Evaluating the Effects of Riboflavin/UV-A and Rose-Bengal/Green Light Cross-Linking of the Rabbit Cornea by Noncontact Optical Coherence Elastography

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METHODS. Low-amplitude (∼10 μm) elastic waves were induced in mature rabbit corneas by a focused air pulse. Elastic wave propagation was imaged by a phase-stabilized swept source OCT (PhS-SSOCE) system. Corneas were then cross-linked by either of two methods: UV-A/riboflavin (UV-CXL) or rose-Bengal/green light (RGX). Phase velocities of the elastic waves were fitted to a previously developed modified Rayleigh-Lamb frequency equation to obtain the viscoelasticity of the corneas before and after the cross-linking treatments. Micro-scale depth-resolved phase velocity distribution revealed the depth-wise heterogeneity of both cross-linking techniques.

RESULTS. Under standard treatment settings, UV-CXL significantly increased the stiffness of the corneas by ∼47% (P < 0.05), but RGX did not produce statistically significant increases. The shear viscosities were unaffected by either cross-linking technique. The depth-wise phase velocities showed that UV-CXL affected the anterior ∼54% of the corneas, whereas RGX affected only the anterior ∼16% of the corneas.

CONCLUSIONS. UV-CXL significantly strengthens the cornea, whereas RGX does not, and the effects of cross-linking by UV-CXL reach deeper into the cornea than cross-linking effects of RGX under similar conditions.

Keywords: corneal biomechanics, cross-linking, optical coherence elastography

The biomechanical properties of the cornea are inherently tied to its health,1 consequently any changes in the biomechanical properties of the cornea can affect vision quality. Keratoconus is a structurally degenerative disease that can pathologically diminish visual acuity because the cornea thins and forms a conical shape due to a significant reduction in its stiffness.2–4 Riboflavin/ultraviolet-A corneal collagen cross-linking (UV-CXL) is an emerging clinical treatment which increases the stiffness of the cornea and improves its ability to resist further degeneration.5 Although UV-CXL has shown great promise,6–8 there are concerns associated with UV irradiation, such as keratocyte cytotoxicity.9–12 In addition, endothelial toxicity restricts the use of UV-CXL for corneas thinner than 400 μm.12 Other side effects of UV-CXL have been reported, such as corneal haze,13 keratitis and corneal scarring,14 and endothelial cytotoxicity, even when the cornea was of sufficient thickness.15 Nevertheless, these side effects are rare, and UV-CXL is gaining momentum as a treatment for keratoconus6,8,10 and other conditions such as infectious keratitis.17,18

Rose-Bengal/green light corneal collagen cross-linking (RGX) has been proposed as an alternative to UV-CXL.19 Because green light is used in lieu of UV irradiation, there is no or only minimal cytotoxicity.19 Moreover, the high absorbance of green light by rose-Bengal dye results in a significantly shorter treatment time than with UV-CXL (30 min for RGX vs. 1 h for UV-CXL).

Currently, UV-CXL treatment has not been individualized to specific cases, and RGX has only recently been investigated as a corneal collagen cross-linking technique. If changes in the biomechanical properties of the cornea due to cross-linking could be accurately assessed, the treatment could be personalized to increase its efficacy and reduce the risk of side effects.
Evaluating the Effects of Cross-Linking With OCE

Therefore, a noninvasive technique which can quantitatively characterize the biomechanical properties of the cornea accurately would provide valuable insight into corneal biomechanical changes induced by cross-linking.

Several techniques have been proposed to assess biomechanical properties of the cornea. Ocular devices such as the Ocular Response Analyzer (ORA; Reichert Technologies, Buffalo, NY, USA) and CorVis ST (Oculus Optikgeräte GmbH, Wetzlar, Germany) have been able to detect differences between elasticity of the healthy corneas and that of keratoconic corneas; however, there have been conflicting reports of their ability to detect changes in the stiffness of the cornea after UV-CXL. Mechanical assessments by extensometry and inflation testing have demonstrated how UV-CXL stiffens the cornea, but these techniques are inherently unfeasible for in vivo investigations. Acoustic techniques have also been used to assess the changes in elasticity after UV-CXL of ex vivo canine and in vivo porcine corneas. However, acoustic techniques require contact with the cornea and use of a transmission medium, such as water or gel, which may not be appropriate for clinical applications.

Brillouin microscopy is a noninvasive optical imaging technique which can provide a depth-resolved map of the Brillouin frequency shift in the cornea. Brillouin microscopy has mapped the Brillouin shift of human corneas in vivo, revealed differences in the Brillouin shift between healthy and keratoconic human corneal buttons ex vivo, illustrated the depth-resolved effects of UV-CXL on the Brillouin shift of ex vivo porcine corneas, and shown depth-resolved changes in the Brillouin shift induced by RGX on ex vivo rabbit corneas. Although it is understood that the Brillouin shift may be related to elasticity, accurately obtaining quantitative biomechanical parameters (e.g., Young’s modulus and viscosity) from the Brillouin shift is still a challenge.

Elastography is an established technique for quantitatively obtaining biomechanical properties of tissue by using an imaging modality to detect displacements. Traditional elastographic techniques such as ultrasound elastography (USE) and magnetic resonance elastography (MRE) are clinically available tools for detecting diseases such as hepatic fibrosis and breast cancer. However, these techniques require relatively large displacement amplitudes and have poor spatial and temporal resolutions, which limit their use for small and thin samples such as ocular tissues.

Optical coherence tomography (OCT)-based elastography (OCE) is a rapidly emerging technique for imaging mechanical contrast of tissues with micrometer-scale spatial resolution. By analyzing the phase of the complex OCT signal, displacement sensitivity can reach nanometer scale. Although the imaging depth of OCT is limited to a few millimeters in scattering media such as tissue, this is not an issue in corneal applications due to the optical transparency and relatively small thickness of the cornea. Hence, OCE is specifically suited for characterizing the biomechanical properties of the cornea.

Previous elastic wave-based OCE techniques have used the group velocity of an externally induced elastic wave to characterize elasticity of the cornea. However, models which translate the group velocity to Young’s modulus often do not account for the thickness of the cornea nor the fluid-solid interface between the aqueous humor and the posterior surface of the cornea. We recently showed that the group velocity changes as a function of thickness when all other parameters are equal, and therefore, the thickness should be taken into account when quantifying the biomechanical properties of the cornea. Furthermore, group velocity-based assessments only provide information about the elasticity of the sample, which is not fully reflective of its biomechanical properties. Therefore, we have developed a robust model for reconstructing the viscoelasticity of the cornea by modifying the Rayleigh-Lamb frequency equation (RLFE) to incorporate the solid-fluid effect at the boundary between the corneal posterior surface and aqueous humor. Moreover, spectral analysis can provide the depth-resolved micrometer-scale phase velocity distribution in the cornea, which has previously been used to reveal the elasticity differences in cornea layers.

In this work, we present the first direct comparison of viscoelastic changes in the cornea induced by UV-CXL versus those by using RGX. A focused air pulse (≤1 ms) induced a low-amplitude elastic wave in rabbit corneas in the whole eye globe configuration at an artificially controlled IOP. The viscoelasticity of rabbit corneas before and after UV-CXL and RGX were quantified by our previously developed modified RLFE. In addition, the differences in depth-wise effects of the cross-linking treatments were revealed by depth-resolved micrometer-scale phase velocity distribution.

METHODS

Cornea Preparation

Mature rabbit eyes (≥6 months old) were obtained fresh (Pel-Freeze Biologicals, Rogers, AR, USA) and shipped overnight on ice. Samples were separated into two groups for the cross-linking experiments (n = 4 samples for each group). Extraneous tissues such as muscles were removed from the eye globe before all experiments, and eyes were visually inspected to ensure no damage had incurred during transportation and preparation. All OCE measurements were completed within 24 h of enucleation. After OCE experiments using the virgin corneas were conducted, the cross-linking procedures were performed, and OCE measurements were repeated.

UV-CXL was performed according to our previous work, which mimicked the most commonly used clinical protocol. Briefly, epithelium was removed, and a 0.1% riboflavin solution in 20% dextran was applied to the corneal surface every 5 min for 30 min. The cornea was then irradiated with UV-A (365 nm, 7-mm-diameter spot on the corneas, 3 mW/cm²) for 30 min. During UV irradiation, the riboflavin solution was topically instilled every 5 min.

Rose-Bengal/green light was performed by applying a solution of 0.1% rose-Bengal dye in 0.9% phosphate-buffered saline every 5 min for 20 min after epithelium was removed. The cornea was then irradiated with green light (560 nm, 7-mm-diameter spot, 0.25 W/cm²) for 10 min.

Phase-Stabilized Swept Source Optical Coherence Elastography (PhS-SSOCE) System

Custom-built PhS-SSOCE system consisted of a phase-stabilized swept source optical coherence tomography system and a focused air pulse delivery device. The PhS-SSOCE system included a broadband SS laser (HSL2000; Santec, Inc., Hackensack, NJ, USA) with a central wavelength of ~1,310 nm, a bandwidth of ~130 nm, an A-scan rate of 50 kHz, an axial resolution of ~11 µm in air, and a phase stability of ~40 nm during the corneal experiments. The focused air pulse delivery device was composed of a controller and an electronically controlled pneumatic solenoid. The air pulse was expelled from an air port, which had a flat edge and an inner diameter of ~150 µm. The air source pressure was controlled by a standard pneumatic valve and monitored by a pressure gauge. The air pulse was positioned precisely with a three-dimensional (3D) linear micrometer stage, and the port was kept at ~400 µm from the surface of the corneas. To image the air pulse-induced elastic wave, successive (n = 501) M-
mode images were acquired in a line over ~6.3 mm (M-B mode), with the apex of the cornea at the middle of the scan. The air pulse excitation was also at the apex of the cornea. By synchronizing the M-mode frame trigger and air pulse with a transistor-transistor logic (TTL) signal, the PhS-SSOCT system effectively imaged the same elastic wave.47

In addition to the intrinsic corneal structure, IOP can have a significant effect on the measured elasticity of the cornea.46,55 Therefore, the IOP of the eyes was artificially controlled by a homemade closed-loop IOP control system.52 Whole rabbit eyes were placed in a customized holder, which had two holes for the IOP control system. Eye globes were cannulated with two 23-gauge needles: one needle was connected by tubing to a microinfusion pump, and the other needle was connected by tubing to a pressure transducer. A physiological IOP of 15 mm Hg was maintained during all experiments. A schematic representation of the experimental setup is illustrated in Figure 1.

The raw unwrapped vertical temporal phase profiles for the surface of the cornea, \(u_{\text{surface}}(t)\), were converted to displacement, \(d_{\text{surface}}(t)\), by:

\[
d_{\text{surface}}(t) = u_{\text{surface}}(t) \times \frac{\lambda_0}{4\pi n_{\text{air}}}\tag{1}
\]

and the raw unwrapped phase profiles within the cornea, \(u_{\text{inside}}(t)\), were translated to displacement, \(d_{\text{inside}}(t)\), after correcting for the corneal surface motion and refractive index mismatch between air and the cornea by:

\[
d_{\text{inside}}(t) = \left[u_{\text{inside}}(t) + u_{\text{surface}}(t) \frac{n_{\text{cornea}} - n_{\text{air}}}{n_{\text{air}}} \right] \times \frac{\lambda_0}{4\pi n_{\text{cornea}}}
\tag{2}
\]

where \(n_{\text{air}} = 1\), \(n_{\text{cornea}} = 1.376\),58 and \(\lambda_0\) was the central wavelength of the OCT system.

**Phase Velocity**

Phase velocities of the air pulse-induced elastic wave were used to quantify the viscoelasticity of the corneas and to obtain the depth-wise heterogeneity of the cross-linking techniques. For each in-depth layer, \(z\), a fast Fourier transform was performed with the temporal displacement profile at each OCE measurement position, \(x\), to obtain a phase shift, \(\Delta \phi_{x,z,f}\), for each a fast Fourier transform bin frequency, \(f\). The elastic wave propagation distance to each of the OCE measurement positions, \(D_{x,z}\), incorporated the curvature of the cornea to ensure accuracy of the phase velocity calculations. The phase velocity for each in-depth layer and each bin frequency, \(c_p(z,f)\), was calculated by linearly fitting the phase shifts to their corresponding propagation distances by:

\[
c_p(z,f) = \frac{2\pi f}{D_{x,z}}\Delta \phi_{x,z,f}.\tag{50}
\]

**Viscoelasticity Reconstruction**

Phase velocities were averaged depth-wise for each sample and fitted to our previously developed viscoelasticity reconstruction model based on a modified RLFE.49 Briefly, the RLFE describes a symmetrical or antisymmetrical wave assuming free boundary conditions.59 However, this does not account for the fluid-solid interface between the aqueous humor and posterior surface of the cornea. Therefore, the boundary condition at the corneal posterior surface was incorporated into the RLFE. The OCE-measured phase velocities were fitted to the analytical solution of the modified RLFE, and the viscoelasticity was determined by an iterative gradient-based procedure until the error between the OCE data and analytical solution was minimized. Figure 2 shows a typical fitting of the modified RLFE to OCE-measured phase velocities from a cornea before (Fig. 2a) and after (Fig. 2b) RGX.

**Depth-Wise Effects of Cross-Linking**

Depth-wise phase velocities were used to obtained the micrometer-scale depth-resolved elasticity of the cornea.50 The turning point of the smoothed depth-wise phase profile (i.e., where \(dc_p/dz = 0\), and \(z\) is depth) (Fig. 3a, blue arrow) was used to determine the region affected by the respective cross-linking techniques. The affected region (AR%) was determined by dividing the thickness of the cross-linked region
(anterior surface of the cornea to the turning point) by the central corneal thickness (CCT) of the corresponding virgin cornea. To demonstrate that the posterior stroma was not affected by cross-linking treatments, the slopes of the phase velocity versus depth for corresponding posterior regions between the virgin and cross-linked corneas were compared and found to be nearly equal in all samples before and after the cross-linking treatments.

RESULTS

Central Corneal Thickness

Central corneal thickness was calculated from the OCT structural image after correction for the refractive index of the cornea and is plotted in Figure 4. Before UV-CXL, the mean CCT of the corneas was 724 ± 6135 µm, which decreased to 506 ± 667 µm after UV-CXL (change of ~30%). The CCT averages of the corneas before and after RGX were 754 ± 636 µm and 728 ± 641 µm, respectively (change of ~3%). Statistical analysis by a 1-tailed paired t-test showed that only the change in CCT after UV-CXL was significant (P < 0.05), which was primarily due to the topical instillation of dextran.

Viscoelasticity

The changes in viscoelasticity as quantified by the modified RLFE are plotted in Figure 5. Figure 5a shows that before UV-CXL, the average Young's modulus of the rabbit corneas was 73.0 ± 615.4 kPa, which increased to 107.5 ± 625.0 kPa after UV-CXL (change of ~47%). The mean elasticity values of the virgin and RGX-treated corneas were 79.0 ± 19.3 kPa and 80.0 ± 26.5 kPa, respectively (change of ~1%). Figure 5b shows that the shear viscosities of the corneas before and after UV-CXL were 1.0 ± 0.3 Pa · s and 1.1 ± 0.3 Pa · s, respectively. The shear viscosity of the corneas before RGX was 1.0 ± 0.4 Pa · s and 1.0 ± 0.4 Pa · s after RGX. Only the change in stiffness after UV-CXL was significant (P < 0.05 by a 1-tailed paired t-test).

Results of Depth-Wise Effects of Cross-Linking

Figure 6 shows the mean affected region (AR%) of the corneas after each treatment. After UV-CXL, the anterior 34 ± 64% of the corneas was stiffened, and the anterior 16 ± 2% of the corneas was affected by the RGX treatment.

DISCUSSION

We have used a noncontact OCE technique to compare the biomechanical changes in rabbit cornea induced by UV-CXL and RGX. By using a previously developed viscoelasticity reconstruction method based on a modified RLFE, we determined Young's modulus and shear viscosity values of the corneas before and after cross-linking using both techniques were quantified. Furthermore, the changes in the depth-resolved elastic wave phase velocities in the cornea after UV-CXL and RGX were compared to reveal the depth-wise heterogeneity of the cross-linking techniques. Our results show that the cornea is significantly stiffened by UV-CXL but not by RGX, and the
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The rapid scanning and acquisition would also reduce the measurement times, and was exceeding the maximum permissible exposure (MPE) for the cornea. Moreover, IOP has a significant effect on the measured elasticity of the cornea after RGX. Although ORA and CorVis ST were unable to discern the effects of UV-CXL on the elasticity of the cornea, the other techniques were able to demonstrate how UV-CXL increases the stiffness of the cornea. Our results showed that UV-CXL increased the stiffness of the cornea by ~47%, which is similar to the ~59% increase in stiffness of in situ porcine corneas, measured by inflation tests, and the ~55% decrease in the tangential strain of in situ canine eyes as assessed by USE. On the other hand, there is a wide variance in published reports of changes in elasticity of the cornea after UV-CXL. For example, strip extensiometry showed that Young’s modulus of human, porcine, and rabbit corneas increased ~450%, ~180%, and ~850% after UV-CXL, respectively. Our previous strip extensiometry results on rabbit corneas showed a ~73% increase in Young’s modulus after UV-CXL. It has been postulated that the elastic wave velocity in a porcine cornea at 15 mm Hg IOP increased ~80%, which would correspond to an increase in the stiffness of ~200%. Supersonic shearwave imaging (SSI) indicated that Young’s moduli of porcine corneas increased between 238% and 760% after UV-CXL. This wide variation can be attributed to the different samples used (human, porcine, canine, and rabbit), the method used to assess the elasticity (mechanical, acoustic-based, and optical techniques), and the testing conditions (in vitro strips, in situ whole eye-globes, and in vivo). Furthermore, IOP has a significant effect on the measured elasticity of the cornea, which is why IOP was controlled in this work.

The overall elasticity of the rabbit corneas increased ~1% after RGX, as assessed by the present technique, which was not statistically significant by a 1-tailed t-test, but extensiometry has shown that the stiffness of the rabbit cornea increased ~3-40% after RGX. However, extensiometry cannot replicate the physiological conditions encountered in the whole eye globe configuration, and Young’s moduli as assessed by OCE are a few orders of magnitude less than those measured by extensiometry. It has been postulated that the equivalent IOP during extensiometry can be a few hundred millimeters of mercury. Combined with the nonlinear stress-strain curve of the cornea, these two factors can explain the

![Figure 4](image-url) Change in CCT after both cross-linking techniques. *Statistical significance (P < 0.05) as calculated by a 1-tailed paired t-test. Error bars represent intersample standard deviations.

![Figure 5](image-url) (a) Young’s modulus and (b) shear viscosity as estimated by OCE-measured phase velocities fitted to the modified RLFE. *Statistical significance (P < 0.05, using a 1-tailed paired t-test). Error bars represent intersample standard deviations.
large discrepancy in Young’s modulus between mechanical testing and our OCE measurements. We are currently investigating alternative techniques with which to obtain the biomechanical properties of the cornea in situ to validate our OCE measurements.

Results showed that the anterior ~1/3 of the cornea was stiffened by the UV-CXL treatment, which corroborates previous investigations using Brillouin microscopy34 and USE.28 The riboflavin solution is applied as a photosensitizer and UV shield. Hence, most UV absorption and subsequent cross-linking occurs in the anterior of the cornea where the riboflavin is present.12 Slit-lamp illumination revealed a distinct demarcation at ~40% of the corneal thickness.71 However, this line only became apparent 2 weeks after the treatment, and the elasticity assessments by Brillouin microscopy, USE, and OCE (including the present work), were performed immediately after the UV-CXL treatment was completed. Future investigations will assess the long-term depth-resolved elasticity changes in the cornea after UV-CXL.

The depth-resolved changes in the biomechanical properties of the cornea after RGX have been investigated by using Brillouin microscopy, which revealed that the anterior ~16% of the cornea was stiffened by RGX treatment.19 Fluorescence analysis illustrated that most of the rose-Bengal dye penetrated only ~100 μm into the anterior stroma. Because cross-linking will occur predominantly where the green light is absorbed, due to the transparency of the cornea, these results are intuitively reasonable and similar to our findings. It has been hypothesized that electrostatic interactions between negatively charged rose-Bengal dye and positively charged amino acids in collagen trap the dye within the anterior stroma. Our future work will entail longer soak times to determine if this can increase the penetrative stiffening of RGX. The relatively small region of cross-linked tissue may also explain the insignificantly small cross-linked region did not significantly contribute to an increase in the overall stiffness of the cornea. Therefore, imaging along different radial directions may increase intra-sample variance of the biomechanical properties. Future investigations will ensure that the elastic wave propagation is imaged along the same direction for all samples. We did, however, ensure that the OCE measurements were taken over the same region before and after cross-linking for a given sample to reduce intra-sample variability. Moreover, SSI showed that the elastic anisotropy becomes apparent only at relatively high IOPs (20 mm Hg), whereas a physiological IOP of 15 mm Hg was maintained during all OCE measurements in this work. Currently, there has been an investigation of the effects of cross-linking on the mechanical anisotropy of the cornea, and this is the future direction of our research. In addition to anisotropy, the age of the samples may have contributed to relatively large variations in viscoelasticity.77,78 Nevertheless, all parameters assessed for the virgin corneas were not significantly different (P > 0.05 by a 2-tailed paired t-test for CCT, Young’s modulus, and shear viscosity) between the two cross-linking groups.

Although UV-CXL has been proven to be an effective cross-linking technique in the clinic,6,8,16 RGX has been proposed as an alternative to UV-CXL without harmful UV-A irradiation.19 Brillouin microscopy and extensiometry showed that RGX increased the stiffness of the cornea by at least 3% without any keratocyte cytotoxicity. However, our results showed that Young’s modulus of the whole cornea in the whole-eye globe configuration at 15 mm Hg IOP increased only ~1%, which was not statistically significant. Nevertheless, RGX may still be an
 effective cross-linking treatment for corneas that may not be eligible for UV-CXL due to insufficient thickness or other complications. There is an extensive number of published reports of experimental and clinical investigations of UV-CXL, but RGX investigations are limited, and our results warrant further work.

**Conclusions**

We have presented a comparison between the viscoelastic changes in rabbit cornea after UV-CXL versus those after RGX by noncontact OCE. In addition, the depth-resolved micrometer-scale phase velocity distribution in the cornea after both of the cross-linking techniques was used to reveal the depth-wise heterogeneity after both of the cross-linking techniques. Results showed that UV-CXL significantly increased Young’s modulus of the cornea by ~47% but that RGX did not significantly alter the stiffness of the cornea. The shear viscosities were unaffected by either of the cross-linking technique. Spectral analysis demonstrated that the UV-CXL treatment increased the stiffness of the anterior 1/3 of the cornea, whereas RGX predominantly affected the anterior 1/7 of the cornea. Because of noncontact excitation and imaging, OCE may be potentially useful for assessing the dynamic changes in biomechanical properties of the cornea before and after cross-linking in vivo.

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