Mechanical outcome of accelerated corneal crosslinking evaluated by Brillouin microscopy

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Purpose: To quantify corneal mechanical changes induced by corneal crosslinking (CXL) procedures of different ultraviolet-A (UVA) intensity and exposure time using Brillouin microscopy.

Settings: University of Maryland, College Park, Maryland, USA.

Design: Experimental study.

Methods: Porcine cornea samples were debrided of epithelia and soaked with riboflavin 0.1% solution. Samples were exposed to a standard 5.4 J/cm² of UVA radiation with varying intensity and exposure time as follows: 3 mW/cm² for 30.0 minutes, 9 mW/cm² for 10.0 minutes, 34 mW/cm² for 2.65 minutes, and 50 mW/cm² for 1.80 minutes. Using Brillouin microscopy, the Brillouin modulus for each sample was computed as a function of radiation intensity/exposure time. For validation, the Young’s modulus was found with the stress–strain test and compared at each irradiation condition.

Results: The standard 3 mW/cm² irradiance condition produced a significantly larger increase in corneal Brillouin modulus than the 9 mW/cm² (P < .05), 34 mW/cm² (P < .01), and 50 mW/cm² (P < .01) conditions. Depth analysis showed similar anterior sections of the standard and 9 mW/cm² conditions but significantly less stiffening in the central and posterior of the 9 mW/cm² condition. The stiffening of the standard protocol was significantly larger in all sections of the 34 mW/cm² and 50 mW/cm² conditions (P < .01). The overall change in Brillouin-derived Brillouin modulus correlated with the increase in Young’s modulus (R² = 0.98).

Conclusions: At a constant UVA light dose, accelerating the irradiation process decreased CXL stiffening. Brillouin analysis showed that accelerated protocols were especially ineffective in the deeper portions of the cornea.


Corneal ectasia resulting from progressive keratoconus or refractive surgery can lead to progressive loss of vision and the need for corneal transplantation. Clinically observed alterations in corneal morphology, a distinguishing characteristic of ectasia, are believed to be the consequence of a non-uniform decrease in stiffness of the corneal stroma. To combat ectasia, the U.S. Food and Drug Administration recently approved corneal crosslinking (CXL). The accepted CXL procedure involves the debridement of the corneal epithelium followed by a 30-minute application of riboflavin solution (riboflavin 0.1%–dextran 20.0%) and an additional 30 minutes of ultraviolet-A (UVA) exposure (3 mW/cm²; 5.4 J/cm²). The photochemical reaction between the riboflavin photosensitizer and UVA light has been shown to increase the covalent bonding within the stroma, thereby increasing the overall stiffness of the cornea and halting ectasia.

Recently, much effort has focused on decreasing the overall treatment time of the CXL procedure. It was proposed that treatment time could be shortened by increasing the radiation intensity because the biological effects should only depend on the total energy dose. Although protocols using intensities up to 50 mW/cm² for less than 2 minutes of exposure time were suggested, the evidence does not conclusively support accelerated CXL in clinical settings. Wernli et al. reported a significant reduction in CXL stiffening when the procedure was performed above a 45 mW/cm² intensity. Hammer et al. found a decrease in CXL effectiveness when comparing the standard protocol with a treatment using a radiation intensity of just 9 mW/cm². In addition, all previous mechanical studies observed the stiffness changes as a function of irradiance regime without spatial resolution.

To assess the spatially varying effects of accelerated CXL, indirect techniques have been used; these include...
fluorescence imaging to assess riboflavin penetration or optical coherence tomography to quantify the depth location of refractive index changes within the stroma (ie, the demarcation line). We used recently developed Brillouin microscopy, which can directly assess corneal mechanics with 3-dimensional resolution to observe the depth dependence of stiffening after accelerated CXL protocols. As expected, we found the standard 3 mW/cm² radiation intensity for 30 minutes of exposure time produced the largest stiffness increase. Depth analysis showed that the 9 mW/cm² intensity condition could match the stiffening of the standard protocol in the anterior stroma but that it produced lower stiffening in the central and posterior sections.

MATERIALS AND METHODS

Corneal Crosslinking

Fresh porcine eyes were obtained from a local slaughterhouse (Frederick, Maryland, USA). For all eyes, the epithelium was care- fully removed by scraping with a razor blade. The eyes were then dissected in order to punch two 5.0 mm corneal disk samples (Integra Miltex Disposable Biopsy Punch). Each disk sample was treated with 1 drop of riboflavin 0.1%–dextran 20.0% solution every 3 minutes for 30 minutes. After undergoing identical procedures, 1 of the 2 samples was set aside as the control while the other sample was exposed to UVA radiation. In all experimental settings, a constant UVA energy of 5.4 J/cm² was provided by a high-power UV Curing Light-Emitting Diode System (Thorlabs, Inc.). Four treatment regimens were performed. Table 1 shows the regimens.

Brillouin Microscopy

After the CXL procedure, the control disks and crosslinked corneal disks were imaged via Brillouin microscopy with a setup and procedures described previously. Briefly, the confocal Brillouin microscope uses a 532 nm laser with an optical power of 10 mW. Light was focused into the sample by a 532 nm laser with an optical power using the known frequency shifts of water and glass. Camera were fitted using a Lorentzian function and calibrated Brillouin shift at each sample location, raw spectra from the Brillouin spectrum was acquired in 0.2 second. To quantify the EMCCD camera (IXon Du-897, Andor Technology Ltd). Each treatment protocol was performed by a high-power UV Curing Light-Emitting Diode System (Thorlabs, Inc.). Four treatment regimens were performed. Table 1 shows the regimens.

<table>
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Brillouin Image Analysis

The corneal samples were set next to each other on the Brillouin microscope and imaged within the same acquisition run. Therefore, each scan imaged the frequency shift of both the control and crosslinked sample as a function of depth. For each scan, a depth cross-section (ZX) was collected, producing a 1000 µm (lateral) × 1400 µm (axial) image of Brillouin shift. A central sliver of each corneal cross-section was chosen for consistent post-processing analysis. In addition to the entire corneal sample, the cross-sections were divided into 3 equal segments (anterior, central, and posterior) for depth analysis. For this analysis, the depth of cornea (d) was normalized for each set of corneal samples to compare the Brillouin modulus of each sample in the anterior (0 < d ≤ 0.33), central (0.33 < d ≤ 0.66), and posterior (0.66 < d ≤ 1) segment.

Compressive Biomechanical Testing

All samples were measured with compressive mechanical testing immediately after Brillouin imaging using a home-built compressive stress–strain instrument. The instrument consisted of a metallic baseplate topped with finely gritted sandpaper to prevent unwanted slippage and a downward-moving plunger containing a force-measuring loading cell (Futek Advanced Sensor Technology, Inc.) that is controlled via a motorized translational stage (Zaber Technologies, Inc.). Before every sample, the plunger was systematically moved downward until a reaction force from the baseplate was detected. The plunger position was then recorded and later used to calculate the total thickness of the sample.

The corneal sample was placed on the bottom plate of the instrument and the plunger compressed the sample at a constant downward rate of 10 µm/s. Using a purpose-designed Labview software program (National Instruments Corp.), the increasing compression force from the plunger and the corresponding material displacement were measured to produce the stress–strain curve of the material. The plunger position at which a reaction force was first sensed was noted in conjunction with the previously recorded baseplate position to accurately quantify the thickness of each sample. To obtain the Young’s modulus of a sample, respective stress (force/area) versus strain (displacement/thickness) graphs were plotted and the slope of the linear segment of the curve following the sleek strain was quantified. Using the stress versus strain curve for each sample, the Young’s modulus was reported by fitting the tangent line at 15% strain. A consistent 15% strain was chosen to observe the linear elastic behavior of the biological tissue as shown by Wernli et al.

To compare with the compressive mechanical test, the Brillouin modulus was calculated from the Brillouin frequency shift and averaged the value over the whole sample. First, as for the traditional modulus analysis, the Brillouin modulus of CXL samples were compared with their respective nonirradiated controls.

Statistical Analysis

First, to characterize individual CXL protocols, a paired 2-tailed t test was performed by comparing CXL samples with their respective controls cut from the same eye. Then, to compare different irradiation conditions, the stiffening effects of CXL protocols were analyzed by comparing the percentage difference of both Brillouin and Young’s moduli. The percentage difference for both the moduli were found per eye using the following equation: Modulus (X) Percentage Difference = 100(X_{CXL} – X_{Control})/(X_{Control}). The respective moduli percentage differences for the samples in each irradiation group were averaged ± SEM. A Wilcoxon rank-sum test was used to assess the significance in comparisons between
samples and/or corneal sections. A $P$ value of 0.05 or less was considered statistically significant.

RESULTS

All CXL protocols produced a statistically significant increase in stiffness compared with nonirradiated control conditions ($P \leq .05$). A comparison of the mechanical outcome of the varying CXL procedures by quantifying the percentage change in Young’s modulus with respect to each control found that the standard protocol, 3 mW/cm² of UVA power for 30 minutes was significantly more effective than the 34 mW/cm² ($P \leq .05$) and 50 mW/cm² ($P \leq .01$), the 9 mW/cm² condition was statistically significantly higher than the 50 mW/cm² ($P \leq .01$), and there was no significant difference between the 34 mW/cm² and 50 mW/cm² conditions. Figure 1 shows the average percentage change in Young’s modulus ± SEM for the 4 irradiation conditions.

A comparison of the Brillouin modulus of CXL samples to their respective nonirradiated controls showed that all CXL protocols produced a statistically significant increase in corneal stiffness compared with their control ($P \leq .05$). Figure 2 shows the stiffness outcome of the CXL protocols as measured by Brillouin microscopy. Figure 2, a, shows the representative cross-sectional Brillouin images of corneas from each irradiation condition with the color encoding the Brillouin frequency shift at each location. Computation of the percentage change in the Brillouin modulus for each irradiation condition showed that the 3 mW/cm² CXL condition resulted in higher corneal stiffening than the 9 mW/cm² ($P \leq .05$), 34 mW/cm² ($P \leq .01$), and 50 mW/cm² ($P \leq .01$); the 9 mW/cm² had significantly higher stiffening than the 34 mW/cm² ($P \leq .01$) and 50 mW/cm² ($P \leq .01$), and the 34 mW/cm² and 50 mW/cm² irradiation conditions did not significantly differ in CXL-induced stiffening. Figure 2, b, shows the stiffening as a function of radiation intensity.

Figure 3 shows the relationship between Young’s modulus and the average Brillouin modulus of the cornea sample. Each of the 4 points in Figure 3 represents the average of all the samples at a given irradiation condition. As radiation intensity decreased, both the Young’s modulus percentage change and the Brillouin modulus percentage change increased at a similar rate. The similar rate of increase resulted in a highly statistically significant linear correlation between the percentage change of Young’s modulus and the percentage change of the Brillouin modulus ($R^2 = 0.985$).

Figure 4 shows the percentage change in Brillouin modulus for each irradiance condition in the anterior, central, and posterior of the cornea. These findings were by obtained using Brillouin microscopy to perform a depth-dependent analysis on the corneas and calculate the percentage change in the Brillouin modulus in the anterior, central, and posterior sections of the cornea. There was no statistical significance in stiffening in the anterior section the 3 mW/cm² and 9 mW/cm² conditions. However, the difference between the 2

Figure 1. The averaged percentage change in Young’s modulus for each irradiation condition from 3 to 50 mW/cm². As the intensity increases, the change in Young’s modulus decreases. Error bars represent the SEM for each condition (** = $P < .01$; *** = $P < .001$).

Figure 2. a: Representative image (700 μm × 100 μm) produced with Matlab software (color map: jet) of the Brillouin shifts for each condition. Because of the relationship between shift and Brillouin modulus, a higher Brillouin shift correlates to a higher Brillouin modulus. The corneal slices are positioned top down from the anterior to the posterior. b: The averaged percentage change in Brillouin modulus for each irradiation condition from 3 to 50 mW/cm². As the intensity increases, the overall change in Brillouin modulus decreases. Error bars represent the SEM for each condition (* = $P < .05$; ** = $P < .01$).
conditions was statistically significant in the central and posterior sections of the corneas ($P \leq .05$). In all sections, the 3 mW/cm$^2$ produced significantly more stiffening than the 34 mW/cm$^2$ and 50 mW/cm$^2$ conditions ($P \leq .01$). The stiffening from the 9 mW/cm$^2$ sample significantly differed from that of the 34 mW/cm$^2$ sample in the anterior ($P \leq .01$) and the 50 mW/cm$^2$ sample in the anterior ($P \leq .01$) and central ($P \leq .05$). There were no significant differences in stiffening at any section between the 2 most accelerated conditions.

**DISCUSSION**

In this study, we evaluated the stiffening effects of accelerated corneal CXL protocols. To minimize the control-to-sample variability, 2 disks were punched from each cornea to act as the nonirradiated control and CXL sample, respectively. Keeping a constant energy dose of 5.4 J/cm$^2$, we performed 4 corneal CXL regimens with varying power and exposure time. To first validate our crosslinking procedures, we compared the resulting stiffness of our crosslinked samples to their respective controls up until a maximum radiation intensity of 45 mW/cm$^2$ for 2 minutes of exposure time. However, unlike our findings and those of Hammer et al., the study found a relatively constant effect of CXL from 3 mW/cm$^2$ through roughly 45 mW/cm$^2$ before a significant decrease in stiffening magnitude occurred. The differences in results could depend on the variability in experimental procedures. For example, Wernli et al. kept the corneas immersed in a pool of riboflavin solution for 30 minutes before UVA exposure. Our protocol more closely resembled that of Hammer et al. in that riboflavin drops were incrementally applied to the cornea for a total of 30 minutes before radiation exposure. Differing from Hammer et al., our protocol used corneal punches rather than the entire globe. This distinction, by
minimizing the solution runoff from decreasing the sample curvature, might have been responsible for our greater stiffening effects.

The results of the stress–strain testing were also used to validate Brillouin microscopy. We previously determined a log–log linear correlation between Young’s modulus and the Brillouin-derived Brillouin modulus. Therefore, our linear correlation between the percentage change in Young’s modulus and Brillouin modulus agrees with the previously found relationship.

Using Brillouin microscopy, we were able to expand on our findings as well as those in relevant studies by observing the effects of CXL as a function of depth. This analysis yielded interesting results when comparing the standard protocol (3 mW/cm² for 30 minutes) with the slightly accelerated protocol (9 mW/cm² for 10 minutes). When the entire cornea was analyzed, the standard condition had a significantly higher Brillouin modulus than the 9 mW/cm² protocol. From depth–dependent analysis, the 2 treatments did not show statistically significant differences in anterior stroma but they showed statistically significant differences in the central and posterior portions of the cornea. This suggests the difference between the 2 conditions was primarily the result of the deeper sections. Our findings on depth–dependent stiffening via CXL are in agreement with those of Al Dahlawi et al., who used the resistance to enzymatic degradation properties of CXL corneas to test the effective depth of the procedure. The study similarly showed that up to a maximum radiation power of 18 mW/cm² for 5 minutes of exposure time, accelerating the CXL procedure had little effect on the anterior of the corneal stroma but reduced the effective depth of CXL.

It has been suggested that oxygen is a key limiting factor in the CXL process because CXL had no significant stiffening results when performed in a low-oxygen environment. Moreover, oxygen is expected to be consumed in the CXL process because of its transformation into the reactive species that catalyze the covalent bonding of collagen and other matrix proteins in the stroma. Thus, at high-radiation intensity, the rate of oxygen depletion could exceed the rate of oxygen replenishment via diffusion. In particular, the available oxygen concentration is a depth–dependent quantity because it is increasingly difficult for the oxygen to diffuse deeper into the cornea. Therefore, the difference between oxygen depletion and replenishment correlates to both depth and radiation intensity. As a result, when comparing 3 mW/cm² and 9 mW/cm² conditions for example, it is expected that the anterior portions of the corneas would have similar results while larger stiffening differences should be observed deeper into the cornea. This phenomenon would explain the lower stiffening we observed at the deeper sections of the accelerated cross-linked corneas. However, this effect was primarily seen in the 9 mW/cm² condition because the 34 mW/cm² and 50 mW/cm² conditions significantly lacked stiffening at all 3 sections of the stroma when compared with the standard protocol. The reported anterior lack in stiffening of the most accelerated protocols might be an effect of the measurement technique used. Brittingham et al., when comparing the standard 3 mW/cm² and 9 mW/cm² conditions, found a significant decrease in the depth of the demarcation line following accelerated protocols. Therefore, it is possible that for our highest accelerated conditions, the most effected depth of the CXL was too shallow to significantly detect using our Brillouin microscopy parameters. However, a similar threshold of stiffening efficiency was also observed by Wernli et al. It is possible that when the procedure is performed at such a rapid rate, oxygen is unable to diffuse back into the cornea to a significant depth. Past a threshold of a sufficiently oxygenated stroma, we would expect to observe very little stiffening effects of CXL because of the low-oxygen environment, as reported by Richoz et al.

In conclusion, our study confirmed the suboptimum effects of accelerated CXL compared with those of the standard 3 mW/cm² condition. Furthermore, using the depth–dependent analysis of Brillouin microscopy, we discovered the lack of CXL stiffening in deeper sections of the cornea, an important difference when comparing the standard methods and accelerated regimens.

WHAT WAS KNOWN
• Accelerated CXL, when compared with the standard 3 mW/cm² intensity for 30 minutes regime, provides sub-optimum results regarding the stiffening effects on the entire cornea.

WHAT THIS PAPER ADDS
• Brillouin microscopy allowed for a depth–dependent analysis after CXL. The confirmed decrease in effectiveness of accelerated CXL was primarily the result of the lack of stiffening deeper in the cornea compared with what occurs with the standard protocol.

REFERENCES

Disclosure: None of the authors has a financial or proprietary interest in any material or method mentioned.