**Use of Nanoindentation in Determination of** **Regional Biomechanical Properties of Rabbit Cornea after UVA Crosslinking**

XiaoBo Zheng 1,2,3,4, Yue Xin 5,4, Chong Wang 4, YiWen Fan 4, Peng Yang 4, LingQiao Li 4, DanPing Yin 4, ErChi Zhang 4, YuXin Hong 4, Han Bao 4, JunJie Wang 2,3,4, FangJun Bao 4,2,3, WeiWei Zhang 1\*, ShiHao Chen 4,2,3\*, Ahmed Elsheikh 6, Michael Swain 7

XiaoBo Zheng and Yue Xin are co-first authors of the article.

1School of Aeronautics, Northwestern Polytechnical University, Xi’an 710072, China

2National Engineering Research Center of Ophthalmology and Optometry, Eye Hospital, Wenzhou Medical University, Wenzhou, 325027, China

3State Key Laboratory of Ophthalmology, Optometry and Visual Science, Eye Hospital, Wenzhou Medical University, Wenzhou, 325027, China

4National Clinical Research Center for Ocular Diseases, Eye Hospital, Wenzhou Medical University, Wenzhou, 325027, China

5 Dalian Medical University, Affiliated Dalian No.3 People’s Hospital, Dalian 116092, China

6 School of Engineering, University of Liverpool, Liverpool L69 3GH, UK

7 AMME, Biomechanics Engineering, The University of Sydney, Sydney, Australia.

**Running title:**

Regional biomechanical effects of CXL on cornea

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## Co-Corresponding author

Professor Weiwei Zhang

No. 127 Youyi West Road, Xi’an City, ShanXi Prov, 710072, China

e-mail: aeroelastic@nwpu.edu.cn

Tel: 86-29-88491342; Fax: 86-29-88491342

**Corresponding author**

Professor ShiHao Chen

No. 270 Xueyuan West Road, WenZhou City, ZheJiang Prov, 325027, China

e-mail: csh@eye.ac.cn

Tel: 86-577-88068862

Fax: 86-577-88824115

**Highlights:**

The effect of CXL on corneal biomechanics decreased gradually from the center to the periphery and beyond the irradiated area.

**Conflict of Interest:**

All authors declare no conflict of interest.

**Abstract**

***Purpose***

Toevaluate the regional effects of different corneal cross-linking (CXL) protocols on corneal biomechanical properties.

***Methods***

The study involved both eyes of 50 rabbits, and the left eyes were randomized to the five intervention groups including standard CXL group(SCXL) and accelerated CXL groups (ACXL1-3), which were exposed to ultraviolet-A at different irradiations(3mW/cm2, 9mW/cm2, 18mW/cm2, 30mW/cm2, respectively) but with the same total dose(5.4J/cm2), and the control group(CO) was not exposed to ultraviolet-A. No surgery was done on the contralateral eye. The corneas of each group were evaluated by the effective elastic modulus(*Eeff*) and the hydraulic conductivity(*K*) within a 7.5mm radius using nanoindentation measurements.

***Results***

Compared with the CO group, *Eeff* (in regions with radii 0-1.5mm, 1.5-3.0mm and 3.0-4.5mm) significantly increased by 309%, 276% and 226% with SCXL; 222%, 209% and 173% with ACXL1; 111%, 109% and 94% with ACXL2; 59%, 41% and 37% with ACXL3, respectively, all P<0.05). *K* also significantly reduced by 84%, 81% and 78% with SCXL; 75%, 74% and 70% with ACXL1; 64%, 62% and 61% with ACXL2; 33%, 36% and 32% with ACXL3, respectively(all P<0.05). For the other regions(with radii between 4.5mm and 7.5mm), SCXL and ACXL1 groups(but not ACXL2 and ACXL3) still showed significant changes in *Eeff* and *K*.

***Conclusions***

CXL had a significant effect on corneal biomechanics in both standard and accelerated procedures which may go beyond the irradiated area. The effect of CXL in stiffening the tissue and reducing permeability consistently decreased with reducing the irradiance duration.

***Keywords***

corneal cross-linking; regional; biomechanical properties; nanoindentation

**Introduction**

The cornea is a significant part of the eye's refractive system, providing approximately 70% of the refractive power1. The corneal stroma accounts for approximately 85% of the entire corneal thickness2. Collagen fibrils interweave into a reticular structure in the stroma, and a liquid unevenly fills the gaps in the collagen fibril grid, forming a porous biological tissue with a fluid content of approximately 80%3, affecting corneal biomechanics, and helping maintain corneal morphology and transparency. In corneal ectatic diseases such as keratoconus (KC), in which progressive steepening of the cornea occurs, the normal parallel organization of collagen fibrils is disrupted resulting in progressive myopia, irregular astigmatism and significant effects on the vision and quality of life of patients4. Corneal cross-linking (CXL) effectively increases the mechanical stiffness of collagen fibrils and their ability to resist collagenase lysis, thereby increasing tissue stiffness and halting or slowing the progression of corneal distortion5.

Over time, CXL has gradually evolved from the early classic cross-linking with long treatment time to accelerated cross-linking protocols with shorter treatment time6. However, the regional changes of corneal biomechanical properties after CXL with different operative protocols are understudied, and the effective treatment range and the degree of local stiffening have not been fully evaluated. To address this need, biomechanical measurements such as inflation tests7 and uniaxial stretch experiments8 were used to evaluate the cornea as a whole, while Brillouin microscopy9 and optical coherence elastography10 were useful in quantifying the changes in elastic modulus at different stromal depths after CXL. Nevertheless, little attention was given to the spatial characterization of corneal stiffness, and to the area outside the irradiation range. In this study, nanoindentation was used to address these gaps. Additionally, due to the high fluid content and porous structure of the cornea, fluid-dependent viscoelasticity (also known as poroelastic viscoelasticity11, permeability12 or hydraulic conductivity12) plays a key role in influencing the tissue’s biomechanical response. This parameter was therefore included in our study while utilizing the photobleaching (FRAP) technique13,14 and permeameter with diffusion chamber15.

The nanoindentation used in this study is a method of measuring mechanical properties by pressing probes of a certain shape and size into materials16. Although traditional nanoindentation techniques were developed for stiff materials17, nanoindentation testing of hydrogels18, soft tissues19 and cells20, has subsequently attracted substantial attention because of the high spatial resolution nanoindentation allows with local testing of mechanical properties of soft matter that is not possible using macroscale techniques. Over the past decade, despite the challenges encountered with substantial inelastic and associated creep deformation of soft biomaterials, nanoindentation technology, especially utilizing spherical tipped indenters along with the classic Hertz model has gradually been used for reliable measurement of soft matter21. However, the measurement approach still needs to meet certain objective preconditions, such as contact strain (contact radius divided by indenter tip radius) < 0.4 for the samples tested22. In corneal measurement, nanoindentation technology has been applied in elasticity measurement23, creep testing23, porous media analysis12 and multilayer structure measurement3, and has become a widely accepted technique for material characterization. This study seeks to assess corneal regional biomechanical properties after UVA crosslinking through the use of nanoindentation technology, leading to measurements of the effective elastic modulus, *Eeff*, and the hydraulic conductivity, *K*, across the corneal surface.

**Materials and methods**

***2.1 Experimental animals***

Fifty New Zeeland White rabbits (3 months old, weight 2.0–3.0 kg) were included in this study. The left eyes were randomly divided into five equal groups named SCXL (standard CXL, 3 mW/cm2 for 30 min), ACXL1 (accelerated CXL, 9 mW/cm2 for 10 min), ACXL2 (accelerated CXL, 18 mW/cm2 for 5 min), ACXL3 (accelerated CXL, 30 mW/cm2 for 3min) and CO (control group, unirradiated but underwent epithelium removal and riboflavin instillation) (**Table 1**), and the contralateral eyes did not undergo any surgical procedure. 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 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 Corneal Thickness Measurement***

Metal rings with radii of 3 mm and 6 mm were stained with dye and gently placed on the cornea to mark the thickness measurement positions. The central, paracentral and peripheral corneal thicknesses (at 0, 3 and 6 mm away from pupil center, respectively) were measured in vivo with an ultrasound pachymeter (PachPen; Accitome, Malvern, PA) along two meridional lines (nasal horizontal and temporal horizontal). Each measurement was taken three times preoperatively (pre) and 1-month post-CXL (pos1m) and the average value was recorded.

***2.3 Preparation and CXL procedure***

Before CXL, the rabbits were premedicated with subcutaneous injection (SU-MIANXIN, Veterinary Institute at University of Munitions, Changchun, China, 0.2 mL/kg). General anesthesia was administered with an intramuscular injection (Pentobarbital sodium, Merck KGaA, Darmstadt, Germany; 30 mg/kg). Additional topical anesthetic (Alcaine eye drops, Alcon Inc., Texas, USA) was instilled into the left eyes, and a wire eyelid speculum was positioned in the same eyes. Prior to UVA (370 nm) irradiation, the central 9 mm of the corneal epithelium was carefully removed using a hockey knife, and the corneas were saturated with 0.1% riboflavin drops (Peschke M; Peschke Meditrade GmbH, Huenenberg, Switzerland) at 3-minute intervals over a total period of 30 minutes. Following this step, the CXL procedure was conducted while performing the protocols explained in **Table 1** using the CXL system CL-01 (SiHaiTong Co., Suzhou, China). The protocols involved subjecting the central corneal area with 9-mm diameter to irradiation (*Di*, **Figure 1a, 1b**) with different intensities and exposure times, but with a constant total energy dose of 5.4 J/cm2. The cornea was kept moist with a balanced salt solution24 (one drop every 2 minutes) during the entire irradiation procedure. 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 calf blood extract eye gel (Xingqi; Shenyang Xingqi Pharmaceutical Co., Ltd., Shenyang, China) to ensure complete re-epithelialization.

***2.4*** ***Specimen preparation***

One month after CXL, a corneal ring with a diameter of 9 mm was placed in the central pupil to mark the corneal irradiation area in vivo, and the corneal diameter (*Dc*, nasal-temporal) was measured at the same time. The rabbits were then over-anesthetized to death, the both eyes were immediately removed and kept in phosphate-buffered saline (PBS, Maixin, China) until used. The corneal epithelium was completely scraped gently using a corneal epithelial scraper, and the remaining corneal tissue, along with a 1-2 mm scleral band, was separated with ophthalmic scissors. Considering that the cornea has a surface curvature and is difficult to be fixed as a whole, it was cut with a blade into a 5 mm wide strip containing the nasal-temporal corneal rim. The corneal strip was then glued to the base of a Petri dish, and the endothelial surface was spread and gently affixed to avoid ruffling of the corneal anterior surface (**Figure 1c**). A Vernier caliper was used to measure the nasal-temporal limbus-to-limbus length of the strip (*Lc*) and the length of the irradiation area (*Li*) using the marked positions of the metal ring (**Figure 1c**). A storage medium of PBS was then injected into the petri dish to maintain hydration during the nanoindentation measurements.

***2.5 Nanoindentation measurements***

Nanoindentation measurements were performed using a Piuma instrument (Optics11, Amsterdam, The Netherlands). A glass indenter with a tip radius of 261-270 µm was used, and the maximum indentation depth was set at 30µm. The indents were performed along the specimen center line (**Figure 1d**) with a constant spacing of 500 µm. The indents were separated into five groups based on their locations – these indents were within distances of 0-1.5 mm, 1.5-3.0 mm, 3.0-4.5 mm, 4.5-6.0 mm and 6.0-7.5 mm from the specimen centre, respectively (**Figure 1d**). All measurements for each eye were completed within 1 hour.

With the cornea considered a porous elastic (poro-elastic) material, two properties were determined from the nanoindentation tests; the elastic modulus and the permeability12. In the analysis, the balance of forces between the indenter and indented structure is represented by

 (1)

Where *F* is the applied force, *Fe* is the elastic resistance given by Hertz equation25, and *Fp* is the resistance provided by permeability and derived from Darcy’s Law12. Therefore, Equation (1) takes the form

 (2)

Where*Eeff*is the effective elastic modulus, *h* is the indentation depth, *R* is the radius of the indenter tip, *K* is the hydraulic conductivity, and *dh/dt* is the indenter displacement rate. The load-displacement response at different indentation rates is indicated in **Figure 2**. Based upon Equation (2) an estimate of *K* may be had by loading at two different indentation rates, *dh1/dt*, and *dh2/dt* (**Figure 2**)

 (3)

Knowing *K,* one can use Equation (2) to determine *Eeff*. The average values of *K* and *Eeff* during loadingwithin an indentation depth between 8~22μm were obtained from the analysis.

***2.6 Statistical analysis***

Quantitative data were presented as the mean ± standard deviation, and all statistical analyses were performed using PASW Statistics 25.0 (SPSS Inc. Chicago, USA). Comparisons of the dimension changes in cornea (*Dc* and *Lc*) and irradiated area (*Di* and *Li*) were performed using paired t test. Comparisons of results obtained for the different specimen groups were performed using analysis of variance (ANOVA) and the Games-Howell post hoc test. Spearman correlations examined relationships among different parameters. p-values of less than 0.05 were indicative of statistical significance.

**Results**

**Table 2** shows the corneal diameters (*Dc*), the length of corneal strip (*Lc*) and the length of strip within the irradiation area (*Li* ) of rabbit eyes in each intervention group – the results show no statistically significant differences among the five groups in all 3 parameters (all p>0.05). During the preparation of corneal strips in vitro, the diameter of the cornea increased by 2.30±0.54 mm from *Dc* to *Lc*(p<0.001), and the diameter of the irradiated area increased by 0.24±0.12 mm from *Di* (9 mm) to *Li* (p=0.001).

**Corneal thickness at different positions**

Corneal thickness measurements, taken at pre CXL treatment, showed no significant differences between all intervention groups (all p > 0.05, **Table 2**). The central corneal thickness after CXL decreased significantly compared to the preoperative values for SCXL and ACXL1 groups (p<0.001, 0.010). The significant decrease was found only in the paracentral regions of the SCXL groups (p<0.001, 0.002). In contrast, the peripheral thickness in all groups did not reduce significantly (all p>0.05).

**Elastic modulus at different** **indentation rates**

Each measurement point was measured twice at two loading speeds of 60 µm/min and 600µm/min – in both cases, the time interval between successive measurements was 10 minutes to ensure tissue recovery. Hertz’s formula was used to fit and obtain elastic modulus values at different indentation rates, denoted as *E60* and *E600*. The mean values of *E60* (20.11±15.78 kPa), *E600* (26.80±23.26 kPa) and *Eeff* (19.31±15.09 kPa) in all five groups are shown in **Figure 3**, *E600* differs from both *Eeff* (p<0.001) and *E60* (p<0.001), no significant difference shows between *Eeff* and *E60* (p=0.395).

**Regional changes of effective elastic modulus**

The *Eeff* of different intervention groups is shown in **Table 3** and **Figure 4**. Compared with the CO group, the *Eeff* of all regions was significantly higher in the SCXL, ACXL1 and ACXL2 groups (all p<0.05) but not in some regions of the ACXL3 groups (p=0.092 for region 4.5-6.0mm; p=0.326 for region 6.0-7.5mm). No significant difference was detected between region 0-1.5 mm and region 1.5-3.0 mm for all groups (p=0.409 for SCXL group; p=0.695 for ACXL1 group; p=0.946 for ACXL2 group; p=0.150 for ACXL3 group; p=0.926 for CO group). No significant difference was also detected between region 3.0-4.5 mm and region 4.5-6.0 mm for ACXL2 group (p=0.085), ACXL3 group (p=0.164) and CO group (p=0.792). For the ACXL3 group and CO group, no significant difference was detected in region 1.5-3.0 mm (p=0.175) and region 3.0-4.5 mm (p=0.322).

**Regional changes of hydraulic conductivity**

The hydraulic conductivity of different intervention groups is shown in **Table 4** and **Figure 5**. Compared with the CO group, the hydraulic conductivity of all regions was significantly lower in the SCXL, ACXL1 and ACXL2 groups but not the ACXL3 group (p=0.332 for region 4.5-6.0mm; p=0.423 for region 6.0-7.5mm). For different regions of each group，no significant difference was detected between region 0-1.5 mm and region 1.5-3.0 mm for all groups (p=0.979 for SCXL group; p=0.830 for ACXL1 group; p=0.946 for ACXL2 group; p=0.359 for ACXL3 group; p=0.997 for CO group). No significant difference was also detected between region 1.5-3.0 mm and region 3.0-4.5 mm for SCXL (p=0.170)，ACXL1 (p=0.118) and ACXL2 (p=0.220) groups. Furthermore, no significant difference was detected between region 3.0-4.5 mm and region 4.5-6.0 mm for ACXL1 (p=0.161)，ACXL2 (p=0.083)，ACXL3 (p=0.773) and CO (p=1.000) groups.

Spearman correlation was used to determine the relationship between the effective elastic modulus (*Eeff*) and the hydraulic conductivity (*K*). For all measurement points, the results showed a negative correlation (rs=-0.745, p<0.001). A simple power law relationship was found using a non-linear model (red line) as shown in **Figure 6**. Further, different regions were analyzed for each group, and the results are shown in **Table 5**, presenting negative correlations between *Eeff* and *K*.

Additionally, there were no statistical differences in all parameters (effective elastic modulus, hydraulic conductivity and corneal thickness) in each region between the six uncross-linked groups, including one control eye group and five contralateral eye groups (all p>0.05, **Table 6**).

**Discussion**

CXL is a minimally invasive surgical technique, which stabilizes the progression of corneal ectasia and postpones the need of lamellar or penetrating keratoplasty26. Comprehending the change of corneal biomechanical behavior is critical to assessing the effectiveness of CXL. Corneal solid components (e.g., collagen fibrils and proteoglycans) primarily determine the elasticity of the cornea as measured by the elastic modulus12. On the other hand, characterization of the corneal mobile liquid components (e.g., interstitial fluid) can be expressed by the tissue’s permeability or hydraulic conductivity27, which also relates to corneal nutrition transport and transparency12, 28. The present study found a highly radial dependence of tissue’s elasticity and permeability after CXL treatment. In terms of corneal elasticity, the strengthening was highest in the central cornea and decreased toward the periphery (**Figure 4, Table 3**). In terms of corneal permeability, an opposite trend was observed (**Figure 5, Table 4**). This uneven radial effect of CXL may be related to the more tightly arranged collagen fibrils in the central region29,31.

CXL enhances stiffness to the tissue by producing additional covalent bonds within and between collagen fibrils. In this study, the increase in stiffness gradually reduces with reducing the UV-A power duration. This trend is compatible with earlier studies in which ACXL led to reduced effectiveness7, 30. Oxygen is necessary to drive CXL process within the corneal stroma, which is impaired in hypoxia31. The smaller effect of accelerated CXL has been attributed to the higher consumption and shortage of oxygen in the stroma32, 33. Kamaev et al.34 noted that oxygen consumption occurs within seconds during UVA irradiation and that following cessation of irradiation, oxygen levels can take several minutes to be restored.

Some previous studies have shown changes of corneal elastic modulus after CXL. Nohava et al.23 used nanoindentation to analyze the regional elastic modulus of human corneal stroma. The results showed that the elastic modulus of the central (radius 0-1.0 mm), paracentral (radius 1.0-2.5 mm) and peripheral (radius 2.5-4.0 mm) regions increased by 108%, 79% and 63% after SCXL, respectively, and the corresponding values were 89.9±42.4 kPa, 65.0±17.9 kPa and 37.7±20.4 kPa. In this study, the effective elastic modulus *Eeff* of region 0-1.5 mm, region 1.5-3.0 mm and region 3.0-4.5 mm in the SCXL group were 47.65±20.58 kPa, 41.12±19.87 kPa and 31.17±16.23 kPa, respectively, which increased by 309%, 276% and 226% compared with the CO group (**Table 3**). As can be seen from **Figure 3, t**he higher the indentation rate, the greater the penetration resistance and thus the greater the elastic modulus. In this study, the effect of indentation rate penetration resistance was excluded, and instead the effective elastic modulus *Eeff* , independent of penetration rate, of the rabbit cornea was obtained, which was not statistically different from the elastic modulus *E60* (**Figure 3**), that is, *Eeff* at a speed of 60μm/min23.

The main reasons for the difference from the previous results are as follows. First, the rabbit cornea does not contain the outer Bowman's layer which is stiffer than the stroma35, and its collagen fibrils are much smaller than those of the human cornea36. Second, the elastic modulus of the normal rabbit cornea is smaller than that of the human cornea37. As a result, while the elastic modulus of rabbit cornea is smaller than that of human cornea, the proportion of increase after cross-linking is higher than in the human cornea. Third, although both studies used the nanoindentation technique, the actual measurement parameters were set differently, such as the size of the spherical indenter. With increasing indenter radius, the contact diameter increases for the same depth of penetration. Also, the volume of the region beneath the indenter that is compressed scales directly with the indenter contact radius. That is, the bigger the indenter radius and contact diameter, the greater the depth of the cornea that is experiencing compressive deformation. As the elastic modulus of the cornea decreases with depth, especially after cross-linking, this gradient structure of the cornea becomes more obvious38. Therefore, the measurement of the human cornea with a bigger indenter (500μm) also incorporates the elastic modulus of the deeper stroma, resulting in a much smaller proportion of the increase of the elastic modulus after cross-linking than that of the rabbit.

The reported progression rate of KC after CXL is up to 23%39, and the reason may be that CXL is commonly focused on the central cornea for irradiation. KC can theoretically occur anywhere in the cornea, and it has been reported that it is mostly found in localized lesions in the inferior temporal part of the cornea40, with cone apexes 1-2 mm off the corneal center and with cone borders up to 3 mm from the center41, the eccentricity values are even higher in pellucid marginal degeneration (PMD)42. Since the position of greatest need for strengthening is not located in the center of the cornea, where CXL is most effective, this may result in an overestimation of the expected outcome of the procedure. In such cases, peripheral compensatory irradiation or customized irradiation centered on the cone apex could achieve better results.

The strengthening range of corneal stiffness was larger than the irradiation range set in this study, that is, the strengthening effect was still evident in the non-crosslinked region. As can be seen from **Figure 4,** *Eeff* increased by 133%, 124% and 62% after SCXL, ACXL1 and ACXL2 in the region 4.5-6.0 mm, respectively (all p<0.001). However, this region contains a small fraction of the irradiated area (0.24 ± 0.12 mm, **Table 2**), so the increase in *Eeff* cannot be used to accurately determine whether the non-crosslinked region has strengthened. It is worth noting that the *Eeff* still increased by 40%, 39%, and 32% after SCXL, ACXL1 and ACXL2 in the region 6.0-7.5 mm, respectively (p=0.002, 0.002, 0.016). Webb et al.43 performed blue light CXL (447 nm) on porcine eyes and shaded the outside of the irradiated region to quantify the changes of corneal stiffness using Brillouin microscopy, and confirmed that the strengthening effect occurs beyond the set range. The factors that influence the biomechanical properties of a non-crosslinked region are various, and may include diffusion of oxygen radicals44 and scattering of UVA in the stroma45 as well as reflection of light through the cornea, plus device and conjunctival capsule (**Figure 1a**) can lead to extensive strengthening of the non-crosslinked region. The strengthening of non-crosslinked region, especially the limbus, may have unintended adverse consequences on the cornea. Limbal epithelial stem cells are considered to be the only cells responsible for retaining the corneal epithelium in a steady state, and their integrity is a key determinant for maintaining a clear, avascular cornea46. Previous studies have reported cases of delayed corneal epithelial healing after CXL47, 48, and the reason has not yet been explored, which may be due to the cytotoxic effects of UVA on epithelial stem cells at the corneal limbus49 or the increase of corneal stromal stiffness that affects the maintenance and differentiation of epithelial stem cells50. Jeyalatha et al.51 suggested that the use of polymethylmethacrylate covering could prevent UVA damage in the epithelial stem cells in the limbus. Otherwise, recent studies have developed customized CXL treatments that changes the irradiation energy and irradiation site according to the specific morphology of the cornea52, 53, and show that the irradiation border of eccentric CXL is closer to the limbus than the conventional CXL, so more attention needs to be paid to the influence of CXL on the limbus.

The cornea’s avascular and solute diffusion becomes an important mechanism for cellular transport of nutrients and wastes54, which depends on the transport of substances by corneal endothelial cells and the structure of the corneal stromal collagen network14. Combined with the results of this study and our recent work7, CXL may change the spatial structure of corneal stroma collagen fibrils and reduce corneal permeability, thereby increasing the resistance of corneal stroma to the diffusion of certain solutes. Previous studies also showed a trend of decreased transport after CXL, with direct measurements of fluorescein diffusion through the corneal stroma showing that solute permeability significantly decreased following cross-linking in rabbit and porcine eyes15, 55. Furthermore, the regional variation of *K* (**Figure 5, Table 4**) indicates that the effect of CXL on solute transport depends on the lateral position from the corneal center point. The reduction of corneal permeability was higher in the central region than in the peripheral region, that is, like elastic modulus, the greatest effect of CXL on corneal permeability occurred in the central region. It is worth noting that the non-crosslinked region was also affected to some extent, resulting in a slight decrease in permeability. This confirmation has important clinical implications and provides a more detailed understanding of the influence of CXL on cornea transport behavior. For instance, drug delivery to certain regions of the cornea would be incorrectly overestimated if a uniform decrease in permeability was present. Again, the present study preliminarily presented a power function relationship between *Eeff* and *K* (**Figure 6, Table 5**), that is the stiffer the region, the less permeable it becomes. This aspect suggests that surgeons should consider the effect of CXL on drug transport when selecting surgical options for patients who require long-term drug use, especially for high-energy, low-irradiance CXL. Apart from that, with further investigations of the quantitative relationship between these factors, *K* has the potential to become a novel and important in vivo biomechanical parameter13 with great diagnostic value for corneal biomechanical weakening diseases in the future.

The differences in biomechanical parameters between treatment groups were primarily caused by the variations adopted in the CXL protocols, which can be able to demonstrate by the comparison between the uncross-linked groups (**Table 6**), but there are still some limits in the current study. Because of the difficulty in obtaining a sufficient number of human donor corneas, rabbit corneas were used instead as they have been shown to be suitable alternatives in previous experiments7, 30. Also, depth dependence and radial variations of corneal biomechanical properties after CXL could not be further explored and will be investigated in future studies. Finally, the regional biomechanical changes after CXL in this study were not confirmed by corresponding histological analysis of collagen fibrils. The results of future studies should address these limitations.

In conclusion, this study confirms the uneven effect of CXL on corneal biomechanical properties. This effect is most evident in the corneal center, and the effectiveness decreases with distance away from the center. In the case if severely eccentric KC, peripheral compensatory irradiation or customized CXL centered on the cone apex may be necessary to ensure maximum impact on corneal biomechanics and ensure stability of the cornea.

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**Table captions:**

**Table 1** Corneal cross-linking settings adopted in different specimen groups

**Table 2** Corneal dimensions (mm) and thickness (μm) measurements in all intervention groups

**Table 3** Regional effective elastic modulus (*Eeff*) for different groups

**Table 4** Regional hydraulic conductivity (*K*) for different groups

**Table 5** Correlation of effective elastic modulus (*Eeff*) with hydraulic conductivity (*K*) in different regions for all the nano-indentation tests undertaken in intervention groups

**Table 6** Corneal regional biomechanical parameters and thickness (μm) measurements in CO group and all contralateral eye groups

**Table 1**

|  |  |  |
| --- | --- | --- |
| Group | No. | Irradiation |
| SCXL | 10 | 3 mW/cm2 for 30 minutes |
| ACXL1 | 10 | 9 mW/cm2 for 10 minutes |
| ACXL2 | 10 | 18 mW/cm2 for 5 minutes |
| ACXL3 | 10 | 30 mW/cm2 for 3 minutes |
| CO | 10 | Unirradiated but underwent epithelium removal and riboflavin instillation |

Note: SCXL=standard corneal crosslinking, ACXL=accelerated corneal crosslinking, CO=control

**Table 2**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Dimensions | | | SCXL | ACXL1 | ACXL2 | ACXL3 | CO | ANOVA P |
| *Dc* | | | 13.14±0.55 | 12.99±0.90 | 12.94±0.53 | 13.17±0.75 | 13.12±0.74 | 0.935 |
| *Lc* | | | 15.22±0.89\*\* | 15.50±1.02\*\* | 15.26±0.73\*\* | 15.46±1.10\*\* | 15.42±1.01\*\* | 0.948 |
| *Li* | | | 9.22±0.13## | 9.28±0.08## | 9.22±0.12## | 9.26±0.13## | — | 0.555 |
| Thickness | | | SCXL | ACXL1 | ACXL2 | ACXL3 | CO | ANOVA P |
| pre | Central | | 369.86±16.30 | 366.93±14.02 | 362.62±14.00 | 368.23±14.11 | 372.78±12.37 | 0.598 |
| Paracentral | N | 363.59±15.02 | 357.26±14.46 | 359.85±23.04 | 365.68±22.33 | 368.49±18.34 | 0.693 |
| T | 351.06±15.69 | 349.50±16.88 | 351.47±22.61 | 355.63±17.22 | 359.30±13.49 | 0.716 |
| Peripheral | N | 341.73±19.75 | 330.05±14.77 | 332.77±18.66 | 349.47±22.36 | 347.30±21.00 | 0.118 |
| T | 335.03±20.59 | 323.83±15.68 | 328.43±21.20 | 328.70±13.68 | 332.03±19.03 | 0.713 |
| pos1m | Central | | 347.51±17.38\* | 359.30±13.42\* | 363.98±12.41 | 363.33±18.23 | 370.93±10.17 | 0.015 |
| Paracentral | N | 341.26±14.51\* | 363.83±10.34 | 366.73±12.43 | 363.35±17.77 | 362.53±17.66 | 0.002 |
| T | 335.44±13.28\* | 352.97±12.39 | 357.73±17.58 | 353.15±18.47 | 356.03±19.87 | 0.031 |
| Peripheral | N | 339.43±24.27 | 333.60±14.21 | 340.48±14.65 | 339.98±13.51 | 339.43±24.27 | 0.916 |
| T | 329.30±13.18 | 325.03±17.83 | 330.37±14.73 | 331.69±27.75 | 337.22±26.74 | 0.777 |

Note: ACXL=accelerated corneal crosslinking, CO=control, SCXL=standard corneal crosslinking; *Dc*=corneal diameter,*Di*=9-mm diameter of the irradiated area, *Lc*=length of corneal strip,*Li*=length of strip in irradiation area; Central-0 mm away from pupil, Paracentral-3 mm away from pupil, Peripheral-6 mm away from pupil; T-temporal, N-nasal; \* p<0.05 difference against pre-CXL**,**\*\* p< 0.001 difference against *Dc*, ## p< 0.001 difference against *Di*

**Table 3**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Group | Effective elastic modulus（*Eeff*， kPa，mean±SD） | | | | |
| 0-1.5mm | 1.5-3.0mm | 3.0-4.5mm | 4.5-6.0mm | 6.0-7.5mm |
| SCXL | 47.65±20.58 | 41.12±19.87 | 31.17±16.23 | 20.70±10.16 | 11.78±5.03 |
| ACXL1 | 37.55±15.50 | 33.88±17.34 | 26.13±12.48 | 19.89±10.77 | 11.68±5.23 |
| ACXL2 | 24.63±13.02 | 22.89±12.84 | 18.59±10.77 | 14.41±7.82 | 10.93±5.28 |
| ACXL3 | 18.53±8.62 | 15.41±6.78 | 13.07±5.61 | 10.95±5.35 | 10.29±6.00 |
| CO | 11.66±4.79 | 10.95±5.12 | 9.57±3.08 | 8.89±3.77 | 8.55±4.05 |

Note: SCXL=standard corneal crosslinking, ACXL=accelerated corneal crosslinking, CO=control

**Table 4**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Group | Hydraulic conductivity （*K*, mm4 ⁄ N∙s, mean±SD） | | | | |
| 0-1.5mm | 1.5-3.0mm | 3.0-4.5mm | 4.5-6.0mm | 6.0-7.5mm |
| SCXL | 1.22±1.05 | 1.65±1.43 | 2.25±1.84 | 3.39±2.69 | 6.91±4.38 |
| ACXL1 | 1.93±1.42 | 2.27±1.83 | 3.11±2.02 | 4.58±2.86 | 7.75±4.00 |
| ACXL2 | 2.83±1.64 | 3.33±1.73 | 3.93±2.83 | 6.25±4.40 | 9.36±6.80 |
| ACXL3 | 5.23±3.19 | 5.62±3.39 | 6.97±4.99 | 8.06±5.62 | 11.47±9.52 |
| CO | 7.78±4.10 | 8.81±4.86 | 10.20±7.43 | 11.98±8.23 | 12.68±7.45 |

Note: SCXL=standard corneal crosslinking, ACXL=accelerated corneal crosslinking, CO=control

**Table 5**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Group | Correlation coefficient(rs) | | | | |
| 0-1.5mm | 1.5-3.0mm | 3.0-4.5mm | 4.5-6.0mm | 6.0-7.5mm |
| SCXL | -0.610\*\* | -0.411\*\* | -0.508\*\* | -0.520\*\* | -0.383\* |
| ACXL1 | -0.622\*\* | -0.675\*\* | -0.567\*\* | -0.734\*\* | -0.392\*\* |
| ACXL2 | -0.591\*\* | -0.579\*\* | -0.503\*\* | -0.559\*\* | -0.336\*\* |
| ACXL3 | -0.546\*\* | -0.533\*\* | -0.302\* | -0.246\* | -0.421\*\* |
| CO | -0.370\*\* | -0.271\* | -0.264\* | -0.430\*\* | -0.454\*\* |

Note: ACXL=accelerated corneal crosslinking, CO=control, SCXL=standard corneal crosslinking; \* p<0.05, \*\* p<0.001

**Table 6**

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Biomechanical parameters (mean±SD) | | | SCXL-CL | ACXL1-CL | ACXL2-CL | ACXL3-CL | CO-CL | CO | ANOVA P |
| Effective elastic modulus (*Eeff*, kPa) | 0-1.5mm | | 12.46±5.69 | 11.86±4.11 | 12.10±4.93 | 12.67±3.32 | 12.58±4.16 | 11.66±4.79 | 0.783 |
| 1.5-3.0mm | | 10.81±4.60 | 11.48±4.47 | 11.76±5.01 | 11.47±3.36 | 11.18±3.35 | 10.95±5.12 | 0.806 |
| 3.0-4.5mm | | 9.67±3.84 | 10.58±4.13 | 10.71±4.02 | 10.62±3.72 | 10.50±3.27 | 9.57±3.08 | 0.500 |
| 4.5-6.0mm | | 9.26±3.34 | 9.44±2.89 | 10.32±3.54 | 10.24±3.51 | 9.47±3.62 | 8.89±3.77 | 0.188 |
| 6.0-7.5mm | | 7.99±3.17 | 7.97±3.14 | 9.00±3.63 | 9.33±3.66 | 8.92±3.16 | 8.55±4.05 | 0.174 |
| Hydraulic conductivity (*K,* mm4 ⁄ N∙s) | 0-1.5mm | | 7.06±3.59 | 7.76±4.34 | 7.89±6.26 | 9.03±4.52 | 7.90±3.36 | 7.78±4.10 | 0.240 |
| 1.5-3.0mm | | 7.95±3.74 | 9.19±4.30 | 8.87±5.27 | 10.15±4.47 | 8.84±4.82 | 8.81±4.86 | 0.139 |
| 3.0-4.5mm | | 9.04±4.97 | 9.58±4.87 | 9.78±4.49 | 11.08±6.28 | 10.37±6.36 | 10.20±7.43 | 0.435 |
| 4.5-6.0mm | | 10.60±9.26 | 11.28±8.72 | 11.49±7.21 | 11.84±8.04 | 12.15±8.49 | 11.98±8.23 | 0.940 |
| 6.0-7.5mm | | 12.77±5.70 | 12.24±7.04 | 13.22±10.11 | 12.36±8.28 | 13.74±7.83 | 12.68±7.45 | 0.891 |
| Thickness (μm, mean±SD) | | | SCXL-CL | ACXL1-CL | ACXL2-CL | ACXL3-CL | CO-CL | CO | ANOVA P |
| pre | Central | | 369.23±13.08 | 368.60±12.98 | 367.60±13.10 | 369.93±13.19 | 370.47±13.36 | 372.78±12.37 | 0.966 |
| Paracentral | N | 362.35±9.46 | 365.58±18.12 | 363.41±19.19 | 360.88±14.38 | 369.41±20.28 | 368.48±18.34 | 0.845 |
| T | 351.73±15.88 | 350.78±13.21 | 355.95±17.90 | 359.70±21.96 | 359.70±13.49 | 359.30±13.49 | 0.715 |
| Peripheral | N | 341.91±23.34 | 343.73±20.93 | 344.19±14.37 | 335.18±22.12 | 342.22±19.32 | 347.30±21.00 | 0.852 |
| T | 343.66±23.73 | 342.93±19.42 | 338.76±17.84 | 335.01±20.72 | 339.43±20.64 | 332.03±19.03 | 0.782 |
| pos1m | Central | | 370.47±13.36 | 369.93±13.19 | 369.23±13.08 | 371.05±13.37 | 372.03±13.15 | 370.93±10.17 | 0.998 |
| Paracentral | N | 369.47±20.28 | 360.88±14.38 | 362.35±9.46 | 369.47±15.97 | 367.46±17.36 | 365.35±17.66 | 0.723 |
| T | 359.70±21.96 | 351.73±15.88 | 350.78±13.21 | 358.25±18.51 | 360.93±16.17 | 356.03±19.87 | 0.734 |
| Peripheral | N | 342.22±19.32 | 335.18±22.12 | 341.91±23.34 | 347.12±10.88 | 348.00±21.10 | 339.43±24.27 | 0.746 |
| T | 337.18±23.08 | 324.28±14.40 | 333.34±20.38 | 329.17±14.07 | 335.69±20.29 | 337.22±26.74 | 0.667 |

Note: ACXL=accelerated corneal crosslinking, CO=control, SCXL=standard corneal crosslinking, CL= contralateral eyes, Central-0 mm away from pupil, Paracentral-3 mm away from pupil, Peripheral-6 mm away from pupil; T-temporal, N-nasal

**Figure captions:**

**Figure 1** UVA cross-linking and corneal specimen preparation. (a. Irradiation diameter was set to 9.0mm for CXL; b. Cross-sectional view of the cornea; c. Fixation of the corneal strip; d. Division of corneal measurement area).

**Figure 2** Schematic diagram of the load-displacement response at two loading rates based upon the analysis developed for an elastic-permeable material.

**Figure 3** Elastic modulus (kPa) at different indentation rates. (The box indicates the lower and upper quartiles and the central line marks the median; whiskers are 0.5 times interquartile range); \*\* difference against with p< 0.001.

**Figure 4** Regional effective elastic modulus (*Eeff*) for different groups. (The box indicates the lower and upper quartiles and the central line marks the median; whiskers are 0.5 times interquartile range). \* indicates a significant difference from the CO group (p<0.05); # indicated difference had no statistical significance (p≥0.05).

**Figure 5** Regional hydraulic conductivity for different groups. (The box indicates the lower and upper quartiles and the central line marks the median; whiskers are 0.5 times interquartile range). \* indicates a significant difference from the CO group (p<0.05); # indicated difference had no statistical significance (p≥0.05).

**Figure 6** The relationship between the effective elastic modulus (*Eeff*) and the hydraulic conductivity (*K*) for all the nanoindentation tests undertaken.