**Long-term effects of riboflavin ultraviolet-A-induced crosslinking with different irradiances on the biomechanics of in-vivo rabbit corneas**

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**Abbreviated title:**

Long-term effects of CXL with different irradiances on cornea

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**Highlights:**

The long-term effect of CXL on corneal biomechanics decreased gradually with increasing UV-A power density.

**Abstract**

***Purpose***

To evaluate the long-term effects of ultraviolet-A crosslinking (CXL) with different irrandiances on the biomechanical properties of rabbit corneas and the corresponding changes in stromal microstructure.

***Methods***

The study involved the left eyes of eighty-five healthy white Japanese rabbits, randomly divided into five groups (n=16 to 18 each). After removing the epithelium, the first four groups were exposed to riboflavin (0.22% concentration by volume) and ultraviolet-A (370 nm) at different CXL irradiations but with the same total dose (5.4 J/cm2). The four groups were defined as the SCXL group (standard CXL, 3 mW/cm2 for 30 min, n=17), ACXL1 group (accelerated CXL1, 9 mW/cm2 for 10 min, n=16), ACXL2 group (accelerated CXL2, 18 mW/cm2 for 5 min, n=17) and ACXL3 group (accelerated CXL3, 30 mW/cm2 for 3min, n=17). The control (CO) group (n=18) was treated with riboflavin without ultraviolet-A exposure. Nine months after CXL, ten corneas from each group were tested ex vivo under inflation, and the tangent modulus (Et) was estimated using an inverse analysis process. The remaining six to eight specimens in each group were examined by electron microscopy to determine the mean fibril diameter and interfibrillar spacing.

***Results***

Compared with the control group, the mean corneal tangent modulus of each cross-linking group and the increased ratio under different stress were indicated in Table 4. The SCXL group and ACXL1 group showed statistically significant differences in all stresses analyzed compared to the CO group (P<0.01), while remained similar in ACXL3 group (P>0.05). For ACXL2 group, there was no statistical difference in the low stress range of 0.005 MPa, while became statistically significant in 0.01 MPa and 0.015 MPa when compared with the CO group.

***Conclusions***

CXL had a significant long-term effect on corneal biomechanics in both standard and accelerated procedures. However, the standard CXL was the most effective, and this effectivness decreased gradually with increasing UV-A power density.

**Introduction**

Keratoconus is a progressive and non-inflammatory disorder of the eye characterised by thinning and protrusion of the cornea1. The pathogenesis of keratoconus includes the increase of lysosome and proteolytic enzyme expression, decrease of protease inhibitor levels, and disruption of stromal collagen lamella arrangement 2-4. Corneal crosslinking (CXL) was first described by Spoerl and Seiler in 1997 with the aim to increase corneal stiffness to stabilise the ectatic cornea 5,6. CXL mainly increases the formation of covalent bonds in and between fibrils through photosensitive oxidation 7, thus increasing mechanical stiffness 8,9. Since keratoconus is a progressive disease, maintaining long-term corneal stability is an essential part of disease management.

In 2003, Wollensak et al published the first clinical study on the use of CXL to halt the progression of keratoconus 7. Early studies on the subject focused mainly on corneal morphologic changes after CXL 10-12. However, in recent years and with the advancement of behavior monitoring technology, evaluation of corneal biomechanics became more promiment, leading to estimates of the stiffness increase caused by the CXL procedure between 21% and 144% 13,14.

Since its introduction in previous study 7, the "Dresden" protocol, which involves dropwise application of 0.1% riboflavin in 20% dextran followed by 30 minutes of ultraviolet-A (UVA) irradiation, has become the standard CXL treatment regime. As this protocol was considered too time consuming, a variety of accelerated protocols were suggested to reduce operating time 15, improve patient’s comfort and decrease the likelihood of complications 16. One of these protocols involved shortening the irradiation time (from 10 min to 1 min) while increasing its irradiation intensity in order to maintain the same energy level of 5.4 J/cm 2 15,17,18. This protocol was developed in accordance with the Bunsen-Roscoe law of reciprocity 7,19. The development of the accelerated protocol (ACXL) was followed by studies on its long-term effects on visual acuity and corneal topography – relative to the standard protocol 20-23, and then, the long-term changes in corneal biomechanics 24,25 also became the focus of ACXL research.

Studies performed on ex-vivo human corneas and porcine corneas showed that the proportion of the stiffness increase was less in accelerated protocols due to a decrease in CXL effect, even though the total energy provided remained constant 15,26,27.These studies relied on uniaxial testing of corneal tissue, which, due to dissection and tissue flattening, subjected the specimens to non-physiologic conditions 28. The current study seeks to confirm and quantify this trend and aims to assess the effect of standard CXL (SCXL) and ACXL protocols on the long-term biomechanical behavior of rabbit corneas using inflation testing – a more representative testing protocol of physiologic conditions than uniaxial testing. The study also seeks to identify, using transmission electron microscopy (TEM), the changes in stromal microstructure that take place due to the SCXL and ACXL procedures. This part of the study was conceived due to the proven dependence of corneal biomechanical behavior on the tissue’s microstructure, and in particular the collagen fibril structure within the stroma 29-33.

**Materials and methods**

***2.1 Experimental animals***

The left eyes of eighty-five, 3 months old, Japanese white rabbits (2.0–3.0 kg) were inclued in this study. The eyes were randomly divided into five groups, each including 16 to 18 rabbits. The groups were named SCXL, ACXL1, ACXL2, ACXL3 and control (CO) (see Table 1). All animals were obtained from the Animal Breeding Unit of Wenzhou Medical University and observed for two weeks before commencing the experimental study. All animals were treated in agreement with the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research, subject to the approval of the Animal Care and Ethics Committee of the Eye Hospital of Wenzhou Medical University.

***2.2 Preparation and CXL procedure***

The rabbits were premedicated with a subcutaneous injection of SU-MIANXIN (Veterinary Institute at University of Munitions, Changchun, China) (0.2 mL/kg). General anaesthesia was administered with an intramuscular injection of pentobarbital sodium (Merck KGaA, Darmstadt, Germany; 30 mg/kg). Additional local anaesthesia consisting of 0.5% propantheline eyedrops was instilled into the left eyes, and a wire eyelid speculum was positioned in the same eyes. Prior to UVA irradiation, the central 9 mm of the corneal epithelium was carefuly removed using a hockey knife, and the corneas were saturated with 0.22% riboflavin drops (VibeX Xtra; Avedro, Inc., Waltham, MA) at 3-minute intervals over a total period of 30 minutes. Following this step, the CXL procedure was performed while performing the protocols explained in Table 1 using the CXL system (CL-01; SiHaiTong Co., Suzhou, China). The protocols involved subjecting the central cornea with 9-mm diameter to irradiation with different intensities and exposure times (to a total energy dose of 5.4 J/cm2).

Immediately after CXL, and for three times a day for one week, the left eyes received tobramycin ophthalmic ointment (Tobrex; Alcon Laboratories, Inc., Fort Worth, TX) and deproteinised calfblood extract eye gel (Xingqi; Shenyang Xingqi Pharmaceutical Co., Ltd., Shenyang, China) to ensure complete re-epithelialization.

***2.3 Corneal thickness***

Central corneal thickness (CCT) was measured in preoperatively (CCT-pre), after epithelium removal but before CXL treatment (CCT-preCXL), 1-week post-CXL (CCT-pos1w), 1-month post-CXL (CCT-pos1m), and 9 months post-CXL (CCT-pos9m). These measurements were taken using an ultrasound pachymeter (SP-3000; Tomey, Inc., Nagoya, Japan). Three thickness readings were taken at each time point, and the mean value was used in analysis.

***2.4 Specimen preparation***

Nine months after CXL, the rabbits were sacrificed by intravenous injection of high concentrations of pentobarbital sodium, and the treated eyes were immediately enucleated. Ten corneas from each group were prepared for inflation testing, while the others were used for histological measurements. The cornea along with a 3 mm-wide ring of scleral tissue was mechanically separated from the eye globe using a pair of curved scissors, followed by the removal of the iris, lens and ciliary body. The specimens were then kept in a storage medium of phosphate-buffered saline (PBS, Maixin, China) for a maximum of 30 min until testing on an inflation rig. It's important to emphasize here that nine months had been the longest term we could manage to get enough samples because the survival rate of our experimental rabbits dropped drastically after this period, largely due to environmental factors such as temperature, humidity and bacteria controls and the anesthesia used during the cross-linking procedures.

***2.5 Instrumentation and inflation testing procedures***

The experimental setup of inflation testing is shown in Figure 1 and detailed in previous studies.34,35 The cornea was mounted on a pressure chamber (Figure 1a) filled with PBS. Each specimen was first subjected to an initial inflation pressure of around 2.0 mmHg to remove any initial wrinkles on the ocular surface. Then, at a loading rate of 0.40 mmHg/s and a maximum pressure of 32 mmHg (increment: 30mmHg), three cycles of loading and unloading were performed to condition the tissue and stabilize its behavior following a process developed in earlier studies 35,36. A relaxation time of 90 seconds was introduced between each two subsequent cycles to enable recovery of specimens’ initial geometry.37 During this recovery period, PBS was sprayed on the surface of the cornea to prevent dehydration. The fourth loading cycle was then applied, the results of which were used in the subsequent inverse analysis process to estimate the biomechanical behavior of the tissue. The pressure was controlled using a custom-built LabView software and was continuously monitored by a pressure transducer (DMP-HS, Hangzhou, China) connected to the chamber. A laser displacement sensor (LK series; Keyence, Ithasca, IL) was connected to the LabVIEW software and continuously monitored the displacement of the corneal apex.

Images of the cornea specimens were recorded during the inflation tests using three digital cameras positioned at 75, 195 and 315 degrees relative to the horizontal direction (Figure 1b). The front shape of the cornea was obtained through processing the camera images with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The central, paracentral and peripheral corneal thicknesses (at 0, 3 and 5 mm away from corneal apex, respectively) were measured with an SP-3000 ultrasound pachymeter. Each measurement was taken three times and the average value was recorded. The thickness measurements and the front shape of the cornea were then used to construct the back surface. The experiment was completed within 3 hours.

***2.6 Inverse analysis***

The material stiffness of corneal tissue was evaluated using an inverse analysis process. In this method, finite element solver Abaqus (Dassault Systèmes Simulia Corporation, Forest Hill, MD) and the LS-OPT optimisation software package (Livermore Software Technology Corporation, Livermore, CA) were used to perform the inverse analysis process described in a previous study 38.

Fifty specimen-specific finite element models representing all inflation test corneas were constructed. Each model incorporated the measured thickness, corneal profile and limbal diameter data, and had 1,728 fifteen-noded continuum elements (C3D15H) arranged in twelve rings and two layers (Figure 1c). The connection of tissue to the mechanical clamps was simulated by assuming encastre boundary conditions along the limbus. A first-order hyperelastic Ogden model 39,40 was used to represent corneal material behavior with a strain energy function in the form:

$W=\frac{2μ}{a^{2}}\left(\overbar{λ\_{1}}^{α}+\overbar{λ\_{2}}^{α}+\overbar{λ\_{3}}^{α}-3\right)+\frac{1}{D}(J-1)^{2}$ Equation 1

where W is the strain energy per unit volume, λk the deviatoric principal stretches = J-1/3×λk (k = 1, 2, 3), λ1, λ2, λ3 the principal stretches, J = λ1λ2λ3. α and μ represent the strain hardening exponent and the shear modulus, respectively. D is a compressibility parameter, whose value is dependent on Poisson’s ratio, ν; D$ =\frac{3\left(1-2v\right)}{μ\left(1+v\right)}$. As Poisson’s ratio for the cornea was estimated earlier between 0.45 and 0.5 41, a value of 0.48 was assumed in this study.

The inverse analysis process was effectively an optimization process where μ and α were varied within pre-set ranges while monitoring corneal deformations under the applied inflation posterior pressure. The process continued until the experimental displacements predicted numerically at corneal apex matched the experimental recorded displacements. Essentially, the analysis determined the optimal values of the material parameters μ and α for each cornea by minimizing the root mean square error (RMSE) between the experimental and numerical displacements at the corneal apex using the following objective function:

$RMSE=\sqrt{\frac{1}{P}.\sum\_{p=1}^{P}(δ\_{p}^{exp}-δ\_{p}^{num})^{2}}$

where *P* is the total number of pressure levels at which the *RMSE* is calculated (i.e. 2, 4, … up to 32 mmHg), and $δ\_{p}^{exp}$ and $δ\_{p}^{num}$ represent the experimental and numerical displacements of the corneal apex at pressure level $p$. The ranges adopted for the variations in μ and α were 0.001 to 0.1 and 10 to 300, respectively, as in all 50 corneas studied, the optimized values of μ and α were within these ranges.

***2.7 Histological analysis***

Six to eight corneas were taken from each group for histological measurements, which were performed in a pressure-free state. The corneas were fixed in freshly prepared glutaraldehyde embedded in Epon (SPIPON 812 Kit; Structure Probe, Inc., West Chester, PA) at 4 °C, sectioned on the sagittal plane and stained with uranyl acetate and lead citrate to semi-quantify the collagen components and interfibrillar spacing. Miron-thick (50 nm) sections were obtained from each cornea at 80 μm below the epithelium and viewed under a TEM (H-7500; Hitachi, Ltd., Tokyo, Japan) at a magnification of ×40000. This depth was selected due to evidence that CXL had a stiffening effect that was most effective in the anterior part of the stroma 42-44. One TEM image was taken from each cornea. A professional pathologist (Shen LJ) performed the histological assessment. TEM images were analysed using the ImageJ image processing software. The original TEM image was converted into an 8-bit grayscale image, which was denoised using a bandpass filter. The resulting image was then thresholded for the color saturation of the fibrils signal and the Watershed algorithms were used to identify borders of fibrils that touch each other 45. After selecting the region of the image, the amount and individual size of the fibrils in the image, displayed by ImageJ as surface area in square nanometers (nm2), were measured. Incomplete fibrils located at the edge of the image were excluded. The mean fibril diameter and mean interfibrillar spacing were then calculated assuming that the fibrils were circular (Figure 2):



The mean values of interfibrillar spacing and fibril diameter obtained from the five groups of TEM images were used in the analysis.

***2.8 Statistical analysis***

Quantitative data were presented as the mean ± standard deviation, and all statistical analyses were performed using PASW Statistics 20.0 (SPSS Inc. Chicago, USA). Comparisons of CCT values measured at different test stages and tangent modulus (Et) obtained for the five specimen groups were performed using analysis of variance (ANOVA) and the Bonferroni post hoc test. Comparisons between measurements of CCT-pre, CCT-preCXL, CCT-pos1w, CCT-pos1m and CCT-pos9m in each specimen group were tested using the MANOVA of repeated measurements. p values less than 0.05 were considered indicative of statistical significance.

**Results**

***3.1 Corneal thickness and pressure deformation behavior***

CCT measurements, taken at the same time points, showed no significant differences between any of the CXL groups and the CO group (all p > 0.05) – the only exception was in CCT-pos1w in the SCXL group (Table 2). The CCT measurements also showed a significant increase (138.4±36.6 μm) between pre-epithelium removal and after riboflavin infiltration (CCT-preCXL vs CCT-pre, p < 0.05), possibly due to swelling. This trend was followed by a significant decrease (115.9±42.1 μm) at 1-week post-CXL (CCT-pos1w vs CCT-preCXL, p < 0.01). CCT then continued to decrease from 1week to 9-month post CXL (CCT-pos1w vs CCT-pos9m, p < 0.01), at which time it became close to the thickness at the pre stage in all groups (CCT-pos9m vs CCT-pre, p=1.00).

***3.2 Experimental behavior throughout and material constitutive models***

As shown in Figure 3, a clear difference was observed in mean pressure-displacement behavior at corneal apex between the five specimen groups. All specimens exhibited nonlinear pressure-displacement behavior with a low initial stiffness at low-pressure levels up to 10 to 18 mmHg, beyond which the behavior became almost linear. Ogden material parameters α and µ were obtained from the inverse analysis process to provide the best possible fit (lowest RMSE) with the experimentally obtained pressure-displacement results. The stress-strain (σ – ε) relationship for each cornea was then determined along with its tangent modulus (Et = dσ / dε).

For comparative purposes, Et is reported at three stresses of 0.005 MPa, 0.01 MPa and 0.015 MPa in Figure 4 and table 4. Compared with the CO group, the mean Et was significantly higher in the SCXL group (by 176%, 150% and 140%, respectively, p < 0.01) and ACXL1 group (by 100%, 100% and 99%, respectively, p < 0.01) but not the ACXL3 groups (by 32%, 32% and 30%, respectively, p =1, 0.785, and 0.679, respectively). For the ACXL2 group, the mean Et was significantly higher at the stresses of 0.01 MPa and 0.015 MPa (by 55% and 54%, respectively, p < 0.05) but not the stress of 0.005 MPa (by 53%, p =0.155).

***3.3 Histological analysis***

No significant differences in fibril diameter were found among the five specimen groups (p = 0.080) after analysing TEM images (see Figure 5 and Table 5). However, for the interfibrillar spacing, there were significant differences between each of the four CXL groups and the CO group (all p < 0.05). Further, the ACXL2 and ACXL3 groups showed significant differences (p < 0.05) from the SCXL group. The interfibrillar spacing was also lower in all four CXL groups than in the CO group (by 23%, 19%, 15% and 8% for SCXL, ACXL1, ACXL2 and ACXL3 groups, respectively, p = 0, 0, 0, 0.013).

**Discussion**

The aim of this study was to evaluate the long-term effects of standard and accelerated CXL on the biomechanics of rabbit corneas. The results indicate an increase in corneal stiffness (as measured by the tangent modulus, Et) after CXL throughout the nine months of follow-up. Although the total irradiation energy remained the same in all protocols, the increase in stiffness was most significant in the SCXL group (3 mW/cm2 for 30 min) where Et at 0.01 MPa stress increased by 150%. The stiffness gain was lower with the accelerated protocols; down to 100%, 55%, and 32% with 9 mW/cm2 over 10 min, 18 mW/cm2 over 5 min, and 30 mW/cm2 over 3 min, respectively.

The stiffness increase after SCXL was higher than the results of previous studies where Et increase after SCXL was 64% 46 and 70% 47 (ex-vivo human cornea), 42% 48 and 58% 49 (ex-vivo porcine cornea), 102% 50 and 113% 51 (ex-vivo rabbit cornea). These differences in stiffening magnitude can be due to a number of reasons. First, the biomechanical measurements were performed at 1 day 49,50, 2 days 48 and one week 51 after CXL in these studies, respectively, whereas our study used measurements at nine months after CXL. Wollensak et al 52 reported a total loss of cells in the irradiated area of rabbit corneal stroma following standard crosslinking, and that this cytotoxic damage could be repaired by re-propagation in 4-6 weeks. Over the same period, α-actin-positive myofibroblasts were identified, especially in the periphery of the irradiated areas. As myofibroblasts are critical components of the healing cascade 53, and play an important role in extracellular matrix remodeling through production of collagen, glycosaminoglycans and collagenases 54-56, they are expected to contribute to tissue stiffening. Second, in this study, CCT was found to have increased significantly one week after both SCXL and ACXL, in line with earlier observations 51, and this increase, which may be due to corneal oedema during epithelial healing, may lead to decreased biomechanical stiffness 57,58.

In addition, the use of corneal tissue from different species may have played a role. Since there is evidence that CXL only affects the most anterior 300 μm of stromal tissue 44, and that human corneas and porcine corneas are significantly thicker than rabbit corneas, a larger percentage of the thickness of human corneas and porcine corneas would be expected to remain unaffected by CXL than in rabbit corneas. This difference could, in turn, lead to lower CXL-related stiffness increases in human and porcine corneas relative to rabbit corneas 59. In addition, the concentration of riboflavin was different between studies, as Matteoli et al 48, Wollensak et al 50 used 0.1% and Bao et al 51 used 0.22%, similar to this study. The concentration of riboflavin has an effect on the cornea post CXL. Under the same irradiance, the higher concentration of riboflavin adopted in our study (0.22%) may increase the absorption of UVA photons, reduce the potential damage of UVA toxicity to intraocular structures (such as endothelium), and enhance the resistance to enzymatic digestion 60; it can also lead to increased corneal stiffness 61, and improve CXL effectiveness 62. On the other hand, a higher riboflavin concentration can reduce the curative effect difference between ACXL and SCXL 60, which is because high-intensity UVA irradiation in ACXL may significantly reduce the concentration of riboflavin in the matrix 62.

More importantly, the experimental platforms used in these studies were not the same. The strip extensiometry used in some studies 46,47,50 involved cutting the cornea into strips and subjecting the strips to uniaxial tension. There is evidence that this test method affects corneal behavior due to flattening of the naturally curved specimens and ignoring the regional variation in corneal thickness and biomechanical properties 28 . In contrast, the inflation testing, used by Matteoli et al 48, kling et al 49 and Bao et al 51, which is also used in the current study, maintains corneal integrity and imposes a load similar to intraocular pressure. In our previous study 51, the corneal tangent modulus increased by 113% at the stress of 0.01MPa one week after SCXL, which is close to the 150% increase in the nine months after SCXL exhibited in this study.

CXL adds tensile stiffness to the tissue by producing additional covalent bonds within and between collagen fibres 63. This process requires oxygen 64, and hence shortening the irradiation time may reduce the effect of CXL even while keeping the irradiation energy unchanged. In this study, the increase in Et decreased gradually with increasing UV-A power density. This trend is compatible with earlier studies in which ACXL was found to lead to reduced effectiveness. Wernli et al 15 and Kanellopoulos et al 65performed accelerated cross-linking of porcine corneas and human corneas in vitro, respectively, and found that the increases in corneal stiffness and the resistance to enzymatic digestion decreased gradually with higher irradiation , especially when the intensity exceeded 45 mW/cm2.

Previous studies investigated the corneal stromal structural changes associated with CXL. TEM images of the human corneas in vitro showed non-significant increases in fibril diameter, and significant decreases in interfibrillar spacing of the anterior-mid stromal region after standard and accelerated CXL 26. In-vitro crosslinking experiments of porcine corneas showed an increase in collagen fibril diameter and no significant changes in interfibrillar spacing 66. Tests on in-vivo rabbit corneas also showed an increase in the diameter of collagen fibrils after 4h of CXL treatment 44, which may have been a transient collagen fibril swelling induced by CXL treatment 18. This study also investigated the possible changes in the diameter and interfibrillar spacing of collagen fibres in the anterior stroma, where CXL is known to be most effective 26,44. No significant differences were found in the diameter of collagen fibrils after CXL, while there was a significant negative correlation between interfibrillar spacing and irradiation time (R2 = 0.52, p < 0.01). This trend is in line with previous studies, which found that CXL increased covalent bonding between collagen fibrils and reducd the hydration of corneal stroma 7,67. This effect would make the fibrils more tightly packed (reduce spacing between the fibrils) and improve material stiffness; a trend that was most evident in SCXL.

At present, there is no established and agreed trend on the change in corneal thickness after CXL, and there is lack of long-term studies on that subject. Some studies reported that corneal thickness of keratoconus patients decreased one year after CXL 11,68,69, while others found no significant change in thickness one year or 18 months after surgery 70-72. Since the operation is performed on patients with keratoconus, it may be difficult to quantify the effect of CXL on the thickness, which varies much across corneal surface. In our study involving animal models, which had a normal distribution of thickness, there was no significant change in corneal thickness at 1-month and 9-months post-surgery. However, our histological analysis found that the interfibrillar spacing reduced significantly after CXL, a trend which should have resulted in a decrease in corneal thickness.

There may be several reasons for this discrepancy. First, CXL is mainly effective on the anterior corneal stroma 44 and therefore, the change in collagen fibril spacing observed in this region may not affect significantly the overall corneal thickness. In addition, different from the clinical studies in which Pentacam or OCT was used to measure the corneal thickness at a fixed position 11,68,69,71 , the ultrasonic pachymeter used in this study required manual positioning – and this may have introduced human errors in the measurements.

The present study has some limitations. Due to the difficulties in obtaining human donor eyes in sufficient numbers, rabbit eyes were considered to be suitable alternatives as shown in previous experiments 73,74. Also, the use of inflation on the cornea (and not the whole eye) may have influenced corneal behaivour as it restricted the limbus from expanding freely. Once more, all rabbit eyes were healthy and without keratoconus, so the findings might be different in keratoconic eyes. Finally，the corneal stroma is a very dense matrix composed of hundreds of highly organized collagen lamellae. The stroma has self-cohesion between corneal lamellae 75, which may be caused by the lamellar interweaving, which varies with depth, and appears maximal at the anterior surface of cornea and decreases posteriorly 76. Repeated shear such as eye rubbing and eye compression may lead to the loosening of this interweaving effect, reduce the shear modulus of corneal tissue against shear strain 77, and initiate or induce the development of keratoconus 78-80. Therefore, it is necessary to understand the effect of CXL on corneal shear properties. However, inflation tests can only represent the in-plane properties of cornea, but cannot describe the transverse properties of corneal shear deformation 75.

In conclusion, this study confirms the long-term effectiveness of CXL in increasing the stiffness of the cornea. This effect is most evident in SCXL, and the effectiveness decreases with accelerated application of irradiance even while maintaining the same UVA energy. In severely progressive keratoconic corneas, SCXL may be necessary to ensure maximum impact on corneal biomechanics to stabilize the cornea.

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**Figure captions:**

**Figure 1** Corneal profile (a) captured before the start of the inflation test by one of the three cameras mounted on the inflation rig (b) and used to construct specimen-specific numerical models (c)

**Figure 2** The measurement of fibril diameter and interfibrillar spacing, where r is the radius of a collagen fibril and D is the interfibrillar spacing between two collagen fibrils

**Figure 3** Fitted pressure-displacement behavior at the corneal apex in the five specimen groups

**Figure 4** Mean and standard deviation of tangent modulus values for corneas included in each specimen group obtained at 0.005 MPa, 0.01 MPa and 0.015 MPa stress levels

**Figure 5** Cross-sectional images of corneal stroma showing collagen fibrils obtained using TEM with 40,000× magnification in the (a) SCXL, (b) ACXL1, (c) ACXL2, (d) ACXL3 and (e) CO groups

**Table captions:**

**Table 1** CXL settings adopted in different specimen groups

**Table 2** CCT measurements in all specimen groups

**Table 3** Constitutive parameters in the five specimen groups

**Table 4** Mean and standard deviation of tangent modulus at 3 stress levels

**Table 5** Interfibrillar spacing and fibril diameter in anterior 50 µm of corneal stroma in different specimen groups