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A review of the epithelial and stromal effects of corneal collagen crosslinking

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Abstract

Induced corneal collagen crosslinking and mechanical stiffening via ultraviolet-A photoactivation of riboflavin (UVA CXL) is now a common treatment for corneal ectasia and Keratoconus. Some effects of the procedure such as induced mechanical stiffening, corneal flattening, and cellular toxicity are well-known, but others remain more controversial. Authors report a variety of contradictory effects, and provide evidence based on individual results and observations. A full understanding of the effects of and mechanisms behind this procedure are essential to predicting its outcome. A growing interest in modifications to the standard UVA CXL protocol, such as transepithelial or accelerated UVA CXL, makes analyzing the literature as a whole more urgent. This review presents an analysis of both the agreed-upon and contradictory results reported and the various methods used to obtain them.

Keywords

Cornea; Crosslinking; Collagen; Keratocytes; Epithelium; Keratoconus

1. Introduction

It has been accepted that the mechanical properties of the corneal collagen matrix greatly affect corneal shape and overall refractive power. Corneal ectatic diseases in which the collagen matrix is weakened such as Keratoconus or post LASIK ectasia lead to corneal thinning, a cone-shaped protrusion, and progressive corneal astigmatism. Keratoconus, specifically, is a non-inflammatory disease characterized by progressive biomechanical weakening of the cornea [1–3], though its non-inflammatory nature has been questioned in recent years [4–6]. Affecting 1 out of every 2000 people in the general population, it is

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the leading cause for corneal transplantation surgery [7–9]. A similar condition, post LASIK ectasia, results from complications of refractive surgery and is estimated to affect 1 in 4500 patients who undergo LASIK surgery [10].

A new treatment has emerged within the last two decades which attempts to prevent and reverse the progression of disease by restrengthening the corneal collagen matrix, known as ultraviolet-A riboflavin induced corneal collagen crosslinking, or UVA CXL. This technique, developed by Spoerl et al. and Wollensak et al. [11,12], utilizes photo-activation of riboflavin within the corneal stroma to induce covalent crosslinking within the corneal stroma [13,14]. UVA CXL has been shown in various animal and human models to produce a 2–3 fold increase in corneal elastic modulus, a lasting, long term increase in the stiffness of corneas up to 300% [11,15–17], and at least one diopter of corneal flattening lasting a year or longer [11,18–26]. The standard UVA CXL protocol, also known as the Dresden Protocol as described by Spoerl et al. and Wollensak et al., requires that the corneal epithelium be removed to allow penetration of the stroma with a photosensitizing riboflavin solution. The solution is dropped onto the corneal surface for 30 min to allow saturation of the stroma by the riboflavin solution. It is then irradiated with 370 nm UVA light at an irradiance of 3 mW/cm^2 for an additional 30 min. This wavelength of light activates the riboflavin molecules to an excited singlet state. If the excited riboflavin undergoes intersystem crossing to an excited triplet state, then a free oxygen radical can be produced, which then induces covalent crosslinks between and within the stromal proteins (collagen) and glycoproteins [13,14]. If intersystem crossing does not occur, the excited riboflavin returns to the ground state energy level, emitting green fluorescence [14]. UVA light excites all of the riboflavin in the optical path, quickly leading to attenuation as a function of depth, and reducing the efficiency of crosslinking deeper within the stroma. Since the main goal of this treatment is to strengthen the biomechanical properties of the corneal stroma, research surrounding the procedure has focused on its effects on the stromal extracellular matrix.

Collagen architecture greatly affects corneal shape, therefore many previous studies have focused on changes to the collagen fibers and/or lamellae post treatment. Various measurements of corneal collagen fibers have been made after UVA CXL, including waviness, fiber diameter, or interfibrillar spacing and the methods for obtaining these measurements are extremely varied. These include computational models, light microscopy, x-ray scattering, and many more [27–33]. In particular, second harmonic generation (SHG) signals, which are the result of frequency doubling of near infrared irradiation through noncentrosymmetric materials, has been useful for observing corneal collagen structure [1,18,27–40]. These studies have attempted to correlate changes in corneal collagen structure with the clinically observed corneal flattening [18–25].

While studies of the effects of UVA CXL on the extracellular matrix are important for the analysis of biomechanical effects, the effects of UVA CXL are not limited to the matrix alone. UVA CXL is also associated with a dose dependent cellular toxicity and corneal haze within the stroma. Epithelial cells are spared damage from UVA exposure only because they are removed prior to the traditional procedure, but stromal keratocytes and endothelial cells are both at risk of damage as cell death occurs within the photo exposed volume [30,41–44]. Post treatment, the epithelial barrier regenerates and corneal fibroblasts migrate

back into the treatment region, but if endothelial cells become damaged due to treatment of a thin cornea, they will never regenerate in the absence of pharmacological manipulation. In vivo confocal microscopy through focus (CMTF) has been used to monitor in vivo changes during the post procedure healing process and provide evidence of changes in corneal thickness, haze, and cellular regeneration [45–47].

Current research is focused on modifying the standard version UVA CXL for a variety of different reasons. For example, accelerated UVA CXL using a higher irradiance (30 mW versus 3 mW) has been attempted to reduce the procedure time from 30 min to 3 min but has been unable to reliably reproduce the results of traditional UVA CXL [48–56]. Various reports suggest this is possibly due to accelerated oxygen depletion during the high-irradiance procedure [14]. Additionally, various methods have been attempted to avoid the lengthy recovery time, patient pain, and risk of corneal infection associated with the epithelial debridement utilized in the approved Dresden protocol [20,57–75]. Similar to accelerated UVA CXL, *trans*-epithelial UVA CXL attempts have been reported as less satisfactory than expected compared to traditional UVA CXL. They have not shown a consistent reduction in disease progression, and in some cases have resulted in epithelial damage from the riboflavin penetration techniques alone [62,76–79]. In contrast, more success has been seen in the alteration of patterns of UVA exposure, such as Photorefractive Intrastromal Crosslinking (PiXL), to produce a more controllable flattening effect for the correction of small refractive errors [23,59,80–82].

While improvements in the traditional UVA CXL procedure would be desirable to avoid unwanted effects, it is necessary to fully understand the effects of corneal crosslinking on all components of the cornea in order to tailor it effectively. For that reason, this review discusses the results and effects of UVA CXL related to both the extracellular matrix and corneal keratocytes and epithelium, and the conclusions that can be drawn from what are sometimes contradictory results within the literature.

2. Corneal collagen

Change to the corneal collagen matrix is a focus of research which is used to study the effects of UVA CXL. This is a consequence of the procedure being directly focused on altering that structure as a means of changing the mechanical properties of the tissue as a whole. The most well established research outlines these mechanical changes utilizing methods such as tensile force measurements [17,83,84], atomic force microscopy (AFM) [85], indentation probing [17,84], and Brillouin microscopy [86–88], ultrasound shear elastography [89], and optical coherence elastography among others [90,91] of treated tissue as a means of assessing the effectiveness of various UVA CXL procedures. Work has also been done in analyzing the microstructure of corneal collagen fibrils via imaging analysis. Such work can be used to understand the mechanism of the microstructural and macrostructural changes, such as corneal flattening, in order to better manipulate the latter.

2.1. Mechanical changes: stiffening

The most well established effect of corneal crosslinking is its ability to produce mechanical stiffening of the corneal stroma up to 300%, halting the progression of Keratoconus [11,15].

This effect is often used as a benchmark for comparison of alternative UVA CXL procedures to the original Dresden protocol. The most common means of measuring mechanical changes is to perform a tensile strength test, as done by Spoerl et al. and Wollensak et al. [12,83] Other groups rely on indentation testing of smaller regions to assess specific regions of interest [17]. Both techniques utilize stress-strain measurements to calculate the elastic modulus of the tissue as a whole. One major flaw in this type of measurement is that it does not provide correction for surrounding, untreated areas of cornea, i.e. the peripheral cornea outside the 8 mm irradiated region for the strip testing or the posterior cornea underneath the 300 μm treatment depth for the indentation testing. For higher resolution measurements, which are less affected by uncrosslinked layers of tissue, atomic force microscopy (AFM) is used to calculate elasticity [85]. Recently a more powerful tool has been developed to optically measure the mechanical properties of biological tissues, known as Brillouin microscopy. This method is non-destructive, label free, contact free, and allows for in vivo 3D measurements [86,87]. These techniques have been outlined in Table 1.

Variations in measurements have been reported between all the different measurement techniques, likely due to variation in testing protocol, species, or the measurement technique used, but the significance of the stiffening effect remains constant. Spoerl et al. and Wollensak et al. reported an increase in Young's modulus of 1.8 fold in porcine corneas and 4.5 fold in human corneas [83]. The indentation technique reported a similar increase of 2.9 fold in rabbit corneas [17]. Interestingly, AFM measurements have shown a 2.6 fold increase in Young's modulus in the anterior porcine stroma and no difference at all at a depth of 200 μm [85], providing further confirmation of treatment attenuation with depth. Unlike other mechanical measurements, Brillouin microscopy does not measure a change in Young's modulus, though the change in the Brillouin modulus has been shown to correlate to the change in Young's modulus measured within the same samples [88].

2.2. Molecular changes

Apart from stress-strain related mechanical testing, gel electrophoresis has also been used to show evidence of collagen crosslinking and compare the size of collagen molecules. Type I collagen is a triple helix structure composed of three polypeptide chains, two α1 chains and one α2 chain, all of which are susceptible to molecular crosslinking. Dimers formed of a combination of any two of these chains are called β components, and trimers of all three are known as γ components. When run through an electrophoresis gel, each molecule moves to a unique depth within the gel corresponding to its molecular weight, with component molecules unable to move as far as the smaller single α chains [92]. UVA CXL, which induces molecular bonding, should increase the size of molecules within treated tissues, and therefore decrease the proportion of monomers seen in electrophoresis gels. Compressed hydrogel sheets of collagen type I that were treated with UVA CXL have been used for this purpose. Both Zhang et al. and our group detailed a significant drop in the intensity of both α and β collagen bands seen in the gels after treatment [16,92]. Wollensak et al. extracted collagen type I directly from treated porcine corneas for a similar experiment. In this experiment, not only did the usual α and β bands of collagen appear weaker in treated samples, an extra 1000 kDa band of collagen appeared. Unlike the natural α and β bands, this larger band was resistant to enzymatic digestion and heat denaturation [93]. The

presence of larger molecules, and the decrease in smaller molecules such as α and β bands provides evidence for crosslinking not just between collagen fibers but also within individual collagen molecules and possibly surrounding glycoproteins.

2.3. Collagen fibrils: TEM and X-ray scattering

Transmission electron microscopy (TEM) is often used for high resolution imaging of UVA CXL treated corneas. Many groups have utilized these images to measure parameters such as collagen fibril diameter, interfibrillar spacing, or fibril density which are all too small to be measured with light microscopy techniques. Wollensak et al. described an increase in fibril diameter using TEM at 89,000× [40]. This study found an increase of 12.2%, or 3.96 nm, in the anterior of rabbit corneas treated with UVA CXL compared to untreated controls. Interestingly, the study also found an increase in fibril diameter in the posterior stroma of 4.6% which was still significantly higher than controls. While the fibril diameter was increased in the posterior stroma, the change was more dramatic in the anterior stroma. Results from Akhtar et al. in human donor Keratoconus corneas showed similar findings, concluding that UVA CXL produced an overall more uniform stromal architecture [94]. Contrary to other reports, Akhtar also reported a slight but significant increase in interfibrillar spacing after treatment. Others reported either no significant difference in spacing, or have simply described the change in spacing as generally more ordered [95,96]. These inconsistencies are likely due to the differences in experimental protocols. For example, Akhtar et al. performed UVA CXL in human corneas, while Chang et al. performed UVA CXL in ex vivo porcine eyes [94,96].

Other researchers have focused on x-ray scattering as a means of analyzing UVA CXL. Similar to TEM, this technique is also used to obtain high resolution measurements of collagen structure such as interfibrillar spacing and fibrillar diameter. Hayes et al. used x-ray scattering to measure both of these properties after UVA CXL in ex vivo porcine, sheep, and rabbit corneas and reported no significant change in either parameter [97]. They suggest that these results here in addition to their inability to measure a significant tilt in collagen molecules within fibrils, and the unpublished reference they've provided that states the normal rate of decrease in intrafibrillar spacing during corneal drying (collected on beamline ID13 at the European Synchrotron Radiation Source, Grenoble, FR) all provide evidence for crosslinking between molecules specifically on the surface of collagen fibrils, and not within or between fibrils. Another reason these measurements may differ from TEM measurements is, again, due to differing protocols. X-ray scattering patterns represent average measurements of every collagen molecule within the path of the beam, while TEM represents measurements at the surface of a cross section. To minimize any error due to this, Hayes et al. chose to take measurements at intervals of 25 μm, the diameter of the beam at the sample [97]. While X-ray scattering reports average measurements throughout the volume of the stroma, TEM is highly localized, and is therefore more prone to selection bias.

2.4. Collagen Fibers: SHG

Another common method of analyzing UVA CXL effects by microscopic imaging post crosslinking is through the use of SHG imaging. This is a well known and widely used method used for studying corneal collagen architecture on a larger scale than the previously

mentioned techniques [1,29–31,34–39,98]. Corneal collagen fibrils produce frequency doubling when exposed to a short pulsed, high energy, infrared laser light is focused into the tissue. In order to produce this signal, a material must have a noncentrosymmetric geometry. Therefore, only fibers in the plane of the image can be detected using SHG, while fibers running perpendicular to the plane of the image are centrosymmetric, and produce no SHG signal. This produces a detailed, map-like image of the collagen architecture of the stromal extracellular matrix, such as in Figs. 1–3. This type of imaging is useful for detailing more macrostructural changes in the overall collagen architecture due to UVA CXL, such as fiber waviness [1,34,37,38,98]. Since corneal shape is highly dependent on corneal collagen architecture, many authors attempt to use their observations of structural collagen changes observed using SHG to explain the mechanism underlying clinically observed corneal flattening.

Researchers have used SHG to study the waviness, or lack thereof, of collagen fibers pre and post crosslinking treatment. While imaging methods for these studies remain fairly consistent, methods for analyzing and quantifying the results differ between groups. Our group compared the traced length of a fiber to a straight line to calculate the degree of crimp within various regions of cornea, anterior versus posterior, treated versus control, and central versus peripheral [98]. We showed that rabbit corneas treated with UVA CXL were 1% straighter in treated regions vs non treated regions at both one and three months after the procedure. We concluded that this data provides evidence which supports the hypothesis that the straightening of the collagen fibers is a main explanation for the reduced corneal curvature seen clinically. Furthermore, the same study found no difference in crimp of fibers in the peripheral region. Since the straightening of fibers in the central treated region is not balanced by an increase in crimp in the peripheral untreated region, we conclude that the fibers must be shortening. Alternatively, other studies often produce very different results and conclusions. For example, Tan et al. reported increased, not decreased, waviness after UVA CXL in ex vivo porcine corneas measured via 2D fast Fourier transform (FFT) of SHG images [37]. The methodology of this study is very different from the previous study, however. The authors performed imaging on unfixed whole corneas in an en face geometry as opposed to imaging cross-sections of corneas fixed under pressure to maintain the in vivo collagen structure. The en face regions of interest are much smaller, with measurements taken within several 9 μm^2 areas of a 45 μm^2 region of interest. In contrast, the previous study measured multiple fibers across the entire width of a 427 μm region of interest, which lowers the possibility for sampling error. Moreover, where the previous study measured waves moving in the x-z direction (anterior to posterior), any crimp measured by Tan et al. would be within the same x-y plane, since any fibers moving with depth into the stroma would not produce a strong SHG signal. Furthermore, these measurements were taken immediately after treatment, when the dehydrating effects of the riboflavin dextran solution are still relevant. This is especially important since SHG imaging has also been used to show increased fiber packing in the anterior corneal stroma immediately after dextran exposure [99]. Taking into consideration all the listed differences of the two studies, it is still possible that the described results are not entirely contradictory, since the imaging was performed from different angles. A fiber that appears wavier when viewed from the top, may appear straighter and shortened when viewed from the side because the observed waves

are secluded to the cross sectional plane. It is also possible that crimping may occur on a molecular scale, within fibrils, to induce wavier, shortened fibers.

Disagreement in this area is prevalent in the literature [100]. For this reason, another study has attempted to explain these discrepancies. Bueno et al. claims results of these studies differ not only due to their many different methodologies of tissue preparation, imaging, and imaging analysis, but because of the different species used [101]. Bueno's study compared SHG imaging analysis after in vivo UVA CXL in both avian and rabbit corneas, Figs. 2 and 3. The rabbit corneas, which are naturally less organized than avian corneas, were more structurally affected by the procedure. The authors qualitatively described the treated rabbit cornea as having collagen bundles which "appeared more delineated and less interwoven," particularly in the posterior region. Similarly the avian cornea, Fig. 2, showed similar interweaving in the anterior and mid stroma in both treated and untreated. In the posterior, however, where the untreated stroma no longer showed such a high degree of initial order, the treated cornea showed a more drastic change, with long collagen fibers running parallel to each other after treatment. When the authors analyzed the images to calculate the degree of isotropy (DOI) of the samples, they discovered that treated rabbit corneas experienced an increase in DOI at every depth, while avian corneas, with much higher initial DOIs, did not experience any significant increase in DOI at any depth. The authors concluded that the more ordered the original collagen structure, the less increase in order seen due to the procedure. Additionally, to our knowledge, all SHG studies have performed imaging within tissue, and no study has been performed in a collagen gel. It could be useful to observe the effects of UVA CXL within a simplified structure. A comparison of the methods and main results of these studies can be seen in Table 2, as well as a diagram to summarize these results in Fig. 4.

3. Cellular effects

3.1. Epithelium

Epithelial debridement is a necessary step in the traditional Dresden protocol for UVA CXL. Without it, riboflavin penetration does not occur in concentrations required for effective crosslinking. Epithelial debridement is painful for the patient, delays visual recovery, and increases the risk of bacterial keratitis and corneal ulceration [102–104] and requires 2 days to heal in rabbits or as long as 3 weeks in humans [43,105].

To avoid epithelial debridement research has focused on the development of a transepithelial UVA CXL protocol. Various methods have been used to enhance transepithelial riboflavin penetration without debridement prior to UVA exposure. Though clinical studies have reported fewer severe complications from experimental transepithelial UVA CXL techniques on average, there has also been minimal success, with reported stabilization of KMax (maximal corneal curvature) of only 43% compared to 93% reported after traditional UVA CXL [20,57]. Additionally, the line of demarcation in the stroma, the presumed zone of transition between treated and untreated tissue often used as a measure of the depth of treatment, was observed to be shallower when the epithelium remained intact [20]. This is likely due to the epithelium acting as a barrier to UVA light as well as riboflavin penetration. Riboflavin within the epithelium could absorb much of the UVA light and reduce the UVA

intensity deeper within the stroma. Research has also shown extensive epithelial damage after transepithelial UVA CXL, in response to both the UVA exposure and the methods used to facilitate riboflavin penetration [62,77,78,106–108]. Benzalkonium Chloride (BAK), commonly used as an excipient, is especially toxic to cells and has been shown to damage the epithelial layer even without subsequent UVA exposure [106,109]. Taneri et al. reported that epithelial defects and perceived pain were common following various transepithelial UVA CXL techniques, though these instances were less common using methods that produced less stromal riboflavin penetration [107]. This indicates an inverse relationship between riboflavin penetration and epithelial disruption. Additionally, Chow et al. reported more extreme epithelial damage following accelerated transepithelial UVA CXL, in which higher intensity UVA light is used to shorten the procedure time [62]. In this study 64% of eyes experienced complications including large epithelial defects and diffuse punctate epithelial erosions, with 100% of patients reporting significant postoperative pain. This indicates that epithelial damage is not only a result of varying riboflavin penetration techniques, but also due to UVA exposure itself. The free oxygen radicals which induce crosslinking in the stroma also damage epithelial cells.

A new technique to imbibe the corneal stroma with riboflavin has shown promise in minimizing epithelial damage prior to crosslinking therapy. By focusing a high pulse energy infrared femtosecond beam into small, widely spaced spots, a pattern of small channels can be drilled into the surface epithelium. At 2 μm in diameter and only 25 μm long, each channel causes minimal cellular disruption while allowing free passage of riboflavin through the epithelium. Microchannels combined with more concentrated riboflavin drops were shown to facilitate similar levels of stromal riboflavin concentration as the standard epithelial debridement method [106]. Additionally, epithelial damage due to the microchannels was undetectable after 24 h of organ culture, as evidenced by phalloidin and propidium iodide cellular staining (Fig. 5).

3.2. Stromal keratocytes

Stromal Keratocyte death occurs due to UVA exposure of around 0.5 mW/cm^2 [42,110], resulting in an acellular zone within the anterior stroma to a depth of 200–300 μm [41,43,84,98,111]. Post treatment activation and migration of keratocytes into corneal fibroblasts from adjacent regions back into the acellular zone and the expression of disordered extracellular matrix produced by those fibroblasts is responsible for the development of haze, as these cells scatter light more than their non-activated counterparts [112]. Wollensak et al. detailed severe haze in rabbit corneas lasting one week, while other studies showed haze peaking at one month [43,84,98]. Clinically, haze has been shown to persist for six to eight months, and over a year in 10% of cases, leading to permanent corneal scarring in 2.9% of cases [44,71,113]. It has previously been observed by Wollensak and Kozobolis via light microscopy that keratocyte repopulation begins around one month post crosslinking in rabbits, with full cellular repopulation reported by six weeks [43,44]. Both reported a continuing presence of acellular areas and apoptotic changes such as apoptotic bodies, shrunken cell nuclei, and chromatin condensation at 4 weeks, especially around the periphery of irradiation. Kruger also observed cellular repopulation by 6 weeks using a combination of confocal laser scanning microscopy and two photon excited

fluorescence, albeit with a lower cellular density than seen in controls [30]. By contrast, we reported persistent acellular regions at three months post UVA CXL, as well as an additional acellular region below the region of crosslinking, using a combination of in vivo CMTF and cellular fluorescence staining, as seen in Fig. 5 [84,98]. Additionally, studies of corneal scrape and freeze injuries have shown stromal cellular repopulation 3–14 days after injury, suggesting the delayed repopulation after UVA CXL may be due to either the biomechanical changes causing fibril stiffening and thinning, or to the UVA exposure itself [114].

4. Conclusion

In the last two decades since corneal UVA CXL has been developed research has focused on discovering the mechanisms that govern the process and the effects it has on corneal tissue. The main objective of the procedure is to enhance stromal mechanical stiffness via alteration of the corneal collagen structure, and for that reason research has been focused on structural changes. There is a general consensus that the procedure mechanically strengthens the corneal stroma and leads to a low degree of corneal flattening, collagen fibril thickening, and halted progression of corneal ectatic disease. The mechanism of these changes are not often agreed upon, however. For example, some authors claim increased fibril waviness plays a role in corneal flattening while others claim the opposite [37,98]. Though collagen structure and mechanical changes are important factors underlying UVA CXL, the procedure can be damaging to certain layers of the cornea. For this reason, safety studies focus on cellular responses to UVA CXL. Studies on the timeline of keratocyte repopulation are plagued with the same inconsistency in results as structural studies, with repopulation being reported anywhere from six weeks to three months or longer [30,43,44,98].

In either case, the truth is likely more complicated than any one study suggests. Taken as a whole it is difficult to compare most studies to each other, due to their varying protocols, measurement techniques, and even species of test subjects used. There is also a lack of consistency in definitions. For example, some studies which describe full cellular repopulation also describe continuing acellular regions or lower than normal cellular density [30]. It is important to take all of these factors into account when comparing various studies to determine whether differing results between individual studies could simply be a case of one question being viewed from many different perspectives.

Finally, as more researchers begin to turn their focus to alterations of UVA CXL it has become necessary to predict how various changes to the standard protocol would ultimately affect the results of the procedure. This has proved difficult, however, since the effects of the original procedure are not wholly understood. Building up a larger body of research focused on the effects addressed in this review would enable researchers to better predict the outcomes of protocol adjustments, allowing for more customized procedures to treat each individual patient’s needs.

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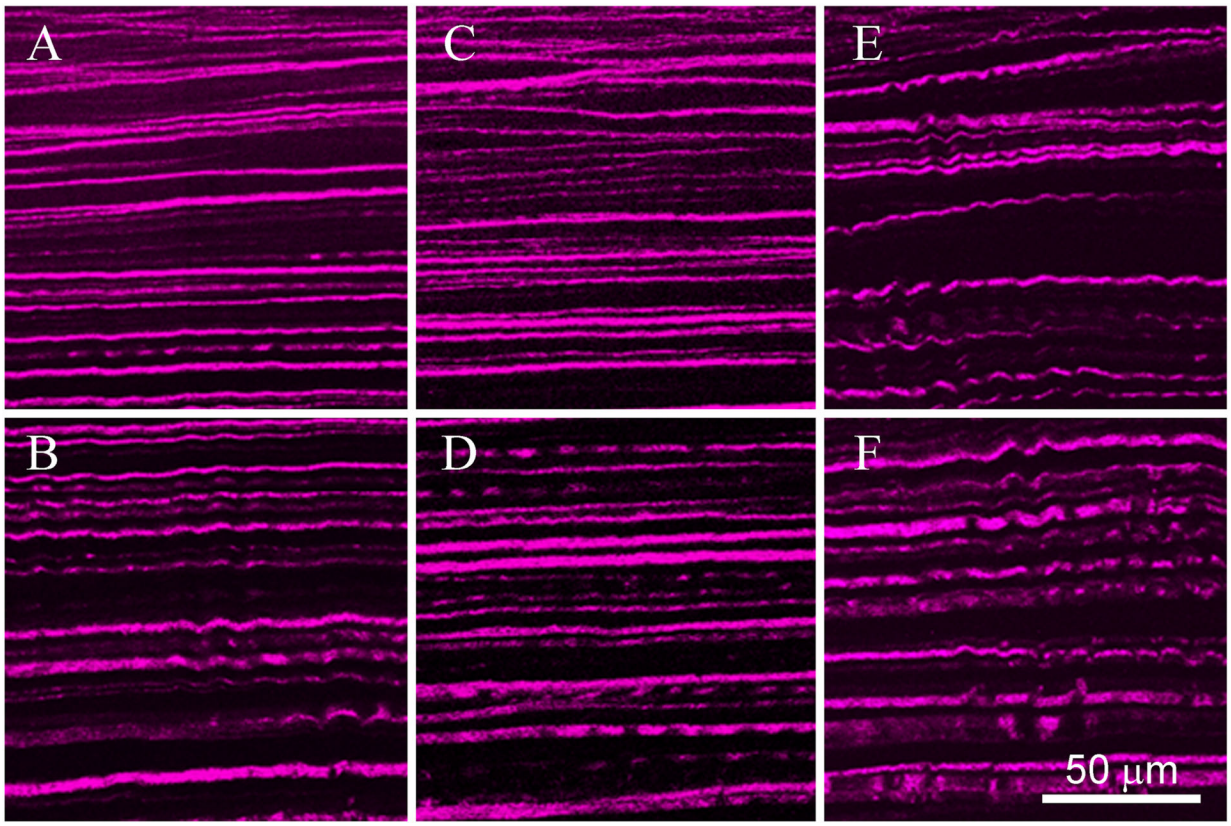

Fig. 1.

SHG Images of Collagen Crimping

Cross sectional SHG imaging revealed significantly straighter collagen fibers in the anterior of treated corneas at both one and three months (A and C respectively) when compared to their own posterior values (B and D) or control anterior or posterior values (E and F). Fibers were visibly wavier in all non-treated areas (B, D, E, and F) [98].

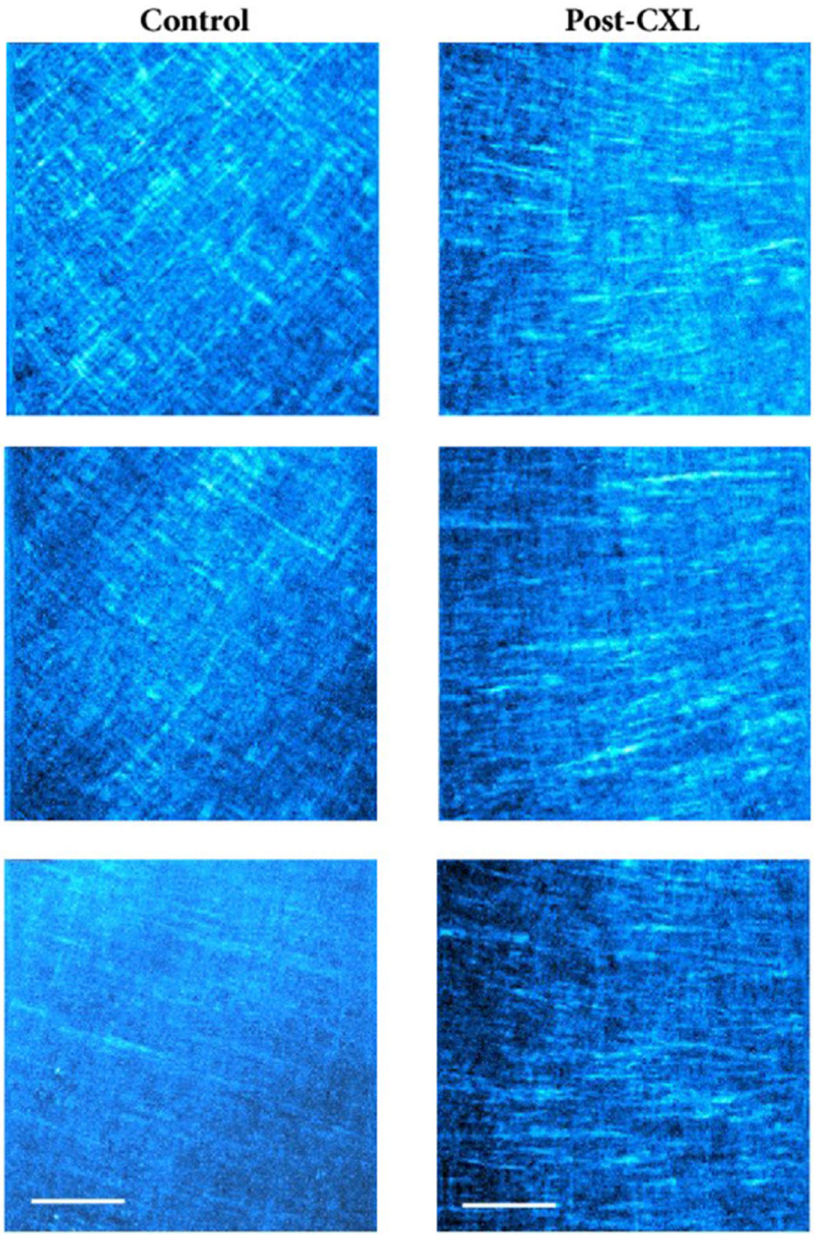

Fig. 2.

SHG images of a control adult chicken cornea (left panels) and a post-CXL cornea CXL (right panels). Depth positions (from top to bottom) correspond to the anterior, mid, and posterior locations. Scale bar: 50 μm [101].

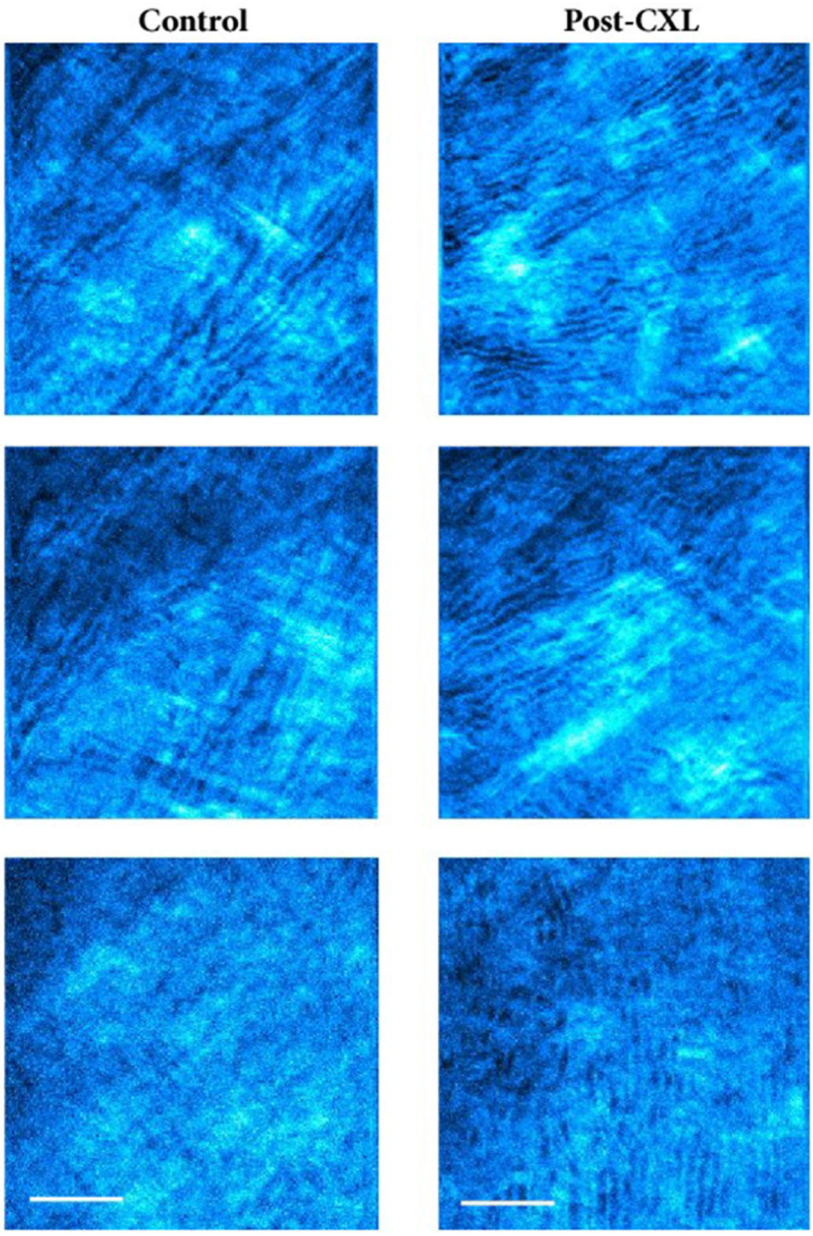

Fig. 3.

SHG images of a control rabbit cornea (left panels) and a post-CXL cornea (right panels). Depth positions correspond to the anterior (40 μm, top), mid (120 μm, middle) and posterior (240 μm, bottom) locations. Scale bar: 50 μm [101].

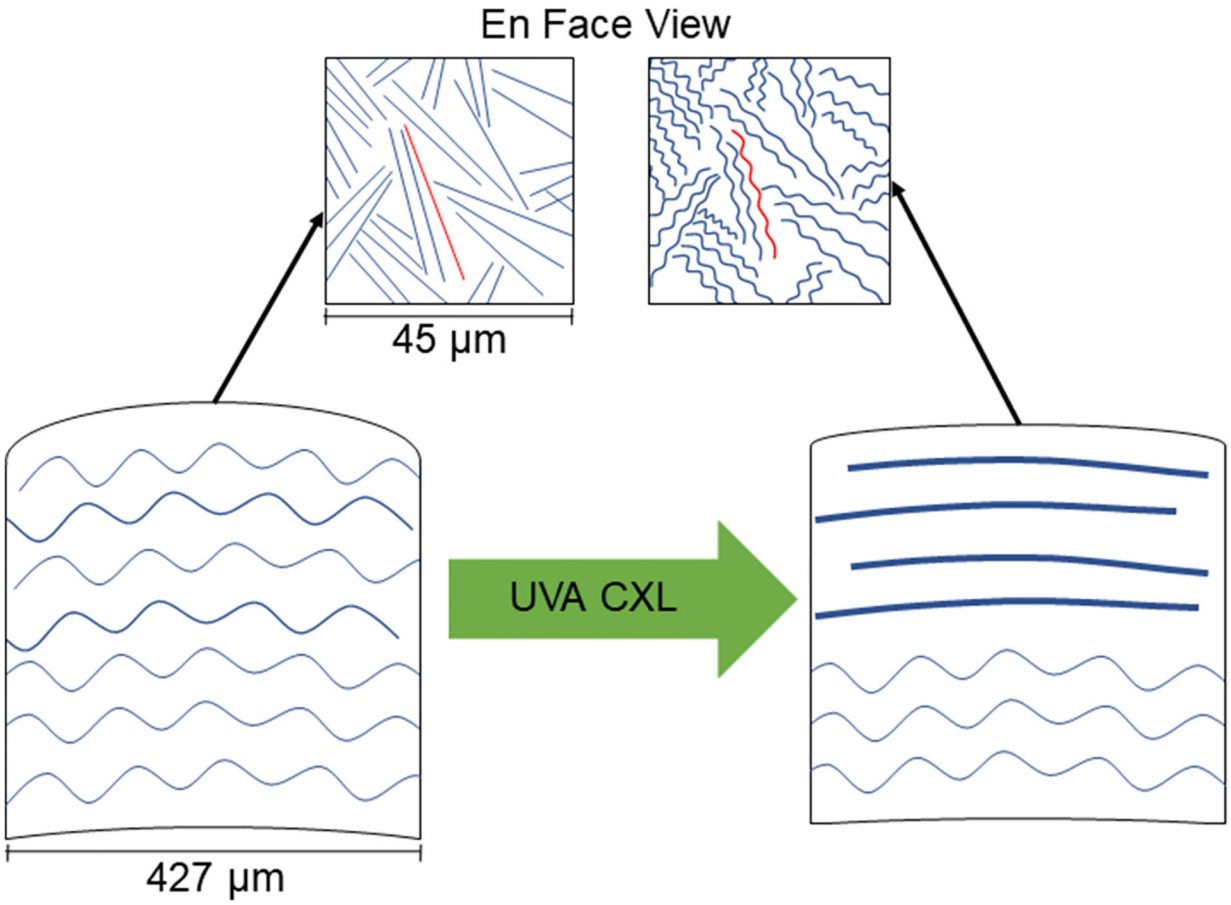

Fig. 4.

Suggestive SHG diagram. Literature reviewed in this article has shown collagen fibers to be straighter after UVA CXL when viewed in a cross section (bottom) [98], and wavier when viewed in an en face orientation (top) [37]. Literature also suggests that observed corneal flattening could be due to the shortening of collagen fibers as they straighten in one orientation, and crimp in another, (Right). The red fibers before and after crosslinking were highlighted to illustrate the transition from a long straight fiber to a shortened wavier fiber after treatment.

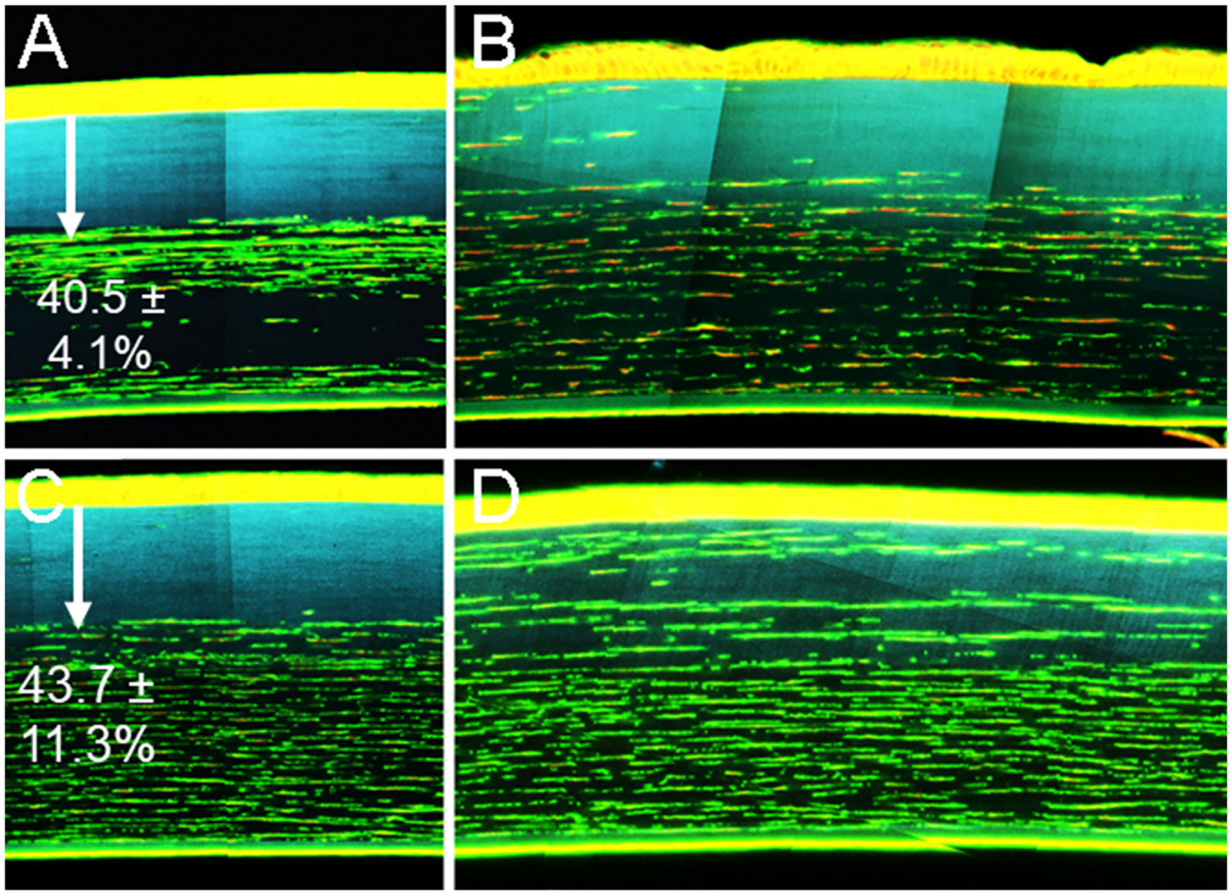

Fig. 5.

Cell Staining

The top row of images represents samples from the central CXL region and the edge of the CXL region bordering the periphery from one month samples (A and B respectively). The bottom row represents corresponding images from three month samples (C and D). Staining with Phalloidin (green; 1:100) and Propidium Iodide (red; 0.01 mg/ml) showed little cellular repopulation into the central CXL region, shown with blue CAF, at either time point. Images from the periphery show migrating cells into the CXL region. The depth of the acellular zone in the central cornea, indicated by arrows, was measured to be 40.5 ± 4.1%, and 43.7 ± 11.3% of the stromal thickness on average for one and three month samples. This corresponds to the measured depth of CAF. Also, in two of the four one month samples, a second deeper acellular region was noted, pictured in A [98].

Table 1

Summary of mechanical testing methods.

Technique	Measurement	Advantages	Disadvantages
Tensile Strength Test	Young's Modulus	• Relatively easy • Most commonly used/comparable	• Destructive • Affected by uncrosslinked tissue in X, Y, or Z
Indentation Testing	Young's Modulus	• Relatively easy • High X/Y resolution	• Destructive • Affected by uncrosslinked tissue in Z
AFM	Young's Modulus	• High resolution • Less affected by uncrosslinked areas in X, Y, or Z	• Destructive • Relies on surface measurements • Specialized equipment
Brillouin Microscopy	Brillouin Modulus	• Measured optically • Non-destructive • Label free • Contact free • 3D in vivo measurements	• Specialized equipment • Slow

Table 2

Fibril Waviness.

Authors	Methods			Conclusion
	Tissue	Imaging	Measurement	
Bradford et al. [70]	Rabbit: in vivo	SHG: cross section	Traced fibrils	1% decreased crimp after UVA CXL
Tan et al. [32]	Porcine: ex vivo	SHG: en face	2D FFT	Decreased waviness after UVA CXL
Germann et al. [72]	Porcine: ex vivo	SHG: en face	Order coefficient	Increased order and straighter fibrils after UVA CXL
Bueno et al. [73]	Avian/Rabbit: in vivo	SHG: en face	Degree of isotropy, preferential orientation, and structural dispersion	The more ordered the original collagen structure, the less increase in order seen after UVA CXL