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Anterior and posterior corneal stroma elasticity after corneal collagen crosslinking treatment

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Abstract

The purpose of this project was to assess anterior and posterior corneal stromal elasticity after corneal collagen cross linking (CXL) treatment in human cadaver eyes using Atomic Force Microscopy (AFM) through indentation. Twenty four human cadaver eyes (12 pairs) were included in this study and divided into 2 groups (6 pairs per group). In both groups, the left eye (OS) served as a control (no riboflavin or CXL treatment was performed) and the right eye (OD) underwent CXL treatment (30 minutes of riboflavin pretreatment followed by 30 minutes of exposure to 3mW/cm² of ultraviolet light). In group 1, the anterior stroma was exposed by manual delamination of approximately 50µm of the corneal stroma including Bowman's membrane. In group 2, the posterior stroma was exposed by delamination of the anterior 50% of the corneal stroma including Bowman's membrane. Delamination was performed after crosslinking treatment in the case of the treated eyes. In all eyes, the stromal elasticity was quantified using AFM through indentation. Young's modulus of elasticity for the anterior cornea (group 1) was 245.9±209.1kPa (range: 82.3 - 530.8 kPa) for the untreated control eyes, and 467.8±373.2kPa (range: 157.4 - 1126 kPa) for the CXL treated eyes. Young's modulus for the posterior cornea (group 2) was 100.2±61.9kPa (range: 28.1 - 162.6 kPa) for the untreated control eyes and 66.0±31.8kPa (range: 31.3 - 101.7 kPa) for the CXL treated eyes. Young's modulus of the anterior stroma significantly increased after CXL treatment (p=0.024), whereas the posterior stroma did not demonstrate a significant difference in Young's modulus after CXL treatment (p=0.170). The anterior stroma was stiffer than the posterior stroma for both the control and CXL treatment groups (p=0.077 and p=0.023, respectively). Our findings demonstrate that stiffness of the anterior corneal stroma after CXL treatment seems to increase significantly, while the posterior stroma does not seem to be affected by CXL.

Keywords

cornea; crosslinking; keratoconus; ectasia; mechanical properties

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INTRODUCTION

Corneal collagen cross linking (CXL) treatment is based on a photo-chemical reaction between ultraviolet light-A (UV-A) and riboflavin (Wollensak et al. 2003a, Wollensak et al. 2003b, Wollensak. 2006). This minimally invasive procedure increases the mechanical strength of the cornea, thereby stabilizing corneal ectatic disorders such as keratoconus and iatrogenic keratectasia (after corneal refractive surgery) (Wollensak et al. 2003a, Wollensak et al. 2003b, Wollensak. 2006). The introduction of CXL in routine clinical practice has changed the management of the above entities; furthermore, it provides a 'true' treatment, by inhibiting the progression of keratoconus and corneal ectasia. Prior to CXL, all interventions (glasses, contact lenses and intra-corneal ring segment implantation (Kymionis et al. 2007)) were used to improve visual function of patients, while they did not treat the underlying pathophysiology of the corneal tissue.

Theoretically, the CXL treatment's ability to increase corneal mechanical strength, and therefore inhibit the progression of corneal ectatic disorders, stems from the treatment's formation of additional crosslinks within the cornea's stromal ultrastructure. Comprehensive knowledge of the precise molecular mechanisms associated with CXL and how it attributes to corneal mechanical strengthening remain unknown (Wollensak. 2006; Hayes et al. 2013; Meek and Hayes. 2013). Studies have shown that the CXL process is dependent upon the presence of carbonyl groups within cornea (McCall et al. 2010; Brummer et al. 2011). In addition, a recent study conducted by Hayes *et al* (2013) demonstrated that the creation of crosslinks produced from CXL most likely occurs at four particular locations, opposing the initial notion of being between collagen fibrils. The four locations are within and between collagen molecules located at the surface of the collagen fibrils as well as within and between proteoglycan core proteins (Hayes et al. 2013). Although such advancements have been made, the exact molecules involved and their reaction pathways have yet to be identified.

Many studies have been published demonstrating CXLs efficacy indirectly, mostly based on clinical findings, such as steady keratometric values and refraction (Kymionis et al. 2012; Vinciguerra et al. 2013). Investigations conducted *ex vivo* have been able to directly quantify the efficacy of CXL by the measurement of corneal mechanical strength (Kling et al. 2010; Kohlhaas et al. 2006, Lanchares et al. 2011, Schumacher et al. 2011, Spoerl et al. 1998, Spoerl and Seiler. 1999, Wollensak et al. 2003b, Wollensak and Iomdina. 2009a, Wollensak and Iomdina. 2009b). A land mark report by Wollensak et al (2003) demonstrates significant increase in corneal stiffness (increased by 328.9%) and in Young's modulus (increased 4.5 times) after CXL assessed using stress-strain measurements (Wollensak et al. 2003b). Ever since then, numerous *ex vivo* studies have been conducted, using various corneal characterization techniques, to confirm such increase in corneal mechanical strength after CXL (Kling et al. 2010; Kohlhaas et al. 2006; Lanchares et al. 2011; Schumacher et al. 2011; Wollensak and Iomdina. 2009a; Wollensak and Iomdina. 2009b; Scarcelli et al. 2013).

Ultraviolet light absorption in the cornea is governed by the Lambert-Beer law of absorption – there is an exponential decrease in UV intensity with increasing depth in the cornea. For this reason, it would be expected that the induction of additional crosslinking bonds within the stroma is not homogeneous, but rather depth-dependent (Kohlhaas et al. 2006). Thus, it is likely that mechanical stiffening induced by crosslinking is much more pronounced in the anterior stromal region (Kohlhaas et al. 2006). In fact, this phenomenon has been demonstrated in the porcine model using strip extensimetry (Kohlhaas et al. 2006) and Brillouin microscopy (Scarcelli et al. 2013). These same conclusions were predicted by an inverse computational model that used *in vivo* data from patients as inputs (Roy et al. 2013).

The purpose of this study was to build upon previous studies and quantify the anterior and posterior stromal elasticity in human cadaver eyes after crosslinking using Atomic Force Microscopy (AFM). Through the principle of nanoindentation and its ability to implement low indentation depths, AFM can independently characterize the distinct layers of corneal samples and perform depth-dependent characterization studies with proper hydration.

MATERIALS AND METHODS

Tissue acquisition

Experiments were conducted on 12 pairs of human cadaver eyes (age range: 39-88 years) retrieved from the Florida Lions Eye Bank (Miami, Florida). The human eye globes arrived from the eye bank in sealed vials placed in Styrofoam containers filled with ice. Upon arrival in the laboratory, the corneal epithelium was removed using a cotton-tipped applicator and the whole globes were placed, cornea side down, in 20% Dextran solution for 24 hours in the refrigerator at 4°C to restore corneal thickness to physiological levels (Borja et al. 2004, Duffey et al. 1989, Swinger and Kornmehl. 1985). The mean postmortem time of the eyes at the time of receipt was 4.58 ± 1.31 days (range: 2 – 7 days), but the actual experiments were performed 24 hours later to enable this initial pretreatment with 20% Dextran. Pachymetry measurements were taken after the pretreatment (DGH 55 Pachmate, DGH Technology Inc., Exton, PA) to ensure the restoration of the corneal thickness within the physiological range of 400-600µm. All human eyes were obtained and used in compliance with the guidelines of the Declaration of Helsinki for research involving the use of human tissue.

Corneal collagen cross linking treatment

For this study, the right eyes (OD) were treated using the standard CXL Dresden protocol and the left eyes (OS) served as untreated controls (with no riboflavin pretreatment). Since the epithelium was removed in for swelling restoration purposes, instillation of 0.1% riboflavin solution (10mg riboflavin-5-phosphate in 10mL Dextran 20% solution) was applied on the bare corneal stroma one drop every 5 minutes for 30 minutes. The cornea was then irradiated using UVA light at 378nm wavelength and with an intensity of 3mW/cm². The irradiance was performed for 30 minutes, corresponding to a total surface dose of 5.4 J/cm². During UVA irradiation, riboflavin solution was applied every 5 minutes to maintain corneal saturation with riboflavin.

Six pairs of eyes (n=12) were prepared for anterior stroma measurements (Group 1) and six pairs (n=12) were prepared for posterior stroma measurements (Group 2). For anterior stroma measurements, a 4mm surgical blade (Alcon, Texas, USA) was used to remove approximately 50µm of corneal tissue from the intact cornea, thereby removing Bowman's membrane (17.7µm thickness; Tao et al. 2011) to expose the anterior stromal region, but leaving it connected to the remaining stromal layer and Descemet's membrane. To expose a lower plane in the cornea, approximately 50% of the restored corneal thickness was removed using the same surgical blade; this plane in the stroma was defined as the posterior stroma. Pachymetric measurements were taken incrementally to monitor the amount of stroma removed. The corneas were then excised from the whole globes, leaving a generous rim of sclera and placed in a custom cornea holder with 15% Dextran solution to maintain corneal hydration during mechanical testing.

Elasticity assessment

Mechanical property measurements were performed using a custom-built atomic force microscopy (AFM) system. The AFM system and experimental procedure have been described in detail previously (Ziebarth et al. 2011; Dias and Ziebarth. 2013). Briefly, tip-

less AFM cantilevers (nominal spring constant: 4.5 N/m, NSC12 series, Mikromasch, San Jose, CA) were modified with glass microspheres (59-74µm diameter, 15926-100, Polysciences Inc). The modified tip was then calibrated to determine its spring constant using a reference force calibration cantilever (nominal spring constant: 10.4 N/m, CLFC-NOBO, Bruker, Camarillo, CA) manufactured specifically for the calibration of other probes. The modified cantilever tips were lowered onto the corneal samples using a piezoelectric mechanism (60µm maximal expansion, P-841.40, Physik Instrumente, Germany) with an approach speed of 15µm/s. When the maximal indentation force of 1000V (<20nN, which corresponds to <6µm indentation) was reached, the cantilever was immediately retracted at the same speed. The voltage detected at the photodiode due to deflection of the cantilever was recorded as a function of piezoelectric displacement. These recordings were used to derive the sample's force-indentation curves, after factoring out the cantilever deflection on a hard surface and incorporating the measured spring constant. With the use of custom MATLAB programs, the indentation force-indentation depth curves were analyzed using the Hertz model for a spherical indenter (Hertz H. 1881):

$$F = \frac{4E\sqrt{R}}{3(1-\nu^2)}D^{3/2}$$

where F [N] is the measured force (N), E [N/m²] is Young's modulus (Pa), ν is Poisson's ratio ($\nu=0.49$ for the cornea (Cabrera Fernandez et al. 2005, Knox Cartwright et al. 2011)), R [m] is the radius of the spherical indenter (m), and D is the measured indentation. These recordings were repeated at least 15 times per sample. All experiments were performed at room temperature. The accuracy of the curve fits was visually verified.

Statistical analysis

Statistical analysis of data was performed by a custom made data base in Excel (Microsoft Office). All parameters followed normal distribution and a paired Student's t-test was used to analyze the Young's modulus of the control versus treated corneas. A p value less than 0.05 was considered to be statistically significant.

RESULTS

Mean central corneal thickness (CCT) of all eyes was 526±62µm (range: 403 - 612µm) after epithelial removal and dextran pretreatment. The eyes in group 1 (defined as anterior stroma) had an average of 41±15µm of tissue removed by manual delamination (range: 17 - 64µm), while the eyes in group 2 (defined as posterior stroma) had an average of 53±9% of their thickness removed (range: 40 - 69%) (Table 1).

Young's modulus of elasticity for the anterior stroma (group 1) was 245.9±209.1kPa (range: 82.3 - 530.8kPa) for the untreated control eyes and 467.8±373.2kPa (range: 157.4 - 1126kPa) for the CXL treated eyes (Figure 1). Young's modulus for the posterior stroma (group 2) was 100.2±61.9kPa (range: 28.1 - 162.6kPa) for the untreated control eyes and 66.0±31.8kPa (range: 31.3 - 101.7kPa) for the CXL treated eyes (Figure 1). The variability of repeated mechanical property measurements on the same corneal sample (standard deviation divided by average) was 15±7%. For each individual eye pair, the elastic modulus of the CXL treated eye was consistently higher than its untreated counterpart for the anterior stroma measurements; however, the posterior stroma was stiffer after CXL treatment in only 3 out of 6 pairs. Young's modulus of the anterior stroma significantly increased after CXL treatment (p=0.024), whereas the posterior stroma did not demonstrate a significant difference in Young's modulus after CXL treatment (p=0.170). The anterior stroma was

stiffer than the posterior stroma for both the control and CXL treatment groups ($p=0.077$ and $p=0.023$, respectively).

DISCUSSION

CXL treatment is based on a photo-chemical reaction between UVA and riboflavin. Riboflavin, also known as vitamin B₂ (belonging to the flavin group), is a photo-sensitizer that is excited into its triplet state when irradiated, generating reactive oxygen species (ROS) (Wollensak. 2006). The ROS can react further with various molecules inducing chemical covalent bonds bridging amino groups of collagen fibrils (type II photochemical reaction), inducing cross links which increase corneal stiffness (Wollensak. 2006). The precise molecular interactions of such ROS within the corneal stroma is not yet fully understood, although some progress has been made to improve insight into the effect of CXL on the cornea at the molecular level (Hayes et al. 2013; Meek and Hayes. 2013; McCall et al. 2010; Brummer et al. 2011). There has been evidence of increase in the area, density, and diameter of corneal stromal collagen; such structural alterations may attribute to the increased corneal stiffness (Choi et al. 2012).

Efficacy of CXL treatment has been generally based on indirect clinical outcomes (Kymionis et al. 2012, Vinciguerra et al. 2013). Most studies enroll patients with progression of their ectatic disorder proven by means of corneal topography and alteration of keratometric values over a 6 month time interval. Efficacy of the treatment has therefore been linked with stable keratometric values and stable refraction over time. Long-term results from European clinical trials indicate a steady and continuous improvement in corneal irregularities up to 24 months postoperatively (Kymionis et al. 2012, Vinciguerra et al. 2013); while the trials supervised by the Food and Drug Administration in the United States have also shown great promise (Greenstein et al. 2011, Hersh et al. 2011). In addition to the clinical outcomes, there have been ex vivo laboratory studies based mainly on stress-strain tests that have demonstrated an increase in corneal stiffness after treatment in both animal models and human corneas (Kling et al. 2010, Kohlhaas et al. 2006, Lanchares et al. 2011, Schumacher et al. 2011, Spoerl et al. 1998, Spoerl and Seiler. 1999, Wollensak et al. 2003b, Wollensak and Iomdina. 2009a, Wollensak and Iomdina. 2009b).

In our study, we found that corneal collagen crosslinking treatment significantly increases corneal stiffness in the anterior corneal stromal region only (increased by ~1.9 times when compared to the untreated corneas) while the posterior corneal stroma (as described in our study as approximately 50% of the total CCT) was not significantly affected by CXL. Since riboflavin serves as the photosensitizing agent to produce ROS, the ability to produce a significant biomechanical CXL effect within the stroma is dependent upon the concentration of the riboflavin that is able to diffuse through the stroma (Spoerl et al. 2007; Raiskup and Spoerl. 2013). Studies have investigated the absorption properties of riboflavin and showed that the riboflavin concentration decreases with stromal depth (Wollensak et al. 2010; Spoerl et al. 2010; Sondergaard et al. 2010). Governing the absorption of UV light in the corneal tissue, the Lambert-Beer law states that there is an exponential decrease in UV intensity with increasing depth in the cornea. With the combined effect of riboflavin concentration and the Lambert-Beer law, it would be expected that the induction of additional crosslinking bonds within the stroma is not homogeneous, but rather depth-dependent (Kohlhaas et al. 2006).

Therefore, the results of our study support the theory that the mechanical stiffening induced by crosslinking is much more pronounced in the anterior stromal region than the posterior. Such findings are similar to the qualitative trends found in the studies of Kohlhaas et al (2006) and Scarcelli et al (2013), both of which characterized the anterior and posterior stromal regions of crosslinked and normal porcine corneas. In addition, the same trend was

predicted by the finite-element computational model of Roy et al (2013). All studies agree with this present one that the stiffening effect of UV crosslinking is depth-dependent and that the majority of the stiffening occurs in the anterior stromal region rather than in the posterior stroma (Kohlhaas et al. 2006; Scarcelli et al. 2013).

Our study assessed the elasticity of normal and crosslinked human cadaver corneas using a custom Atomic Force Microscopy (AFM) system. Conceptually developed by Binnig et al (1881), AFM has become an established characterization technique in the arenas of non-biological and biological mechanical testing. AFM technology enables localized mechanical testing of samples in aqueous solutions (Karrasch et al. 1994, Lal and John. 1994) and is a suitable characterization technique to conduct studies on distinct layers within the corneal stroma for depth-dependent analysis. Using this technique, standard mechanical properties like Young's modulus can be derived by the application of a controlled vertical compressive force. The force applies stress to the sample, which will indent as an indication of the strain experienced. The Young's modulus obtained from these measurements is therefore defined as the ratio of stress (force) to strain (indentation), along the same vertical axis. Although the versatility of AFM enables the determination of time-dependent properties associated with viscoelasticity and poroelasticity, these measurements were not performed in the current study. All experiments were performed with the exact same experimental parameter settings (ie, indentation rate and maximal voltage) and encompassed the application and immediate retraction of maximal force unto the sample. In this particular study, we used spherical indenters to probe network layers of the corneal stroma, as opposed to individual collagen fibers. With the spherical indenter geometry used, the Hertz model was used to calculate Young's modulus of elasticity from the force and indentation information. However, this model assumes that the sample is isotropic, homogeneous, linearly elastic, and infinitely thick, none of which accurately describe the cornea because of its heterogeneous, viscoelastic, and anisotropic nature. Therefore, the use of the Hertz model limits our results to only be linear elastic modulus estimates, as opposed to reflecting the absolute elastic modulus values.

It is possible that several experimental constraints may have affected the biomechanical measurements obtained. The variation of corneal edema between the samples resulted in a different initial total corneal thickness for each sample, despite the 24-hour dextran treatment. Although all samples had a thickness within the normal physiological range, these slight differences in thickness may account for some of the measurement variability between samples. Furthermore, the use of manual delamination to access the corneal stromal regions restricted the creation of reproducible corneal tissue excisions. Consequently, there lied the inability to perform elasticity assessments on the exact same anterior stromal depths for all the corneas, which can be evidenced by the large range of the amount of stroma removed to expose the anterior stroma (17-64 μ m). Previous studies have shown structural differences in the collagen fiber interconnectivity at increasing depths within the anterior stromal region (Winkler et al. 2011). Although no significant trend was found between anterior stromal elastic modulus and stromal depth, such structural differences may have contributed to the variation of elastic modulus values obtained. Other limitations of this study include the small sample size of eye pairs used as well as the *ex vivo* experimental model, since *ex vivo* human corneas are affected by factors such as post mortem, collagen autolysis, stromal swelling, and lack of healing response. The effect that these experimental constraints had on the results can be evidenced by the high between sample variability, since the standard deviation of the moduli between samples was on the same order as the mean. Despite the between sample variability, the results for each eye pair consistently showed that the stiffening effect of CXL was significant in the anterior region but not in the posterior region.

In conclusion, our study validates previous experimental evidence of increased corneal stiffness after CXL treatment, using the characterization technique of AFM. The results of this study describe an anterior-posterior stromal depth dependency concerning CXL efficacy.

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Highlights

- We measured human stroma elasticity after UV-B2 crosslinking using AFM.
- There is a significant increase in anterior stroma stiffness after crosslinking.
- There is no significant change in posterior stroma stiffness after crosslinking

Figure 1.

Bar graph of Young's modulus expressed in kilo Pascals for corneas that underwent corneal collagen cross linking treatment (CXL) compared to control corneas (no CXL), both for anterior and posterior corneal stroma.

Table

The age, post-mortem time (PMT), and central corneal thickness (CCT) of human cadaver eyes used in each group. The values reported are average $\pm$ standard deviation (range).

Group		Age (years)	PMT at Time of Experimentation (Days)	CCT after Dextran Pretreatment (μ m)	Manual Delamination (μ m)	Tissue Removed (percentage)
Anterior Corneal Stroma	Treated	70 $\pm$ 19 (39 – 87)	6.3 $\pm$ 0.8 (6 – 8)	509 $\pm$ 83 (403 – 612)	33 $\pm$ 7 (26 – 43)	10.3 $\pm$ 2.3 (7.3 – 14.1)
	Untreated			528 $\pm$ 61 (430 – 599)	50 $\pm$ 17 (17 – 64)	9.7 $\pm$ 3.7 (2.9 – 12.5)
Posterior Corneal Stroma	Treated	78 $\pm$ 14 (56 – 88)	4.8 $\pm$ 1.3 (3 – 6)	535 $\pm$ 59 (480 – 595)	182 $\pm$ 46 (131 – 238)	53.4 $\pm$ 11.4 (40.2 – 69.8)
	Untreated			531 $\pm$ 55 (466 – 591)	282 $\pm$ 52 (229 – 354)	53.0 $\pm$ 5.9 (48.8 – 56.4)