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Biological and Biomechanical Responses to Traditional Epithelium-Off and Transepithelial Riboflavin-UVA CXL Techniques in Rabbits

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Abstract

PURPOSE—To compare the biological effects of riboflavin-ultraviolet A (UVA) corneal cross-linking (CXL) performed with a traditional epithelium-off method to several transepithelial methods in a rabbit model. Preliminary experiments on biomechanical rigidity were also performed.

METHODS—Four treatment groups were included: (1) standard epithelium-off, (2) tetracaine transepithelial, (3) benzalkonium chloride-ethylenediaminetetraacetic acid (BKC-EDTA) transepithelial, and (4) femtosecond laser-assisted transepithelial riboflavin-UVA CXL. Six eyes from each treatment group and the untreated control group were analyzed at 24 hours and 2 months after treatment in wound healing studies. The TUNEL assay was performed to detect the extent of stromal cell death. Optical density was measured with a Scheimpflug analyzer. The corneal stiffening effect was quantitated in three eyes from each group using optical coherence elastography performed 2 months after treatments.

RESULTS—Twenty-four hours after CXL, stromal cell death extended full corneal thickness with both standard epithelium-off CXL and femtosecond laser-assisted CXL, but only approximately one-third stromal depth after BKC-EDTA transepithelial CXL. Negligible stromal cell death was detected with tetracaine transepithelial CXL. Cell death results were statistically different between the BKC-EDTA transepithelial CXL and standard epithelium-off CXL groups ($P < .0001$). Significant corneal opacity differences were noted. Standard epithelium-off CXL had the greatest density and tetracaine transepithelial CXL had the least density compared to the control group after treatment. As measured with optical coherence elastography, a trend toward greater mean stiffening was observed with BKC-EDTA transepithelial CXL than with epithelium-off

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AUTHOR CONTRIBUTIONS

Study concept and design (BKA, MPL, ASR, WJD, SEW); data collection (BKA, MPL, MRF, MRS, VS, GHG, VA, ASR, WJD, SEW); analysis and interpretation of data (BKA, MPL, MRF, MRS, VS, GHG, ASR, RSB, WJD, SEW); drafting of the manuscript (BKA, MPL, MRF, WJD, SEW); critical revision of the manuscript (BKA, MPL, MRF, MRS, VS, GHG, VA, ASR, RSB, WJD, SEW); statistical expertise (RSB); obtained funding (WJD, SEW); administrative, technical, or material support (SEW); supervision (WJD, SEW)

CXL, femtosecond laser-assisted CXL, or tetracaine transepithelial CXL, but the result did not reach statistical significance. All of the CXL treatment groups exhibited significantly smaller variance of stiffness compared to the control group.

CONCLUSION—In the rabbit model, BKC-EDTA transepithelial CXL produced less stromal cell death and less risk of endothelial cell damage than standard epithelium-off CXL or femtosecond laser-assisted CXL. Additional study is needed to determine whether biomechanical stiffness is significantly different between the epithelium-off CXL and transepithelial CXL groups.

Riboflavin-ultraviolet A (UVA) cross-linking (CXL) of the cornea was first proposed as a method to halt the progression of the corneal ectatic disease keratoconus¹ and was later applied to the treatment of ectasia occurring after refractive surgical procedures.² Subsequent studies have demonstrated the efficacy of the treatment,^{3,4} including improvement in steepening and topographic regularity of the cornea in some patients.^{5–7} Biomechanical studies have suggested that CXL increases the stiffness of the cornea,^{8,9} although the mechanism through which it works remains controversial.^{10–12}

The original riboflavin-UVA CXL method described by Wollensak et al.¹ included the removal of the epithelium prior to the administration of a riboflavin-containing solution and exposure to UVA light, and most reports in the literature relate this to the “epithelium-off” CXL method. Others have proposed less invasive transepithelial riboflavin-UVA CXL methods that purportedly stiffen the cornea without removal of epithelium.^{9,12–14}

The purpose of the current study was to compare the corneal wound healing and biomechanical effects of standard epithelium-off riboflavin-UVA CXL compared with tetracaine transepithelial, benzalkonium chloride-ethylenediaminetetraacetic acid (BKC-EDTA) transepithelial or femtosecond laser-assisted transepithelial riboflavin-UVA CXL in a rabbit model.

MATERIALS AND METHODS

Animals

Rabbits were selected for this study because of their well-characterized corneal wound healing response to surgery and traditional epithelium-off riboflavin-UVA CXL.^{15–17} One eye of sixty 12- to 15-week-old female New Zealand white rabbits weighing 2.5 to 3.0 kg each were treated in this study. Rabbits were divided into four different treatment groups, and corneas were analyzed at 24 hours and 2 months. Twenty-four hours was selected as a time period because cell death and other early wound healing effects of corneal manipulations are prominent in the first 48 hours after manipulations or surgery that injures the corneal epithelium.¹⁴ Two months was selected because biomechanical effects and corneal cellular regeneration have been noted by this time after CXL and other corneal surgeries.^{1,7,8} There were six rabbits at 24 hours after treatment in each group and nine rabbits at 2 months after treatment in each group (Table 1). The untreated eye of each rabbit served as an untreated control. The Institutional Animal Care and Use Committee at the Cleveland Clinic approved this study. Animals were treated in accordance with the tenets of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Treatment Groups and Techniques

The treatment groups were: (1) standard epithelium-off riboflavin-UVA CXL, (2) femtosecond laser-assisted transepithelial riboflavin-UVA CXL, (3) BKC-EDTA transepithelial riboflavin-UVA CXL, and (4) tetracaine transepithelial riboflavin-UVA CXL.

General anesthesia was administered by intramuscular injection of ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg) prior to CXL in all groups and a wire eyelid speculum was positioned in the treated eye.

Rabbits in the standard epithelium-off riboflavin-UVA CXL group underwent treatment by the method of Wollensak et al.¹ using riboflavin photosensitizer solution produced by Seros Medical (Menlo Park, CA) containing 0.1% riboflavin-5-phosphate and 20% dextran T-500, except the riboflavin solution was applied for 10 minutes prior to beginning UVA irradiation instead of 5 minutes in the original protocol.¹ One percent proparacaine hydrochloride (Alcon Laboratories, Inc., Fort Worth, TX) was applied to each eye just before treatment. Central epithelial debridement to within 1 mm of the limbus was performed with a #64 blade (Beaver; Becton-Dickinson, Franklin Lake, NJ). The photosensitizer solution was dropped onto the exposed stromal surface every 3 minutes starting 10 minutes before the irradiation was begun and every 5 minutes during the irradiation treatment. UVA irradiation (370 nm) was applied continuously using a double UVA diode laser (Roithner Lasertechnik, Vienna, Austria) with an irradiance of 3 mW/cm² for a total of 30 minutes at a fixed 1-cm distance from the cornea. The radiance for this group, and other groups, was controlled using a hand-held UVA light meter (HHUVA1; Omega Engineering Inc., Stamford, CT) before and after every treatment.

Rabbits in the femtosecond laser-assisted transepithelial CXL group underwent CXL with delivery of the 0.1% riboflavin-0.5% carboxymethylcellulose (RF-CMC) solution into a corneal stromal lamellar pocket created with the WaveLight FS200 femtosecond laser (Alcon Laboratories, Inc.). The eye was pretreated with two drops of 1% proparacaine hydrochloride before being positioned on the femtosecond laser bed. The laser dock was brought into apposition with the cornea and maintained with posterior pressure by the surgeon without obtaining true suction. The suction line was clamped to simulate obtained suction. The laser was focused on the pupil to center the treatment and approximately 10 seconds of laser was applied in a pattern to create an intrastromal pocket and tunnel. Pocket depth was 90 μ m (without a side-cut) and the diameter was 7 mm. The channel width was 3 mm and the spot separation was 7 μ m. Bed energy was 0.75 mJ. The pocket was dissected with a blunt sterile instrument under an operating microscope and RF-CMC solution was infused into the corneal pocket using an olive tip cannula until the pocket was colored bright yellow with the riboflavin solution (approximately 50 μ L). After 10 minutes, UVA irradiation (370 nm, 3 mW/cm²) was applied for a total of 30 minutes identically to the epithelium-off CXL group. The intrastromal pocket was re-instilled with riboflavin solution at the 15-minute mark during irradiation.

Rabbits in the BKC-EDTA riboflavin transepithelial CXL group underwent CXL with 0.1% riboflavin-5-phosphate, 0.02% benzalkonium chloride, 0.01% sodium ethylenediaminetetraacetic acid, and 0.5% carboxymethylcellulose in hypotonic tris buffer,

pH 7.2. This formulation was similar to that reported by Kissner et al.¹⁴ and was compounded by Leiter's Pharmacy (San Jose, CA). One drop of proparacaine hydrochloride 1% and one drop of BKC-EDTA-riboflavin solution were applied 1 minute apart every 5 minutes for 15 minutes prior to irradiation and every 5 minutes during irradiation. UVA irradiation (370 nm, 3 mW/cm²) was applied for a total of 30 minutes identically to the epithelium-off CXL group.

Rabbits in the tetracaine transepithelial CXL group underwent CXL with topical RF-CMC solution containing 0.1% riboflavin, 0.5% carboxymethylcellulose (Seros Medical) without epithelial removal. The eyes were pretreated with both 0.5% tetracaine (Alcon Laboratories, Inc.) and RF-CMC 1 minute apart every 5 minutes for 15 minutes. UVA irradiation (370 nm, 3 mW/cm²) was applied for a total of 30 minutes identically to the epithelium-off CXL group. During irradiation, a sponge soaked with 0.5% tetracaine was applied to the cornea for several seconds every 10 minutes and a sponge soaked with RF-CMC was applied to the cornea for several seconds every 3 minutes.

Each animal in each group received two drops of ciprofloxacin ophthalmic 0.3% solution instilled into the operative eye immediately after surgery. Corticosteroids were not used to avoid the potential confounding effect of this drug on the corneal wound healing response.

Euthanasia was performed at 24 hours or 2 months with an intravenous injection of 100 mg/kg pentobarbital, while the animal was under ketamine/xylazine general anesthesia.

The corneoscleral rims of six eyes from each group at each time period were removed with 0.12 forceps and sharp Westcott scissors and embedded in liquid optical coherence tomography (OCT) compound (Sakura FineTek, Torrance, CA) within a 24 × 24 × 5 mm mold (Fisher Scientific, Pittsburgh, PA) for use in immunohistological testing and quickly frozen on a block of dry ice and stored at -85°C. The corneoscleral rims were centered in the mold prior to freezing so the block could be bisected and transverse sections cut from the center of the cornea.

Central corneal sections (8-mm thick) were cut with a cryostat (HM 505M; Micron GmbH, Walldorf, Germany). Sections were placed on 25 × 75 × 1 mm microscope slides (Superfrost Plus; Fisher Scientific) and maintained at -85°C until staining was performed.

TUNEL Assay, Immunohistochemistry, and 4',6-Diamidino-2-Phenylindole Staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay detects DNA fragments generated by apoptosis and necrosis was rarely performed as previously described using ApopTag (Cat No: S7165; Intergen Co, Purchase, NY).¹⁵

Immunohistochemistry was performed to detect alpha-smooth muscle actin (αSMA), the most commonly used marker for myofibroblasts as previously described.¹⁵

Coverslips were mounted with Vectashield containing 4',6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA) to allow visualization of all nuclei in the tissue sections. Negative controls were included with secondary antibody alone, because no antigen is available for pre-absorption controls.

The sections were viewed and photographed with a LeicaDM5000 microscope equipped with Q-Imaging Retiga 4000RV (Surrey, British Columbia, Canada) camera and Image-Pro 6.0 software (MediaCybernetics, Inc. Bethesda, MD).

Cell Counting

In each cornea, all of the stained cells in seven full-thickness non-overlapping 400× (49,700 μm^2 for 400× field) columns that extended from the anterior to the posterior stromal surface were counted as previously described.^{16,17} The columns in which counts were performed for TUNEL+ or α SMA-stained cells were randomly selected from the central cornea of each specimen.

Densitometry Measurement of Central Corneal Optical Density

Measurements were performed with the Galilei Dual Scheimpflug Analyzer (Ziemer, Port, Switzerland) to compare central corneal stromal optical densities between the different groups 2 months after treatment. Imaging was performed in both the treated and untreated eyes immediately after euthanasia and prior to removal of the corneoscleral rims. The measurements obtained in arbitrary density units (0 to 100) were performed over four axes in each cornea and were averaged to yield a value for that cornea.

Optical Coherence Elastography

Treated and untreated eyes of three rabbits from each treatment group were enucleated at the 8-week time period and placed in Optisol preservation medium (Bausch & Lomb, Rochester, NY). Elastography was performed within 20 minutes of enucleation.

The experimental set-up of the optical coherence elastography apparatus and the method used to measure corneal stiffness has been previously described.¹⁸ Briefly, the technique is based on high-resolution OCT imaging of the displacement of intracorneal optical features tracked with a two-dimensional cross-correlation algorithm that allows precise estimation of local and directional corneal material properties. Intraocular pressure of the enucleated globe was maintained at a physiologic level of 15 mm Hg, while the cornea was vertically displaced with a gonioscopy lens in 20- μm intervals for a total of 140 μm of displacement. The displacement was controlled with an electronic computer-controlled microstage (TLA-28 Linear Actuator; Zaber Technologies Incorporated, Vancouver, Canada). The OCT scan parameters were set to oversample the lateral spot size by a factor of 10 to maximize capture of the speckle pattern in the image. The OCT data stream was then processed to extract the two-dimensional displacement of the cornea across the 4-mm scan width and entire corneal thickness. Color-encoded maps of local axial and lateral displacement were obtained with corresponding maps of cross-correlation strength, an indicator of the fidelity of the displacement tracking. The slope of the lateral displacement divided by the imposed axial displacement was calculated—a unitless measure of corneal resistance to lateral strain—with a smaller ratio indicating greater corneal stiffness. Six regions of interest (3 anterior stromal and 3 posterior stromal) were defined and analyzed in aggregate using analysis of variance (ANOVA) and general linear regression (Minitab Statistic Software Inc., State College, PA).

Statistical Analysis

Statistical comparisons were performed using ANOVA tests for TUNEL Assay, α SMA, and stromal density data were conducted using Microsoft Excel and STATA (Stata-Corp LP, College Station, TX). Statistical pairwise comparisons of biomechanical effects of the different methods of CXL were performed using methods of repeated measures ANOVA with Tukey-Kramer corrections for multiple comparisons. A *P* value less than .05 was considered statistically significant. Pairwise analysis of the variances of the distribution of the biomechanical results of the different CXL methods and the untreated controls was performed using Levene's test with Bonferroni corrections for multiple comparisons.

RESULTS

Corneal healing appeared to proceed normally in all animals and no corneal infiltrates or infections were noted in the corneas after any of the corneal CXL treatments. Variable corneal stromal edema was noted in corneas in the traditional epithelium-off CXL and femtosecond laser-assisted CXL groups at 1 week. Two months after treatment, however, no corneas that had CXL with any of the techniques had stromal or epithelial edema at the slit lamp and the corneal endothelial layers appeared to have normal cell density using specular reflection with the slit lamp in all groups.

Stromal and Endothelial Cell Death at 24 Hours and 2 Months After CXL Treatments

At 24 hours, the standard epithelium-off CXL method has the greatest extent of corneal stromal cell death, with full thickness or nearly full thickness death of stromal keratocytes in the central corneal stroma (Figures 1A and 1B) in all corneas of this group. This was followed by the femtosecond laser-assisted transepithelial CXL group (Figures 1C and 1D) that had nearly full-thickness stromal cell death in all corneas from the group. Conversely, the BKC-EDTA transepithelial CXL group consistently showed TUNEL+ stromal cells extending to a depth of less than one-third of the total stromal thickness 1 week after treatment (Figures 1E and 1F). Few TUNEL+ keratocytes were noted in any corneas in the tetracaine transepithelial CXL group (Figures 1G and 1H) or the untreated control group (Figures 1I and 1J) 1 week after treatment. The quantitative results for TUNEL+ cells per 400 $\times$ stromal column are shown in Figure 2 and the ANOVA *P* values in Table 2. It is important to note that TUNEL+ cells were counted in real time at the microscope and not from photographic images. Thus, 1 week after surgery, the standard epithelium-off CXL, femtosecond laser-assisted transepithelial CXL, and BKC-EDTA transepithelial CXL groups were different from each other and all three of these were different from the tetracaine transepithelial CXL and untreated control CXL groups in TUNEL+ cells per 400 $\times$ column 1 week after treatment. Stromal cell death was not significantly different between the tetracaine transepithelial CXL and untreated control groups.

Corneal endothelial cell death at 24 hours after treatment varied extensively between the different groups. Five of the six corneas in the standard epithelium-off CXL group (Figures 1A and 1B) and two of the six corneas in the femtosecond-assisted transepithelial CXL group (Figures 1C and 1D) had endothelial cell death. Conversely, no corneas in the BKC-EDTA transepithelial CXL (Figures 1E and 1F), tetracaine transepithelial CXL (Figures 1G

and 1H), or non-CXL control groups (Figures 1I and 1J) had endothelial cell death noted with the TUNEL assay at 24 hours after treatment.

Two months after treatment, only rare TUNEL+ stromal cells were noted in corneas from any of the groups and there were no significant differences between the groups (data not shown). No corneal endothelial cells were TUNEL+ at 2 months after treatment in any of the groups and the endothelial monolayer appeared to be normal in all corneas in all groups at this time period with 4',6-diamidino-2-phenylindole staining (data not shown).

Myofibroblast Generation at 24 Hours and 2 Months After Treatment

None of the corneas in any group had SMA+ cells 24 hours after treatment (data not shown). Two months after treatment, few SMA+ cells were detected in the central corneas of any of the CXL treatment groups or the control group (data not shown). Positive control rabbit corneas 1 month after -9 diopters photorefractive keratectomy showed large numbers of anterior stromal SMA+ myofibroblast cells (data not shown).

OCT and Scheimpflug Imaging of Central Corneal Opacity

Figure 3 shows a representative OCT and Scheimpflug image from a cornea in each CXL treatment group and the non-CXL control group. A demarcation line corresponding to the laser pocket was visible in the corneas in the femtosecond-assisted transepithelial CXL group. The mean densitometry of the central cornea 2 months after CXL treatment was significantly increased in all CXL groups (Figure 4 and Table 3). The standard epithelium-off CXL and femtosecond laser-assisted transepithelial CXL groups had the highest mean densitometry measurements (70.7 ± 3.4 , 78.5 ± 3.7 , $P = .148$). Central corneal density in the BKC-EDTA transepithelial group (55.0 ± 1.5) was less than that in the standard epithelium-off CXL or the femtosecond laser-assisted transepithelial CXL groups (Figure 4 and Table 3), but greater than that found in the tetracaine transepithelial CXL group (38.9 ± 3.1). Corneas in the tetracaine transepithelial CXL group had significantly higher central density than corneas in the non-CXL control group, and were significantly less dense than corneas in the other CXL groups (Figure 4 and Table 3).

Corneal Stiffness at 2 Months After Treatment

Analysis of OCT elastography data from all of the CXL groups and non-CXL controls found that the mean lateral-to-axial displacement ratio for the BKC-EDTA transepithelial CXL group was lower (higher corneal stiffness) 2 months after treatment than the traditional epithelium-off CXL, femtosecond laser-assisted transepithelial CXL, or tetracaine transepithelial CXL (Figure 5). However, a statistical comparison across the different methods of CXL yielded no statistically significant differences between the various treatment mechanical effects by ANOVA with a Tukey comparison.

A graph of the probability density functions of the biomechanical properties measured for the various groups 2 months after treatment is shown in Figure 6. Each curve represents the probability density function for a given treatment group calculated using the treatment group's or control group's measured mean and variance. The normality of the distribution of

the measured data was verified prior to this calculation. The normal probability density functions were calculated from

$$\frac{1}{\sigma\sqrt{2\pi}}e^{-\frac{(x-\mu)^2}{2\sigma^2}}$$

where σ^2 is the variance and μ is the mean. It is important to note that the peaks in Figure 6 were not based on a single displacement ratio for each eye, but on measurements from several regions on each eye that were incorporated into these results using the applied statistical methods. ANOVA of the distribution of the results using Levene's test indicated that there was a significant difference in the variance of the mechanical responses of the eyes to the various CXL methods ($P < .0001$) when compared to the non-CXL eyes. All of the treatments exhibited significantly smaller variance than the control group and the treatment group with the smallest variance (BKC-EDTA group) was significantly smaller than the tetracaine CXL group. The differences in these values can be seen in Figure 6.

DISCUSSION

Riboflavin-UVA CXL appears to have use for stiffening and, in some cases, flattening the cornea in eyes that have keratoconus and ectasia after refractive surgery.¹⁻⁷ Since the development of epithelium-off riboflavin-UVA CXL, several other methods have been proposed in an attempt to lessen patient discomfort and decrease the incidence of complications associated with removal of the central corneal epithelium.¹⁹⁻²⁷ Alternative techniques reported have included transepithelial CXL methods using tetracaine,²⁸ BKC-EDTA^{9,14,29} to lessen the barrier function of the corneal epithelium to increase the penetration of riboflavin into the corneal stroma, and femtosecond laser-assisted CXL methods in which a pocket is produced in the anterior stroma to allow riboflavin solution to be infused beneath the epithelium.³⁰⁻³² In the current study, we aimed to compare the injury/wound healing effects of traditional epithelium-off riboflavin-UVA CXL with three different transepithelial methods. We also made preliminary biomechanical measurements in eyes from each of the groups. It is important to emphasize that this study was performed in rabbits and the results could be different in human eyes for all the measured parameters.

The average central corneal thickness in native un-manipulated New Zealand White rabbits measured with ultrasonic pachymetry was 407 μm , with a standard deviation of 20 μm , similar to previous reports.³³ The extensive stromal and endothelial cell death found in this study with riboflavin-UVA CXL have been reported in prior studies in rabbits.^{16,34,35} These results point out the danger to the corneal endothelium of performing traditional epithelium-off CXL and femtosecond laser-assisted transepithelial CXL in thin corneas, as noted in prior clinical studies of human corneas less than 400 μm .^{36,37} Importantly, the human corneal endothelium does not effectively regenerate³⁸ as it does in the rabbit, as noted in this study where there appeared to be at least partial regeneration of the endothelium in all of the traditional epithelium-off CXL corneas 2 months after treatment. From this perspective, BKC-EDTA transepithelial CXL could have a safety advantage over traditional epithelium-off CXL or femtosecond laser-assisted CXL, even if the biomechanical effects of these

treatments were similar, because the stromal cell death from BKC-EDTA transepithelial CXL never exceeded approximately 150 μm from the corneal surface and in no case was endothelial damage noted with the TUNEL assay. Rabbit corneas also do not have a Bowman's layer. The effects of this on the CXL process, if any, are not known.

Importantly, UVA exposure of the level used in CXL is not sufficient to trigger stromal cell death, at least when the epithelium is present, because few TUNEL+ stromal cells were noted in corneas in the tetracaine transepithelial CXL group that had the same level and duration of UV light exposure as the other CXL groups. Thus, the presence of the epithelium in transepithelial CXL methods blocks some, but not all, UV light penetration into the cornea.³⁹

In the rabbits in this study, there also appeared to be complete restoration of stromal cellularity at 2 months after treatment in all groups where keratocyte cell death was noted—even in the traditional epithelium-off CXL treated corneas where there was essentially full-thickness depopulation of the stroma 24 hours after surgery in the majority of corneas. This restoration of keratocyte density has also been noted to occur in humans over several months after CXL using confocal microscopy,^{40,41} although it is not known whether the repopulating cells also have complete restoration of normal keratocyte function.

Many clinicians have the impression, based on clinical observations, that the efficacy of CXL correlates with increases in stromal haze (opacity) after treatment. In the current study, all CXL groups had significantly greater density than controls (Figures 3 and 4 and Table 3) measured with Scheimpflug tomography and noted with OCT 2 months after treatments. Greater optical densities in traditional epithelium-off CXL and femtosecond laser-assisted transepithelial CXL compared to the other transepithelial CXL groups and control group are likely attributed to a more extensive stromal wound healing response that is proportional to the level of stromal cell death and associated with the generation of corneal fibroblasts with decreased intracellular corneal crystalline production⁴² and alterations in the regular structure of the stromal matrix that is responsible for optical transparency of the cornea.⁴³ Our results show that the increase in stromal opacity is not attributed to myofibroblast generation, as it is after photorefractive keratectomy.¹⁵ This provides an explanation for (1) the more transient nature of stromal opacity noted in CXL,⁴⁴ measured in months rather than the years it often persists after photorefractive keratectomy,¹⁶ and (2) the tendency for the opacity to extend deeper in the stroma rather than be confined to the subepithelial stroma, as it is in photorefractive keratectomy.¹⁵ It is important to note that myofibroblasts could be generated in complicated CXL cases with atypical wound healing and result in more severe and persistent haze generation.

The surprising biomechanical effect noted in this study 2 months after treatment was that the lateral-to-axial displacement ratio for the BKC-EDTA transepithelial CXL group was lower (corneas stiffer on average) than the traditional epithelium-off CXL method, the femtosecond laser-assisted transepithelial CXL, or the tetracaine transepithelial method. If the means of all groups are normalized to the mean for the non-CXL control group, the BKC-EDTA transepithelial CXL group had 3.6 $\times$ stiffening compared to the non-CXL control group (Figure 5). This compares to 1.8 $\times$ stiffening for the femtosecond laser-assisted

transepithelial CXL group, 1.6× stiffening for the standard epithelium-off CXL group, and 1.3× stiffening for the tetracaine transepithelial CXL group (Figure 5). However, the comparison of the mean stiffening for the different groups did not reach statistical significance with ANOVA analysis—likely due to the low number of animal eyes in each group and the variation in stiffness in all groups. Power analysis of these results revealed a dozen animals will need to be included in the standard epithelium-off and BKC-EDTA transepithelial CXL groups to have a reasonable chance of finding significance between the CXL methods in stiffness measurements between these groups, if one exists, due to the high variation in stiffness within each group. A direct comparison study of biomechanical effects for these two groups in rabbits is currently being performed.

Each of the CXL methods except tetracaine transepithelial CXL produced a narrowing of the stiffness distribution (Figure 6). This effect was especially pronounced in the BKC-EDTA transepithelial CXL group. There was a highly significant difference in the variance of the mechanical properties of the eyes after the various CXL methods ($P < .0001$) when the stiffness distributions were evaluated using the Levene's test.

The method used in this study to monitor biomechanical changes induced by the different corneal CXL techniques, OCT elastography, is a well-characterized approach that provides directionally resolved corneal displacement measurements in response to a stress.^{45–47} These changes were measured 2 months after CXL in this study. However, a previous study showed that the biomechanical effects of epithelium-off riboflavin-UVA CXL could increase over time for at least 8 months after treatment⁸ and it is not known whether the biomechanical stiffening effects of the different transepithelial CXL methods similarly increase over time after treatment.

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Biography

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Figure 1.

Representative TUNEL assay images of the central cornea from a rabbit cornea 24 hours after cross-linking (CXL) with (A and B) standard epithelium-off CXL, (C and D) femtosecond-assisted transepithelial CXL, (E and F) benzalkonium chloride-ethylenediaminetetraacetic acid (BKC-EDTA) transepithelial CXL, (G and H) tetracaine transepithelial CXL, and (I and J) non-CXL control corneas. TUNEL+ cells are stained red (white arrows indicate some of the TUNEL+ cells in each panel). In some samples, epithelium was artifactually displaced or lost during tissue sectioning and staining (ie, 1F). Note that some TUNEL+ cells may have already disappeared completely by 24 hours after treatment and would not be labeled with the TUNEL assay. Cell nuclei are stained blue with 4',6-diamidino-2-phenylindole. The larger arrows indicate corneal endothelial cells. Note in (A) standard epithelium-off CXL corneal endothelial cells are also TUNEL+, whereas in (B) the corneal endothelial cells are TUNEL- (other sections in that the cornea had TUNEL+ endothelial cells). Similarly, in (C) some of the femtosecond-assisted transepithelial corneal endothelial cells on the left side of the specimen are TUNEL+, whereas those on the right side are TUNEL-. In (D), most endothelial cells were TUNEL-, although one or two TUNEL+ endothelial cell can be seen adjacent to (D). No TUNEL+ endothelial cells were noted in any corneas in the BKC-EDTA transepithelial, tetracaine transepithelial, or non-CXL control groups. In femtosecond-assisted transepithelial CXL figures, the images were obtained in the mid-peripheral cornea where there was a transition between the central cornea in the area of the femtosecond laser pocket where TUNEL+ cells were noted and the more peripheral cornea outside the femtosecond pocket where more posterior keratocytes were TUNEL-. (Original magnification $\times 200$)

Figure 2.

Quantification of TUNEL+ cells in central cornea 24 hours after standard epithelium-off cross-linking (CXL), femtosecond-assisted transepithelial CXL, benzalkonium chloride-ethylenediaminetetraacetic acid (BKC-EDTA) transepithelial CXL, tetracaine transepithelial CXL, and non-CXL control rabbit corneas. Six corneas were examined per time period for each group. Data are expressed as the mean TUNEL+ stromal cells per 400× column from the anterior stromal surface to the posterior stromal surface. Error bars denote the standard error of mean.

Figure 3.

Optical coherence tomography (OCT) images obtained during OCT elastography and Scheimpflug densitometry images of representative corneas from each group 2 months after treatment. Note that the opacity (arrows) attributed to the femtosecond laser pocket can be clearly noted in the cornea from the femtosecond laser-assisted cross-linking (CXL) group in the top panel. (Original magnification $\times 40$) Epi-off = epithelium-off, BKC = benzalkonium chloride

Figure 4. Graph of mean central corneal Scheimpflug densitometry measurements 2 months after treatment in the groups. Error bars represent the standard errors of the mean. Epi-off = epithelium-off, Femto = femtosecond, Transepi = transepithelial, BKC = benzalkonium chloride

Figure 5.

Optical coherence tomography elastography analysis of the average relative stiffness of cornea cross-linked (CXL) with the standard, femtosecond, benzalkonium chloride (BKC), or tetracaine technique against the non-CXL control 2 months after treatment. Relative stiffness is derived from lateral-to-axial displacement slope of CXL over untreated control. Epi-off = epithelium-off; Femto = femtosecond

Figure 6.

Graph of probability distribution versus lateral/axial displacement ratio obtained with optical coherence tomography (OCT) elastography 2 months after treatment for the different riboflavin-ultraviolet A cross-linking (CXL) groups (standard epithelium-off [Epi-off] CXL, femtosecond laser-assisted transepithelial CXL, benzalkonium chlororide [BKC] transepithelial CXL, and tetracaine transepithelial CXL) and the untreated control group (non-CXL control). Note that there is a peak shift toward lower lateral/axial displacement ratio (greater stiffness) for BKC transepithelial CXL, standard epithelium-off CXL and femtosecond laser-assisted transepithelial CXL; however, the shift is greater compared with the untreated control and the variance is less for the BKC transepithelial CXL group.

TABLE 1

No. of Eyes in Each CXL Group at Each Time Point

Technique	24 Hours	8 Weeks	Biomechanical Analysis
Standard epithelium-off CXL	6	6	3
Femtosecond-assisted transepithelial CXL	6	6	3
BKC-EDTA transepithelial CXL	6	6	3
Tetracaine transepithelial CXL	6	6	3

CXL = cross-linking; BKC-EDTA = benzalkonium chloride-ethylenediaminetetraacetic acid

TABLE 2

ANOVA *P* Values for TUNEL+ Cells 24 Hours After Treatment

Group	Standard Epithelium-off CXL	Femtosecond	Transepithelial CXL	BKC-EDTA	Transepithelial CXL	Tetracaine	Transepithelial CXL	Non-CXL Control
Standard epithelium-off CXL	—	.0030		< .0001		< .0001		< .0001
Femtosecond transepithelial CXL	—	—	.0459			< .0001		< .001
BKC-EDTA transepithelial CXL	—	—	—			< .0001		< .0001
Tetracaine transepithelial CXL	—	—	—	—		—		.9291
Non-CXL control	—	—	—	—		—		—

ANOVA = analysis of variance; CXL = cross-linking; BKC-EDTA = benzalkonium chloride-ethylenediaminetetraacetic acid

TABLE 3ANOVA *P* Values for Scheimpflug Central Stromal Density 2 Months After Treatment

Group	Standard Epithelium-off CXL	Femtosecond Transepithelial CXL	BKC-EDTA Transepithelial CXL	Tetracaine Transepithelial CXL	Non-CXL Control
Standard epithelium-off CXL	–	.15	.0001	< .0001	< .0001
Femtosecond transepithelial CXL	–	–	.045	< .0001	< .0001
BKC-EDTA transepithelial CXL	–	–	–	< .0001	< .0001
Tetracaine transepithelial CXL	–	–	–	–	.0006
Non-CXL control	–	–	–	–	–

ANOVA = analysis of variance; CXL = cross-linking; BKC-EDTA = benzalkonium chloride-ethylenediaminetetraacetic acid