Biomechanical properties of corneal tissue after ultraviolet-A–riboflavin crosslinking

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Photodynamic collagen crosslinking (CXL) using ultraviolet-A (UVA) irradiation combined with the photosensitizer riboflavin has been introduced as a new treatment for progressive keratoconus. The results of clinical studies are promising, but the efficacy of the treatment in halting the progression depends on the stability of the induced biomechanical effects. The effects of corneal CXL on corneal rigidity; collagen fiber diameter; and resistance to heat degradation, enzymatic digestion, and swelling due to hydration are reviewed in this paper. The collective results indicate that CXL using UVA and riboflavin enhances the biomechanical properties of the corneal tissue, which remain stable over time. Therefore, this treatment could become the future standard therapy for keratoconus or used to halt the progression of keratoconus and postpone the need for corneal transplantation. The increase in availability and popularity of the CXL technique accentuates the requirement for reliable and accurate techniques for measuring corneal biomechanical properties before and after treatment.

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Keratoconus is a progressive, noninflammatory corneal thinning disorder that affects 1 in 2000 in the younger working-age population.[1] It usually causes high myopia and irregular astigmatism, which lead to vision impairment.[1] Various treatment options exist including conservative methods (soft and rigid contact lenses)[2] and surgical interventions (intrastromal corneal ring segments).[3] However, in some people, profound steepening and corneal scarring require corneal transplantation for visual rehabilitation.[4,5]

The available surgical treatments change the shape of the cornea or replace the keratoconic cornea with a donated healthy cornea in its entirety or in part, but none modify the deteriorated biomechanical parameters in the keratoconic corneas.[6] Photodynamic collagen crosslinking (CXL) using ultraviolet-A (UVA) irradiation combined with the photosensitizer riboflavin has been proposed as a treatment for keratoconus.[7,8] It is considered a less invasive and less costly option than corneal transplantation, and unlike other treatments, CXL aims to modify the intrinsic biomechanical properties of the collagen fibers.[8] Studies have shown the efficacy of arresting the progression of keratoconus using the CXL standard protocol[7,9] and, more recently, the efficacy of transepithelial corneal CXL[10,11] in improving the corrected visual quality[9,11,12] and reducing the associated corneal steepening,[7,9,11,12] astigmatic power[9,12] and corneal wavefront aberrations.[9] No decrease in endothelial cell density has been reported after treatment.[9,13] Thus, CXL could become the standard treatment for keratoconus and could be used to postpone the need for corneal transplantation. However, the efficacy of this treatment is measured by its ability to modify the biomechanical properties of corneal tissue. The purpose of this paper is to summarize the biomechanical effects of CXL reported in the published research.

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BIOMECHANICAL EFFECTS OF COLLAGEN CROSSLINKING

Corneal Rigidity

Because keratoconic corneas lose their rigidity,[14–17] the stiffening effect of CXL treatment has been

investigated by several research groups on human,[8,18] porcine,[18–21] and rabbit[22–24] corneas and on collagen hydrogels.[25] Studies have confirmed a stiffening effect of UVA–riboflavin CXL on corneal tissue,[8,18–21,23,26,27] which is believed to be due to the induced covalent bonds within and between collagen fibers.[28] Studies that have investigated the stiffening effect of CXL using UVA–riboflavin are reviewed.

The elasticity of any tissue can be determined by measuring the stress (force on a cross-section point) and its relative strain (proportional deformation).[15] The Young's modulus is the proportion between the stress and strain values and reflects the elasticity of the tissue.[15] A small Young's modulus value reflects more elasticity of a material and is represented in units of Newton/m^2.[15]

Various methods have been used to calculate the stress–strain measurements and the Young's modulus of elasticity of corneas after CXL.

Strip Estensometry Technique Most studies[8,18,20,21,23,26] have used the strip-extensometry technique to calculate the modulus value of the cornea. In this technique, corneal strips with a certain width and length are taken from the central part of the cornea and clamped individually, horizontally, or vertically between the 2 jaws of a microcomputer-controlled biomaterial tester (Minimat, Rheometriv Scientific GmbH).[8,18,20,21,23,26] The strain (ε) is then increased linearly at a certain velocity, and the relative stress (σ) is measured. The stress–strain values are then fitted by an exponential function $\sigma = A \exp (B \times \varepsilon)$ to calculate the Young's modulus.[8,18,23,26]

Spoerl et al.[21] used strip extensometry and were the first to report increased corneal stiffening after CXL using UVA–riboflavin treatment and other CXL agents, such as glutaraldehyde and Karnovsky solution (0.1% glutaraldehyde, 0.1% paraformaldehyde, 0.1 m Na-phosphate buffer pH 7.4), in vitro on a relatively large sample of 160 porcine eyes. However, the toxicity of these agents (if any) was not assessed in the study. The second important finding was that UVA or the photosensitizer (riboflavin) alone is not capable of inducing crosslinks in corneal collagen. This finding was confirmed later by Ahearne et al.[25] and Wollensak and Iomdina.[26] The Spoerl et al.[21] study was done on porcine corneas and they were not crosslinked with the techniques that are currently used. Instead, the authors used a UVA wavelength of 365 nm or 436 nm for 45 minutes and 0.5% riboflavin for 45 minutes. The current treatment protocol uses a UVA wavelength of 370 nm for 30 minutes and 0.1% riboflavin for 45 minutes.[7,8,23,26,29] The results of the Spoerl et al. study were presented as stress–strain graphs only; no Young's modulus values were reported.

Wollensak et al.[8] studied and compared the stress–strain measurements using the strip extensometry technique on human and porcine corneas before and after applying a "standard" CXL protocol (epithelium removal, riboflavin application for 45 minutes, and UVA irradiation for 30 minutes); they used a sample of 5 human corneas and 20 porcine corneas. They reported a significant increase in the biomechanical rigidity and stiffness of human and porcine corneas after the treatment, which agrees with what Spoerl et al.[21] had found. The most interesting finding by Wollensak et al. was that the increase in the rigidity and Young's modulus was 328.9% and ×4.5, respectively, in the human corneas and only 71.9% and ×1.8, respectively, in the porcine corneas (Table 1). This difference in stiffening between the human and porcine corneas was confirmed later by Kohlhaas et al.[18] The difference in results between the human and porcine corneas is believed to result from the different central thicknesses of the 2 corneas (550 µm versus 850 µm on average) and the different UVA absorption coefficients (higher in the human corneas), which means a larger portion of the human corneas was crosslinked.[8] The UVA–riboflavin stiffening effect is concentrated in the anterior 200 µm of the cornea and in 20% of the next 200 µm, which means a higher CXL effect occurs in the anterior portion of the cornea (Table 1).[18] Other factors may also contribute to this between-species difference, such as the different organization of the collagen fibers, which provide different structural properties.[30] Additionally, the anterior stroma in human corneas is normally much stiffer than the posterior stroma,[31] whereas the anterior and posterior stromal flaps of porcine corneas do not appear to differ significantly.[18] Also, in the human corneas in the study by Wollensak et al.,[8] the control group was not given a placebo treatment and this might have led to a reduction in stiffness in the control corneas due to the time these corneas were left in culture medium as opposed to the treated corneas. This methodology difference could have resulted in the increased difference in corneal stiffening effect recorded in human corneas. Future studies should be done on matched corneal pairs and the control group should have a placebo treatment with saline or riboflavin to make the comparisons more reliable.

The fact that the CXL effects largely concentrates in the anterior portion of the cornea (200 µm of the anterior cornea and 20% of the next 200 µm) suggests a minimal negative influence (toxicity or cell death) on the corneal endothelium and the posterior structures of the eye (crystalline lens, vitreous, and retina), which is crucial for the safety of the CXL technique.

Previous studies have proved that corneal CXL increases the corneal rigidity immediately after the treatment.[8,18,21] However, the success of CXL depends on

the long-term stability of the induced stiffening. Wollensak and Iomdina.[23] used the extensometry technique to study the long-term stability (up to 8 months) of the increased rigidity in 9 rabbits after CXL. The ultimate stress increased by 69.7% immediately after treatment, by 106.0% at 3 months, and by 69.7% at 8 months. The ultimate strain decreased by 78.4% immediately after treatment, by 57% at 3 months, and by 45.9% at 8 months. The Young's modulus increased at a steady rate during this study (78.4% to 87.4%) (Table 1). Similar modulus values at baseline had been reported in rabbits by the same research group.[26] The increase in the Young's modulus in rabbit corneas after CXL was by a factor of ×1.6.[23] Similar results were reported by Spoerl et al.[22] 12 weeks after treatment (×1.3) and by McCall et al.[24] immediately after treatment (×1.5). Further studies are needed to confirm the stability of the induced corneal rigidity in human corneas and for a longer follow-up period.

Overall, the increased rigidity of rabbit corneas found after CXL supports the idea that CXL increases the rigidity of the corneal tissue. However, differences in the increase in the Young's modulus after CXL have been detected between rabbit, porcine, and human corneas. The modulus increased by factors of ×1.3,[22] ×1.6,[23] and ×1.5[24] in rabbit corneas, by ×1.8 in porcine corneas, and by ×4.5 in human corneas.[8] This difference is explained by the different intrinsic parameters of the collagen matrix of each species. The Young's modulus of the untreated human, porcine, and rabbit corneas at 6% strain was 1.3, 1.5, and 1.8 megapascals, respectively (Table 1).[8,23] Additionally, the 3 species have different UVA-absorption coefficients; human corneas have a higher coefficient (70) than porcine corneas (59) and rabbit corneas (63)[32] and consequently more CXL would be expected in human corneas than porcine and rabbit corneas.[23]

Lanchares et al.[20] used another approach to calculate the stress–strain measurements and Young's modulus of corneal strips. The corneal strips were clamped individually between the 2 jaws of a microtester (Illinois Tool Works, Inc.) and gripped from both ends gradually until rupture occurred. The displacement of the jaws was noted. The stretch data (λ) were calculated as $\lambda = (L_0 + \Delta L)\ L_0^{-1}$, where L_0 is the initial strip length and ΔL is the displacement of the clamp; the resultant stress (σ) was calculated as $\sigma = N\ \lambda / A_0$, where N is the used load, λ is the calculated stretch value, and A_0 is the initial strip cross-section (Table 1).[20] The stress–stretch values were then plotted and the slope of this curve corresponded to the Young's modulus value.[20] However, only the stretch data were presented in the paper.

Several research groups[8,18,20,21,23,26] have used the strip extensometry technique, which seems a simple and a popular technique for stress–strain measurement of tissues. However, some of the technique's limitations have been found to affect its reliability. Extensometry flattens the corneal strips as they are naturally curved and thus neglects the natural variation in the length of the center line and the edges of the corneal strip. This technique also neglects the corneal thickness variation between the center and periphery. Additionally, extensometry destroys corneal tissue due to cutting the stromal lamellae.[25] These factors affect the accuracy and reliability of the extensometry results.[33] Mathematical modifications of the extensometry results were suggested by Elsheikh and Anderson[33] to deal with these factors and thus improve the technique's reliability.

Spherical Microindentation Technique Because of the limitations of the extensometry technique in examining the biomechanical effects of CXL, scientists have developed or used different techniques to evaluate the rigidity of the CXL treatment. Ahearne et al.[25] developed a nondestructive technique to examine the mechanical effects of CXL of collagen hydrogels and used them as a model of corneal tissue. The technique has the advantage of using collagen hydrogels or corneal-engineered tissue for laboratory-based research, especially important when there is a lack of human corneas because of high demand for corneal transplantation. The long-working-distance microscope spherical microindentation technique examines the mechanical rigidity of collagen hydrogels after CXL at different UVA exposure times (15, 30, 45, and 60 minutes). The instrument consists of 2 main parts: a sample holder with an indentation sphere and an image analysis system.

The increase in the Young's modulus (200% to 240%) was significantly higher in the UVA–riboflavin-treated collagen hydrogels than in the control group at the various exposure times, and the increased rigidity was stable after a week. The optimum CXL effect occurred between 30 and 45 minutes.[25] The Young's modulus value increased by ×3.2 after 30 minutes of CXL (Table 1). This value is lower than that reported in human corneas (×4.5) and higher than that reported in porcine corneas (×1.8) and rabbit corneas (×1.6). This finding has been confirmed recently in porcine corneas by Lanchares et al.[20] in vitro using the strip extensometry technique. The control hydrogels, which were treated by UVA only, showed a decrease in modulus in the first 15 minutes of the UVA exposure and they converted to liquid after 30 minutes of irradiation, which indicates that UVA degrades the collagen fibers rather than crosslinking them in the absence of riboflavin. This finding confirmed what Spoerl et al.[21] found.

Table 1. Summary of literature on changes in stiffness with CXL.

Study	Crosslinking Procedure	Groups
Spoerl et al.[21]	1. UV-A (λ = 254 nm), 20 min 2. UV-A (λ = 365), 20 min; 0.5% riboflavin, 45 min 3. Blue light (λ = 436 nm), 45 min; 0.5% riboflavin, 45 min 4. Sunlight, 120 min; 5% riboflavin, 45 min 5. Riboflavin 0.5%, 45 min 6. Glutaraldehyde 1%, 10 min 7. Glutaraldehyde 0.1%, 10 min 8. Karnovsky solution 0.1%, 10 min	Porcine corneas Treated n = 80 (10/group) Control no treatment n = 80
Wollensak et al.[8]	Epithelial removal, 0.1% riboflavin, 5 min before the irradiation and at 5 min interval during the UV-A (λ = 370 nm), 30 min 2 strips taken from each human cornea and 1 strip taken from each porcine cornea	5 human/20 porcine corneas
Kohlhaas et al.[18]	0.1% riboflavin, 5 min before irradiation and at 5 min interval during the UV-A (λ = 370 nm), 30 min Controls:epithelium removal with no treatment 2 strips (200 μm) taken from each cornea; the first called anterior strip and the second, posterior strip	5 human/20 porcine corneas Control: 5 contralateral human/20 porcine corneas
Ahearne et al.[25]	Collagen hydrogel immersed in 0.1% riboflavin solution for 5 min, then exposed to UV-A (λ = 370–350 nm) at different times (15, 30, 45 and 60 min); other collagen hydrogels treated with UV-A only. Controls: No treatment Masurements taken immediately and repeated after 1 week	Collagen hydrogels. Collagen type I served as a collagen source for the hydrogels
Wollensak and Iomdina[23]	Cross-linking done in-vivo; 0.5% of proparacine applied before epithelium was removed; 0.1% riboflavin applied 5 min before irradiation and during irradiation (UV-A, λ = 370), 30 min in 5 min intervals. Measurements taken at 3 stages: immediately after treatment (T1) and after 3 (T2) and 8 (T3) months	Left eyes of 9 rabbits treated/right eyes served as controls
Wollensak and Iomdina[26]	Standard treatment (Group 1): epithelium removal; 0.1% riboflavin every 3 min for 20 min; UV-A (λ = 365), 30 min and riboflavic instillation every 3 min and oxybuprocine every 5 min C3-R treatment (Group 2): no epithelium removal, 2 drops of 0.5% proparacine applied every 5 min for 30 min followed by 2 drops of 0.1% riboflavin every 3 min for 30 min and then UV-A irradiation and riboflavin instillation every 3 min and proparacine every 5 min. Group 3: no epithelium removal, 2 drops of oxybuprocine (without benzalkonium chloride) every 5 min for 30 min; 2 drops of 0.1% riboflavin every 3 min for 30 min; UV-A irradiation and riboflavin instillation every 3 min and oxybuprocine every 5 min	Left eyes of 14 rabbits treated/ right eyes served as controls 5 rabbits (Group 1) 5 rabbits 4 rabbits

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Table 1. (Cont.)

Stress-Strain Method	Strips Characteristics (mm)	Stress Measurements	Stress Measurements (MPa)	Young Modulus Values (MPa)
Strip extensometery	Width, 5 Length, 9	4% 6% 8%	—	—
Strip extensometery	Human: width, 4; length, 14 Porcine: width, 5; length, 14	4% untreated Treated 6% untreated Treated 8% untreated Treated 4% untreated Treated 6% untreated Treated 8% untreated Treated	$34.3 \pm 5.5 \times 10^{-3}$ $135.7 \pm 61.4 \times 10^{3}$ $53.0 \pm 11.5 \times 10^{-3}$ $227.3 \pm 95.7 \times 10^{3}$ $79.3 \pm 21.2 \times 10^{-3}$ $344.7 \pm 141.9 \times 10^{3}$ $33.7 \pm 9.3 \times 10^{-3}$ $55.8 \pm 17.6 \times 10^{-3}$ $57.3 \pm 17.3 \times 10^{-3}$ $98.5 \pm 29.7 \times 10^{-3}$ $86.5 \pm 29.9 \times 10^{-3}$ $151.8 \pm 44.7 \times 10^{3}$	0.8 3.0 1.3 5.9 2.2 11.8 0.8 1.4 1.5 2.7 2.6 5.3
Strip extensometery	Human: width, 5; length 7 Porcine: width, 5; length, 7	5% Ant. untreated Ant. treated Post. untreated Post. treated Ant. untreated Ant. treated Post. untreated Post. treated	 $108.4 \pm 34.2 \times 10^{3}$ $306.5 \pm 97.6 \times 10^{3}$ $53.0 \pm 16.2 \times 10^{-3}$ $89.3 \pm 34.4 \times 10^{-3}$ $104.1 \pm 52.7 \times 10^{3}$ $261.7 \pm 133.2 \times 10^{3}$ $103.7 \pm 61.8 \times 10^{3}$ $105.0 \pm 55.8 \times 10^{3}$	 3.6 6.0 1.3 1.0 2.9 6.3 2.8 2.7
Long-working-distance microscope, spherical micro-indentation technique			—	—
Strip extensometery	Width, 4 Length, 10	T (1) 52.3% untreated 11.3% treated T (2) 52.3% untreated 52% treated T (3) 52.3% untreated 28.3% treated	3.3 5.6 3.3 6.8 3.3 5.6	11.1 19.9 11.1 19.8 11.1 20.8
Strip extensometery	Width: 4 Length: 10	Group (1) 50.38 ± 2.71% untreated 50.62 ± 1.77% treated Group (2) 50.38 ± 2.71% untreated 48.88 ± 2.93% treated Group (3) 50.38 ± 2.71% untreated 51.4 ± 2.98% treated	 2.42 ± 0.5 5.84 ± 0.62 2.42 ± 0.5 3.06 ± 0.63 2.42 ± 0.5 2.3 ± 0.37	 9.81 ± 1.36 19.86 ± 1.04 9.81 ± 1.36 11.9 ± 1.22 9.81 ± 1.36 9.58 ± 0.89

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Kling et al.[19]	Epithelium removal; 0.125% riboflavin every 3 min for 30 min; UV-A (λ = 365), 30 min and riboflavic instillation every 3 min Controls: Epithelium removal; 0.125% riboflavin every 3 min for 30 min Measurements taken immediately after treatment and after 24 hours	38 porcine corneas: 23 treated, 15 controls Control: 15
Lanchares et al.[20]	0.1% riboflavin every 5 min for 30 min;n UV-A (λ = 370) for 30 or 60 min; 1 drop of 0.1% riboflavin every 5 min during the irradiation Controls: 0.1% riboflavin, 30 min	17 porcine eyes. 6 corneas: 30 min UV-A exposure 5 corneas: 60 min UV-A exposure 6 corneas: control

MPa = megapascal; UV-A = ultraviolet A

The spherical microindentation technique can be useful in measuring the biomechanical effects of CXL on collagen hydrogels or corneal-engineered tissues before and after treatment, which allows a better comparison and monitoring of the modulus over time and for long-term follow-up studies.[25] Additionally, it can be used widely in laboratory research, such as examining the effect of using different concentrations of the photosensitizer (riboflavin) or using other crosslinking agents (eg, glutaraldehyde).[25] However, the technique cannot be used to examine real corneal tissues. An alternative indentation technique has been developed to examine the mechanical properties of whole human corneas in vitro.[34] Examining the mechanical effects of CXL on real corneas gives more accurate results as epithelial and endothelial damage from the UV light, which leads to swelling and inflammation,[35,36] can be evaluated.[25]

Inflation Procedure Another technique was used by Kling et al.[19] for stress–strain measurement of the whole eye rather than of corneal strips—a simple model of inflating the whole globe in 23 crosslinked and 15 control porcine eyes. The model is based on the idea that the corneal thickness and curvature change in response to the change in intraocular pressure (IOP). The IOP was increased by injecting the eye with saline, and thus the corneas were stretched and flattened and their thickness was decreased. At the same time, corneal stretching induced a stress to prevent additional extension to the corneal surface.[19] Stress (σ) and strain (ε) measurements were calculated using the following equations:

$$\sigma = R \times p/2 \times d$$

where R is the mean of the posterior, anterior, vertical, and horizontal radii of curvature of the cornea, p is the IOP value, and d is the corneal thickness, and

$$\varepsilon = \Delta R/R$$

where ΔR is the difference in the corneal radius of curvature in relation to the initial measurements and R is the initial radius of curvature. The stress–strain measurements were then plotted and the slope of the curve represents the Young's modulus.[19]

The Young's modulus was reported to be significantly higher by ×1.58 in the porcine crosslinked corneas than in the control corneas (Table 1).[19] Using this technique, CXL stiffened the porcine corneas by 36.86%,[19] while it was reported to be higher (71.9%) using strip extensometery.[8] The difference in results using these 2 techniques may be due to using strips of cornea versus the whole cornea and using different ways of inducing the stress. However, Elsheikh and Anderson[33] reported that similar results after applying both techniques could be obtained only if the mathematical modifications which they suggest are applied to the extensometry results.

The stress–strain measurement using the inflation procedure may be closer to the in vivo studies as it uses the whole globe ex vivo rather than using strips and it solved many of the problems caused by the extensometry. However, this technique requires constant monitoring of corneal hydration during the measurements to mimic the in vivo measurements. Furthermore, with this technique, changes in the curvature radii are not considered as this model takes the average of the horizontal and vertical radii in the calculations. The results of this method have not been replicated in human corneas. Differences in the human and porcine corneas are expected due to the thickness and elasticity differences in these corneas.[37,38]

Nanoindentation and Scanning Acoustic Microscopy Inflation and strip extensometry techniques measure the modulus of elasticity of the entire cornea,[19,20,23] while only the anterior cornea (anterior 200 μm and 20% of

Inflation				After 24 hours: Untreated, $0.692 \pm 0.30 \times 10^{-3}$ Treated, $1.096 \pm 0.30 \times 10^{-3}$
Strip extensometery	Width: 2 Length: 20	30 min 6%	0.79 ± 0.73	—
		8%	1.36 ± 0.94	
		12%	2.90 ± 1.28	
		60 min 6%	0.16 ± 0.04	
		8%	0.41 ± 0.06	
		12%	1.24 ± 0.21	
		Controls 6%	0.19 ± 0.10	
		8%	0.45 ± 0.15	
		12%	1.36 ± 0.34	

the next 200 μm) is crosslinked.[18] Ideally, to assess the effect of CXL, the modulus of elasticity should be measured across different regions of the cornea to study the extent and efficacy of the CXL treatment. A technique that can detect local changes in rigidity is likely to help improve our understanding of why the results of CXL are different between species. Novel techniques such as nanoindentation and scanning acoustic microscopy (SAM) have been used successfully on human tissue[39,40] to measure the mechanical properties, and these techniques might be suitable for use on human corneas.

Scanning acoustic microscopy allows a nondestructive evaluation of the biomechanical properties of tissues quantitatively and qualitatively.[40,41] It provides a point-by-point analysis of the tissue sample with high spatial resolution (around 1 μm at 1 GHz).[40–42] Scanning acoustic microscopy comprises a transducer, which emits and absorbs acoustic (ultrasound waves); coupling fluid, which is mainly water; and a lens.[41] The transducer transforms the electrical pulses into ultrasound waves (100 MHz-1 GHz)[40] that pass through the coupling fluid and are then focused by the lens on the test sample. The ultrasound waves then propagate within the tissue and reflect back with different vibrations according to the different stiffness values of the tissue tested, which can be used to calculate the Young's modulus of each point of the sample.[40–42]

Scanning acoustic microscopy resolves many of the limitations of strip extensometry. Using SAM, multiple readings can be taken of each cornea, which provides more accurate and precise measurements. This technique has been widely used in industry[43] and on biological tissue, such as bone, teeth,[44,45] and blood vessels.[40] A pilot study has shown that this technique can be applied to corneal tissue.[46]

Nanoindentation is known as a powerful tool for measuring and mapping the micromechanical and elastic properties of materials and tissues.[47] It has been applied to bone and teeth tissue[48] but not to corneal tissue. In nanoindentation, a controlled load is applied to the specimen's surface to induce local deformation.[39,48] Load and displacement are monitored and used to calculate the hardness and stiffness of the tested material. The small tips used allow nanoindentation to measure thin and small specimens.[47] Nanoindentation is also able to measure the hardness of heterogeneous samples and map the biomechanical properties across the surface layer of the tested sample.[47]

These 2 proposed techniques could be good alternatives to the techniques used previously in the CXL literature. Nanoindentation and SAM are more reliable and more accurate as they can measure the biomechanical properties of the cornea locally, not globally, as CXL takes place primarily in the anterior part of the cornea.

Corneal Hysteresis and Corneal Resistance Factor

Other corneal rigidity measurements include corneal hysteresis (CH) and the corneal resistance factor (CRF). The CH is an indication of the viscosity of the cornea, while the CRF represents the cornea's resistance to deformation.[49,50] These factors have been widely examined in vivo by the Ocular Response Analyzer (ORA) (Reichert, Inc.), which is a noncontact applanation tonometer.[50] The instrument assesses the corneal response to indentation (change in shape) as a result of an air pulse. When the air pulse is released and hits the cornea, the cornea moves inward, passing an applanation point (P1). The pressure then decreases gradually until the cornea recovers its original shape, passing through an applanation point (P2).[50] The electro-optical system, which is attached to the ORA, monitors the whole process and measures the CH and CRF parameters according to 2 formulas: CH = (P1–P2), and CRF = (P1–*K*P2), where *K* is a constant.[50]

Changes in the CH and CRF parameters after UVA–riboflavin CXL were studied by Sedaghat et al.[27] and Goldich et al.[49] on keratoconic corneas in vivo. Both studies measured the CH and CRF factors using the ORA before and after treatment for up to 6 months. Goldich et al.[49] did their experiment on a small sample of 10 eyes of 10 keratoconic patients, while Sedaghat et al.[27] used a larger sample of 56 eyes of 51 patients. The baseline measurements of CH and CRF in both studies are similar. The follow-up evaluation at 6 months showed that the CH values decreased slightly and the CRF values remained almost the same in Goldich et al.'s[49] study, while the CH and CRF values increased slightly in Sedaghat et al.'s study.[27] However, the CH and CRF values reported in the 2 studies were not significantly different before and after corneal CXL. Spoerl et al.[51] used a sample of 50 eyes of 46 keratoconic patients and found no change in CH and CRF values. However, using the new version 3.0 ORA software, Spoerl et al. found a statistically significant increase (35%) in the area under peak 2 (the second curve) after CXL, which suggests that changes in this area are more sensitive to CXL treatment than changes in absolute CH and CRF values. Measurements of CH and CRF in both studies are summarized in Table 2. The ORA results contrast with what other researchers[8,18,20,21,23,26] have reported regarding the increased rigidity of the corneas after CXL in vitro, which may be due to the different methodologies used (in vitro strip extensometry versus in vivo ORA) or because of the corneal curvature, which in keratoconus is not homogenous, and thus taking the mean of the variable measurements may neglect the subtle changes in these parameters.[27] Terai et al.[52] suggest that the lower measurements of CH and CRF factors after CXL are not related to fewer crosslinks being induced by the treatment but to the dynamic method used by the ORA, which measures the biomechanics of the collagen fibers and the viscous ground substance (proteoglycans and glycosaminoglycans)[52] while CXL changes only the collagen fibers.[53] Using a static method may provide a better evaluation of the CXL effects on the cornea.[54]

Collagen Fiber Diameter

Collagen fibers are expressed throughout corneal tissue, especially in the stromal layer, and they are responsible for providing corneal strength and rigidity.[55,56] A normal collagen fiber in a healthy cornea has the ability to load 10 kg of weight.[56] Furthermore, the regular organization and orientation of the collagen fibers within the cornea give it the required transparency, which is important to corneal function.[55] In keratoconus, the elasticity of the collagen fibers is reported to decrease by 36%, causing a general corneal weakness.[14] Thus, one of the most important morphological changes after CXL that scientists have examined is the change in collagen fiber diameter.

Collagen fiber diameter was evaluated after CXL in normal healthy rabbit corneas[57] and more recently in human keratoconic corneas[8] in vitro. Wollensak et al.[57] used the electron microscope and morphometric computer software to calculate the collagen diameter of 10 crosslinked and the contralateral control eyes in 10 rabbits. The collagen diameter in the anterior and posterior stroma was evaluated and was significantly increased in the crosslinked corneas compared with the normal controls.[57] This increase in collagen diameter occurs because the regenerated crosslinks push the fibers apart and thus induce an increase in the intermolecular spacing and collagen diameter.[59] Moreover, in the treated eyes, the collagen diameter was significantly larger in the anterior stroma than in the posterior stroma,[57] which may be due to localization of the CXL effect in the anterior portion of the stroma.[18]

Table 2. Preoperative and postoperative CH and CRF measurements in 3 previous studies.

			Postoperative			
Study	Preoperative	1 Wk	1 Mo	3 Mo	6 Mo	1 Y
Goldich et al.[49] (N = 10)						
CH (mm Hg)	8.44 ± 1.82	8.62 ± 1.56	8.22 ± 1.50	7.88 ± 1.57	8.14 ± 1.32	—
CRF (mm Hg)	7.15 ± 1.77	8.48 ± 1.55	7.91 ± 1.54	7.1 ± 1.51	7.16 ± 1.45	—
Sedaghat et al.[27] (N = 56)						
CH (mm Hg)	7.99 ± 1.5	—	—	—	8.20 ± 1.5	—
CRF (mm Hg)	7.36 ± 1.4	—	—	—	7.59 ± 1.5	—
Spoerl et al.[51] (N = 50)						
CH (mm Hg)	7.38 ± 1.42	—	—	—	—	7.37 ± 1.26
CRF (mm Hg)	6.16 ± 1.42	—	—	—	—	6.16 ± 1.50

CH = corneal hysteresis; CRF = corneal resistance factor
All measurements are mean ± SD.

The collagen diameter in the treated rabbit corneas increased by 12.2% anteriorly and by 4.6% posteriorly.[57] Later, Mencucci et al.[58] used the immunohistochemical analysis to evaluate the collagen fiber diameter in 5 normal, 5 keratoconic, and 5 crosslinked keratoconic human eyes. They evaluated the collagen fiber diameter in the anterior and posterior stroma and confirmed the results reported by Wollensak et al.[57] The increase in the collagen diameter in the human treated corneas was 22.6% anteriorly and 16.1% posteriorly.[58] The difference between the 2 species may be due to the higher percentage of the generated crosslinks in human corneas.[8,26,32]

Thermomechanical Behavior

Thermal damage was expected to occur after CXL because the treatment duration is long (30 min) and UVA is highly absorbed in the corneal tissue in the presence of the photosensitizer riboflavin. Corneal surface temperature was measured using an infrared thermocamera and ThermaCam Researcher Software in 6 keratoconic human eyes during the CXL procedure.[60] A constant temperature increase was observed during the treatment, and it did not exceed the threshold of thermal damage for corneal collagen fibers (50°C).[61] Using the riboflavin helped keep the corneal temperature down, as the instillation of riboflavin drops cooled the corneal surface by 4°C ± 0.3°C (SD) throughout the procedure.[60] This study suggests that UVA–riboflavin CXL is a safe procedure and does not cause thermal damage to the corneal collagen fibers.

Spoerl et al.[62] studied the hydrothermal shrinkage effect of porcine corneas after CXL. Crosslinking using UVA–riboflavin increased the corneal tolerance to shrinkage temperature up to 75°C in the anterior portion of the corneas, while CXL using 0.1% glutaraldehyde increased the corneal resistance to shrinkage temperature up to 90°C. However, the maximal shrinkage temperature in the posterior stroma and untreated corneas was up to 70°C. The shrinkage of the corneas is believed to occur as a result of collagen denaturation and strands uncoiling because of the increased heat.[62] The UVA–riboflavin CXL effect was localized to the anterior portion of the cornea in this study, which implies it is less toxic to the corneal endothelium, crystalline lens, and retina.

Enzymatic Digestion

Increased collagenase activity in keratoconus is believed to play an important role in the corneal thinning pathogenesis.[63] This has led researchers to study the influence of UVA–riboflavin CXL on corneal resistance to collagenases and other enzymatic factors. Increased resistance after CXL to collagenases pepsin and trypsin digestion was shown on 80 porcine corneas by Spoerl et al.[64] The highest corneal resistance was to collagenases and the lowest, to trypsin. Trypsin could not digest any of the control or treated corneas until they were denatured by heat. Other interesting observations were the corneal resistance to digestion, which was UVA-level dependent; the higher the irradiation level (3 mW/cm^2), the more corneal resistance. Moreover, the digestion of the treated corneas commenced in the posterior stroma and moved to the anterior stromal portion, while no localization preference of digestion was observed in the control corneas.

Wollensak and Redl[65] found that collagen type I developed a high molecular polymer band after corneal CXL with UVA and riboflavin. The intense molecule proved to be chemically stable as it was resistant to pepsin, mercaptoethanol, and heat treatments.[65] This biochemical finding explained the corneal resistance to collagenases pepsin and trypsin digestion and the increased collagen fiber diameter after CXL, and it may also be a factor in the biomechanical changes after CXL.

Corneal Swelling Behavior

Stromal swelling affects corneal transparency and thus vision.[66] Therefore, it was important to examine the hydration of corneas after UVA–riboflavin CXL. Wollensak et al.[67] studied the alteration of corneal swelling behavior after UVA–riboflavin treatment in porcine corneas (20 corneas were crosslinked and 5 untreated corneas were used as controls). The corneas were first crosslinked and then put in a moist chamber for 24 hours to ensure maximum hydration.[67] The study showed that the swelling behavior was dependent on the degree of CXL; the higher the CXL, the lower the corneal swelling behavior.

The average thickness of the porcine corneas before hydration was 851 ± 25 μm. After 24 hours of hydration, the thickness of the untreated corneas was 2300 ± 165 μm, which means they thickened approximately ×2.7, and the thickness of the crosslinked corneas was 1900 ± 180 μm. The difference between the treated and untreated corneas was statistically significant ($P < .01$).[67] Using light microscopy, the crosslinked cornea was divided into 3 zones depending on the induced CXL percentage. The first was the anterior 242 μm of the cornea, where the CXL was primarily localized; the second was the intermediate 238 μm, where CXL was partially induced; and the third was the posterior 1355 μm region, where no CXL was induced.[67] No change in thickness was observed in the first 242 μm after hydration, while the second zone and third zone were thickened by ×2.2 and ×2.7,

respectively, after hydration.[67] This study identified the different behavior of the treated and untreated corneas against hydration. Similar results might be expected in the in vivo human situation, where the corneal endothelium is intact and functioning.

The increase in availability and popularity of the CXL technique emphasizes the need for reliable and accurate techniques for measuring corneal biomechanical properties before and after treatment. Different methodologies such as strip extensometery,[8,18,20,21,23,26] inflation,[19] and ORA[27,49] have been described. However, other techniques have been used to characterize these properties in other tissues (eg, hard tissues such as bones and teeth,[48] cells, and soft tissues[39,40]) in humans and in animal models. Some of these techniques may be adaptable to characterize the biomechanical properties of the cornea in future. Possible approaches include SAM and nanoindentation, which may be less destructive and more accurate than traditional techniques.

SUMMARY

Collagen crosslinking using UVA irradiation and riboflavin was introduced as a new treatment for keratoconus. This paper reviewed the biomechanical effects of CXL on corneal tissues. Research has primarily studied the short- and long-term biomechanical effects of CXL on human, porcine, and rabbit corneas using different methodologies. Postoperative results such as the increase in corneal rigidity, collagen fiber diameter, and the increased resistance to heat degradation, enzymatic digestion, and swelling due to hydration are positive indicators of the efficacy of the treatment. Most of these studies suggest that UVA–riboflavin CXL is localized to the anterior portion of the stroma. This suggests there are minimal effects of UVA irradiation (toxicity or cell death) on the corneal endothelium and the posterior parts of the eye (crystalline lens, vitreous, and retina), which is important for the safe use of this technique. Most studies have been done on animal (porcine and rabbit) corneas or on healthy human corneas, but differences have been found between the corneas of these 3 species. Further research on human corneas to support the animal model studies and for longer follow-up periods is needed. Moreover, more research should be done on keratoconic corneas after CXL as they are biomechanically different from normal corneas and different results may be obtained.

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