Therefore, some surgeons modify the standard protocol and perform the treatment without removing the epithelium by using BAC, tetracaine, or pilocarpine containing BAC and EDTA.

The first systematic study concerning the CXL procedure without epithelial debridement was performed by Wolensak et al, who tested iso-osmolar riboflavin (with 20% dextran) + 0.005% BAC in rabbit eyes and observed a slight biomechanical effect.<sup>50</sup> Kissner et al tested several BAC concentrations with 0.1% riboflavin in 0.44% NaCl solution on rabbit eyes and observed good UV absorption and a biomechanical effect with a solution containing 0.02% BAC.<sup>51</sup>

An osmotic gradient from the apical to the basolateral side increases paracellular conductance with no ionic selectivity. Basolateral hypo-osmolarity increases paracellular conductance, and apical hypo-osmolarity also moderately increases paracellular conductance. A hydrostatic pressure gradient works as a driving force for water movement. Whereas an osmotic gradient also works as the driving force for water movement, ie, if the permeability of water is adequately larger than a certain solute, the concentration difference of the solute works as the driving force for water movement. Dextran inhibits the increase in paracellular conductance. These inhibitions are dependent on the concentration of dextran, regardless of the side of administration. For this reason, the riboflavin solution used in transepithelial CXL procedures should not contain dextran. However, the solution should contain 0.01% BAC and 0.44% NaCl to increase permeability of the epithelium, which will allow the riboflavin to reach a high concentration in the corneal stroma.<sup>52</sup>

A promising method seems to be the use of channel-forming peptides (NC-1059) to transiently open the intact epithelium barrier to allow the permeation of riboflavin into the stroma. This method should not be too toxic for epithelial cells. The first experimental studies testing this peptide for transepithelial CXL are very encouraging.<sup>53-55</sup>

The permeability of the epithelium can also be increased by mechanical modification, such as the creation of pockmarks in the epithelium with a Daya disruptor.<sup>56</sup>

### 3. Direct Application

Daxer proposed the direct application of riboflavin into the stroma by making a pocket in the stroma and instilling the riboflavin into it.<sup>57</sup> The same technique is used if intracorneal rings are implanted. Thus, the canal is filled with riboflavin, and the cornea is crosslinked.<sup>58-60</sup>

Another possible approach is to deliver the riboflavin through a hole micro-needle array, which is inserted

through the epithelium into the anterior stroma. This technique is currently under investigation.<sup>61</sup>

### 4. Iontophoresis

Iontophoresis is a noninvasive technique in which a weak electric current is used to enhance the penetration of electrically charged molecules into tissue. Riboflavin is negatively charged and suitable for iontophoresis. During the 5-minute iontophoresis, a sufficient riboflavin concentration is achieved in the stroma for CXL. Thus, this technique not only leaves the epithelium intact but also shortens the pretreatment time.<sup>62</sup>

### D. Absorption of the UV Radiation in the Cornea

The CXL effect can be confined to the corneal stroma if the majority of the UV light is absorbed there, ie, radiation has an effect only where it is absorbed and thus transfers energy to that tissue (first law of photochemistry).

For two reasons, a riboflavin concentration of 0.1% was chosen for the treatment of a stroma with a thickness of approximately 400  $\mu\text{m}$  (this value corresponds approximately to the average for keratoconus).<sup>63</sup> First, the biomechanical effect in a large area of concentration (0.015 to 0.5%) is independent of the concentration.<sup>64</sup> Second, low concentration avoids UV damage.<sup>40</sup> A concentration of 0.1% produces a large absorption coefficient,<sup>65,66</sup> so that 90% of the UV radiation is absorbed in the stroma, while the endothelium, the ocular lens, and the retina remain largely protected from the UV light. Because of the high absorption coefficient ( $\mu = 50 \text{ cm}^{-1}$ ), the irradiation intensity of the UV light is decreased enough on its way through the cornea that with a radiation strength of 3  $\text{mW/cm}^2$ , the destruction threshold for the endothelium is not reached.<sup>42</sup> However, the absorption coefficient and concentration must also not be too high so that a layer of the stroma as thick as possible is crosslinked.

In a first approximate model, the concentration of riboflavin and the UV dosage can be used to calculate the CXL strength at a certain depth in the stroma.<sup>43</sup>

## III. UV DEVICES

Riboflavin has two absorption maxima at 365 nm and 430 nm; radiation with 365 nm achieves a greater CXL effect ( $W = h \cdot c / \lambda$ ) because of the higher energy content ( $\lambda = \text{wavelength}$ ,  $c = \text{velocity of light}$ ,  $h = \text{Planck's constant}$ ).<sup>67</sup> Because of the absorption maximum of riboflavin at 365 nm, this wavelength was specially chosen for the treatment with UV light. This achieves 90% absorption of the UV light in a 400- $\mu\text{m}$ -thick de-epithelialized cornea without endangering the lens or the cornea.

The irradiation procedure is independent of the nature of the riboflavin application.

Today, various systems that use UV light emission diodes and supply a homogeneous irradiation strength of 3  $\text{mW/cm}^2$  on a circular area 8 mm in diameter on the cornea are available as sources of UV radiation (eg, CCL-365, PESCHKE Meditrade GmbH, Huenenberg, Switzerland; UV-X<sup>TM</sup>, IROC GmbH, Zurich, Switzerland; CBM VEGA

X-linker; Construzione Strumenti Oftalmici, Florence, Italy). Some devices make it possible to choose the size of the area to be irradiated. The choice of an area 8 mm in diameter guarantees CXL in the central cornea only, without irradiation of the limbus, the sclera, or the goblet cells. The high degree of homogeneity of this radiation can, for example, be achieved with the irradiation device UV-X<sup>TM</sup> with a special radiation homogenizer, which prevents local radiation peaks ("hot spots" at individual diodes), which could cause local damage to the endothelium.

Studies on the biomechanical effect of irradiation produced at an irradiance of 3 mW/cm<sup>2</sup> for periods of 5 to 60 minutes showed an optimal irradiation time of 30 minutes.<sup>68</sup> A significant biomechanical increase in stiffness begins at 15 minutes, and an irradiation longer than 45 minutes achieves no further stiffening. Lanchares et al found that in porcine corneas, even after 60 minutes of irradiation, there was no longer any significant stiffening effect.<sup>69</sup> This phenomenon is known as the photochemical effect of ionizing or ultraviolet radiation.<sup>70</sup> There are two opposing photochemical processes: the accumulation of crosslinkages and the reduction of crosslinkages,<sup>71</sup> and oxygen in the tissue could be responsible. In the beginning, oxygen is still present in a quantity sufficient to make the formation of crosslinkages possible. However, if oxygen is used up, the light energy is used to interfere with CXL.<sup>32</sup>

All parameters (irradiation intensity, irradiation time, and riboflavin concentration) have been tested in experiments,<sup>67</sup> including animal experiments,<sup>40</sup> and have proven themselves in clinical studies.<sup>15</sup>

In accordance with the photochemical law of reciprocity (Bunsen-Roscoe law), one achieves the same photochemical effect with a reduced irradiation time and a correspondingly increased irradiation intensity, such that the total dose remains the same. Such devices have been offered from companies such as IROC AG (UV-X<sup>TM</sup> 2000, Zurich, Switzerland), PESCHKE GmbH (CCL-VARIO, Huenenberg, Switzerland) and Avedro, Inc. (KXL<sup>TM</sup>, Waltham, MA, USA). In biomechanical studies, no difference could be noted between different irradiation times (irradiation intensities) when the radiation dose was the same.<sup>72</sup> Early clinical results confirm this relationship.

#### IV. EFFECTS AND EVIDENCE OF CXL

##### A. Ex Vivo Evidence

Currently, the photochemically induced crosslinks in the cornea cannot be made visible directly by means of coloring methods or microscopic techniques. However, CXL causes changes to numerous physicochemical properties of the collagen-containing tissue, from which one can draw the indirect conclusion that CXL has taken place. Some CXL-produced changes to the corneal stroma include:

- 1) *Increases in the stiffness, the bending stiffness, and the modulus of elasticity.* The cornea that has been cross-linked with riboflavin/UVA is firmer than the normal cornea by a factor of 1.7; the firming effect is thus sufficient to compensate for the decreased stability

seen in eyes with keratoconus.<sup>67,73,74</sup> The firming effect is greater in corneas in which there is a higher collagen content and in older corneas.<sup>68,73</sup>

- 2) *An increase in the shrinkage temperature.* The shrinkage temperature of the cornea is increased after riboflavin/UVA treatment from 63°C to 70°C. This also constitutes proof of the induction of CXL because the shrinkage temperature is positively correlated with the degree of CXL.<sup>4,25</sup>
- 3) *A decrease in the swelling percentage.* Crosslinked collagen exhibits a decreased tendency to swell,<sup>75</sup> which could be exploited in the treatment of bullous keratopathy and decompensated Fuch's endothelial dystrophy.<sup>76</sup>
- 4) *An increase in the thickness of the collagen fibers.* The treatment also causes a 4.5% increase in the diameter of the collagen fibers in the anterior stroma.<sup>77</sup>
- 5) *An increase in the resistance to enzymatic degradation processes.* In keratoconus, the proportions of collagen-degrading enzymes in the tear liquid are increased, which can also contribute to a thinning of the corneal stroma.<sup>78,79</sup> A cornea that has been treated with riboflavin/UVA is not as quickly enzymatically degraded by collagenase as an untreated cornea.<sup>4</sup> This delayed degradation process also results in a lengthening of the turnover time of the collagen.
- 6) *The creation of molecular aggregates with greater molecular weights.*<sup>80</sup>
- 7) *A decrease in permeability.*<sup>81,82</sup> The degree of CXL increases with age and is cumulatively increased in long-lived proteins (eg, collagen). All of these changes to the physicochemical properties provide evidence that CXL has been induced in the cornea by the riboflavin/UVA treatment.

##### B. Collagen CXL: A Nonthermal Process

If radiation is absorbed by tissue, it is transformed into other forms of energy, usually heat. With combination of riboflavin and UV light, the result is transformation of radiation energy into various forms of energy, including fluorescent radiation, chemical energy (binding energy crosslinks), and, to a small extent, heat. The CXL process is energetically comparable to photosynthesis, in which radiation energy is transformed into chemical energy (glucose) with the aid of pigments (chlorophyll).

In CXL of the cornea, light energy leads to a chemical modification of the collagen. For this reason, there is a maximum temperature increase of 2-3°C during riboflavin/UV CXL,<sup>83</sup> and a thermal process is not possible. This temperature increase is well under the threshold for thermal damage to the collagen. If the applied irradiation intensity of 630 W/cm<sup>2</sup> (time=200 ms; diameter of the irradiated area=0.2 mm; power= 200 mW) used for photocoagulation is compared with the irradiation intensity of 3 mW/cm<sup>2</sup> used in UV CXL, which is 200,000 times weaker, it becomes clear that the thermal effect is negligible in the photochemical method of CXL.

### C. Greater CXL in the Anterior Cornea

With the photo-oxidative method, CXL is not homogeneous over the entire thickness of the cornea. Due to the strong absorption coefficient, the UV intensity decreases with increasing depth, which also suggests a decrease in CXL. A smaller increase in the fiber diameter of the posterior cornea,<sup>77</sup> a greater enzymatic degradation,<sup>4,84</sup> and a greater shrinking effect<sup>85</sup> in the posterior crosslinked cornea point to this decrease in CXL. In an experimental study on enucleated porcine eyes, it was shown that a stiffening effect was present only in the first third (approximately 200  $\mu\text{m}$ ) of the corneal stroma.<sup>74</sup> In these studies, two flaps with a thickness of 200  $\mu\text{m}$  were cut from one eye in each case. The stiffness of the first of the treated flaps was significantly higher than those of the second treated flap and the corresponding control flaps. The second treated flaps were only minimally stiffer than the second untreated flaps. Analogous results were also seen in biochemical studies of the CXL in stroma layers at different depths.<sup>84</sup>

The decreasing CXL effect with increasing depth can be explained as follows. The riboflavin concentration decreases linearly with increasing corneal depth, in accordance with the diffusion gradient.<sup>44</sup> Additionally, the exponential decrease of the UV intensity is much greater, in accordance with the Beer-Lambert law. Because of this decrease, 65% of the UV radiation is absorbed in the first 200  $\mu\text{m}$ . Because the CXL effect is primarily dependent upon the intensity of the UV irradiation and, to a lesser extent, upon the concentration of riboflavin, the stiffening effect is greatest in the first 200-250  $\mu\text{m}$ .

### D. The Influence of CXL on Intraocular Pressure Measurement

In applanation tonometry, the corneal thickness and stiffness influence the measurement of intraocular pressure (IOP). Thus, it is to be expected that after CXL and stiffening of the cornea, the IOP measurement would be too high.

In an *in vitro* model on human corneas, an overestimation of the IOP by Goldmann applanation tonometer after CXL was in the range of 1.3-3.1 mm Hg.<sup>86</sup> However, because the cornea becomes firmer by only a factor of 1.7, ie, the Young's modulus increases by a factor of 1.7, the IOP is measured as, at most, 1.6 mm Hg too high, according to a calculation by Liu.<sup>87</sup> In a study involving a large number of cases, this effect—an IOP value that is too high after CXL—was demonstrated with statistical significance.<sup>88</sup> However, the influence of CXL on IOP measurements in individual cases is insignificant because of variations in IOP.

### E. Cellular Changes after CXL

In addition to the biomechanical stiffening effect of the CXL procedure, the reaction of the corneal cells, especially with regard to safety, was also investigated. The keratocytes, endothelial cells, limbal epithelial stem cells, and goblet cells were investigated immediately after CXL and at follow-up, using histological methods and *in vivo* confocal microscopy.

#### 1. Epithelial Cells

The removed epithelium was replaced by the remaining epithelial cells from the periphery in the 3-4 days following CXL. The limbal stem cells were not damaged during CXL if they were protected by the epithelial cells, which kept the riboflavin away.<sup>89</sup>

*In vivo* scanning laser confocal analysis revealed that epithelium was very thin (10-20  $\mu\text{m}$ ) 1 month after the CXL procedure in the apex region of keratoconus, and normal epithelium thickness, resembling preoperative data, was detected between 3 and 6 months after CXL.<sup>90</sup>

#### 2. Keratocytes

As a result of riboflavin/UVA treatment, apoptosis of keratocytes occurs in the anterior stroma (250-300  $\mu\text{m}$ ).<sup>91-93</sup> This was extensively investigated by histology and *in vivo* confocal laser scanning microscopy.<sup>94,95</sup> Keratocyte apoptosis is accompanied by a lacunar edema that is anterior to the apoptotic keratocytes because of entrapped edema.<sup>96</sup> In experimental studies with standard CXL, a toxic threshold of 0.5 mW/cm<sup>2</sup> for 30 min (dosage 0.65J/cm<sup>2</sup>) was measured for keratocytes in rabbits.

In the weeks following CXL, new, activated keratocytes migrate from the periphery to the center.<sup>97,98</sup> These activated keratocytes or fibroblasts did not contain  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)<sup>99</sup> and did not exhibit a myofibroblast phenotype.

A major finding of *in vivo* confocal microscopy in the process of stromal healing was increased density (hyper-reflectivity) of the extracellular matrix combined with the presence of keratocyte nuclei between 3 and 6 months after CXL; this was detected consistently at later follow-up. Another major finding was evidence of collagen compaction by new structured fibers in the anterior-mid stroma after CXL, expressed by late hyperdensity of the extracellular matrix, with activated (hyper-reflective) keratocyte nuclei and elongated cell processes.<sup>89</sup> CXL induces cellular wound healing mechanisms and alters the normal structure and cellularity of the cornea for up to 36 months.<sup>100</sup>

#### 3. Endothelial Cells

The cytotoxic effect of CXL was investigated in porcine endothelial cell cultures<sup>101</sup> and in rabbits<sup>102</sup> to determine the toxic threshold of 0.35 mW/cm<sup>2</sup> for 30 min to prevent irradiance of the endothelium in treatment. A statistically nonsignificant reduction in endothelial cell count with respect to physiological reduction was observed after treatment (approximately 2% per year). Endothelial cell density and morphology were unaltered at corneal confocal microscopic examination at 1-year post-CXL treatment.<sup>90</sup>

#### 4. Nerves

After CXL, the subepithelial nerve plexus disappears.<sup>97,98,100</sup> Xia et al identified significant alteration of the morphology of the corneal nerves immediately after CXL, with regeneration occurring after 7 days.<sup>103</sup>

## V. CXL IN THIN CORNEAS

Thin corneas with a stromal thickness of less than 400  $\mu\text{m}$  are excluded from the standard treatment so as to avoid damaging the endothelial cells with the UV radiation.<sup>42</sup> Because many patients with keratoconus have a corneal thickness of less than 400  $\mu\text{m}$ , various methods of CXL have been developed to treat this patient population with varying degrees of success. Methods are described below.

- 1) Corneal thickness can be increased to 400  $\mu\text{m}$  by applying a hypo-osmolar riboflavin solution to produce swelling without reaching the toxicity threshold of the endothelium.<sup>104</sup> The CXL effect is comparable to that in a cornea with a thickness of 400  $\mu\text{m}$  because the anterior stroma barely swells while the posterior stroma swells greatly.<sup>105</sup> Using this technique, it is possible to treat corneas with a minimum stromal thickness of 320  $\mu\text{m}$ .<sup>106</sup> Positive clinical results from this technique have been recorded.<sup>107</sup>
- 2) It has been suggested that the irradiation dose can be decreased in accordance with the thickness of the stroma (shorter irradiation time at an irradiation intensity of 3  $\text{mW}/\text{cm}^2$ ) so as not to exceed the toxicity threshold of 0.63  $\text{J}/\text{cm}^2$  for the endothelium. This method has been used primarily for the CXL of thin corneas when no hypo-osmolar riboflavin solution was available for topical application.
- 3) To protect the endothelium from very high irradiation intensity, the epithelium is not removed from the thinnest place on a surface with a corneal thickness of <400  $\mu\text{m}$ .<sup>108</sup> However, with the "customized epithelial debridement technique," not enough riboflavin penetrates below the epithelium; thus, no keratocyte changes and no clear line of demarcation (as is present with complete epithelium removal) could be found, which suggests an insufficient biomechanical effect.
- 4) With a brief application of riboflavin to the surface, a sufficient concentration in the anterior stroma should be achieved without riboflavin reaching the endothelium. Because the toxicity threshold of the endothelial cells is much higher without riboflavin, the endothelial cells would thus be protected, even in thin corneas (KXL™ Technique). However, a prerequisite for this procedure is a short irradiation time with a high irradiation intensity (equal dose) to keep the diffusion time short. Unfortunately, no clinical experiences with this technique can currently be cited.
- 5) An increase in the concentration of riboflavin to 0.2% leads to a greater absorption of UV light in the anterior stroma and a decrease in UV exposure of the endothelium.

Combinations of various techniques described above, for example 1 + 5 or 3 + 5, increase safety during the CXL of thin corneas.

## VI. SAFETY OF CXL

If possible, exposure of the eye to UV light should be avoided. Health and safety regulations allow a daily UVA irradiation intensity of 1  $\text{mW}/\text{cm}^2$  without a photosensitizer for the unprotected eye.<sup>109</sup> Of this irradiation, approximately 35% is absorbed by the cornea, and it can be assumed that the endothelium is exposed to 0.65  $\text{mW}/\text{cm}^2$ . In animal experiments, the damage threshold of the endothelial cells for a 30-minute UVA irradiation without a photosensitizer was found to be 4  $\text{mW}/\text{cm}^2$ .<sup>102</sup> For collagen CXL with riboflavin and UVA light, an irradiation of 3  $\text{mW}/\text{cm}^2$  following the application of riboflavin is used. In a layer of stroma approximately 400  $\mu\text{m}$  thick, 90% of the UV radiation is absorbed in the cornea that has been treated with riboflavin, and the endothelium is exposed to only 0.18  $\text{mW}/\text{cm}^2$ .<sup>42</sup> This value is under the damage threshold of 0.35  $\text{mW}/\text{cm}^2$  established in animal experiments for the endothelium<sup>101</sup> and is also under 0.65  $\text{mW}/\text{cm}^2$ , which is referenced in health and safety regulations. For this reason, there is no danger to the lens or the retina.

Further safety for the retina can be achieved via the choice of irradiation equipment. Because of the short distance from the source of irradiation to the eye and the divergent irradiation (Koehler irradiation principle), the UV radiation is not focused on the retina. Thus, only very shallow irradiation densities, which lie well under the damage threshold, reach the lens and the retina. Using Scheimpflug imaging, Grewal<sup>110</sup> and Vinciguerra<sup>111</sup> found no significant difference in crystalline lens density before and 12 months after CXL. No retinal morphology changes after CXL were observed. Looking directly at the sun results in very high retinal irradiation densities because of the focusing effect.

## VII. SUMMARY AND CONCLUSIONS

The CXL procedure performed by means of riboflavin and UVA light have resulted in significant increases in biomechanical rigidity in porcine and human corneas. From other experiments on the diameter of corneal collagen fibers, resistance to enzymatic digestion, and keratocyte loss after the CXL procedure, we could see that the CXL and cytotoxic effects are significantly higher in the anterior portion of the corneal stroma. This is caused by the significant increase in UVA absorption by riboflavin, leading to a rapid reduction in UVA irradiation and collagen CXL across the cornea. The anterior localization of the main CXL effect has the advantage of allowing us to achieve a relatively high increase in corneal rigidity in human eyes because of the relatively small thickness of the human cornea and to spare the endothelium, lens, and retina from cytotoxic damage. Other CXL methods that have been proposed and tested in vitro have a biomechanical effect on the cornea comparable to that of riboflavin-UVA treatment, but they cannot be used clinically because they produce corneal haze and scarring.<sup>73</sup>

In summary, safe clinical application of CXL must adhere to the following requirements. 1) To facilitate

diffusion of riboflavin throughout the corneal stroma, the epithelium should be removed or a sufficient permeability of the epithelium must be guaranteed. 2) 0.1% riboflavin solution should be applied for at least 20 min before UV exposure (during UV exposure, the riboflavin serves as both a photosensitizer and a UV blocker). 3) The UV dosage of 5.4 J/cm<sup>2</sup> with a wavelength of 370 nm must be homogeneous. 4) The cornea to be crosslinked must have a minimal thickness of 400 μm to protect the endothelium.<sup>42</sup>

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