High-irradiance CXL combined with myopic LASIK: flap and residual stroma biomechanical properties studied ex-vivo

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ABSTRACT

Background/aims To evaluate ex vivo biomechanical and enzymatic digestion resistance differences between standard myopic laser in-situ keratomileusis (LASIK) compared with LASIK+CXL, in which high-irradiance cross-linking (CXL) is added.

Methods Eight human donor corneas were subjected to femtosecond-assisted myopic LASIK. Group A (n=4) served as a control group (no CXL). The corneas in LASIK+CXL group B were subjected to concurrent prophylactic high-irradiance CXL (n=4). Saline-diluted (0.10%) riboflavin was instilled on the stroma, subsequently irradiated with UV-A through the repositioned flap. The cornea stroma and flap specimens were separately subjected to transverse biaxial resistance measurements; biomechanical differences were assessed via stress and Young's shear modulus. Subsequently, the specimens were subjected to enzymatic degradation.

Results For the corneal stroma specimen, stress at 10% strain was 128±11 kPa for control group A versus 293±20 kPa for the LASIK+CXL group B (relative difference Δ=+129%, p<0.05). The stress in group B was also increased at 20% strain by +68% (p<0.05). Shear modulus in group B was increased at 10% strain by +79%, and at 20% strain by +48% (both statistically significant, p<0.05). The enzymatic degradation time to dissolution was 157.5±15.0 min in group A versus 186.25±7.5 min in group B (Δ=+18%, p=0.014). For the flaps, both biomechanical, as well as enzymatic degradation tests showed no significant differences.

Conclusions LASIK+CXL appears to provide significant increase in underlying corneal stromal rigidity, up to +130%. Additionally, there is significant relevant enzymatic digestion resistance confirmatory to the above. LASIK flaps appear unaffected biomechanically by the LASIK+CXL procedure, suggesting effective CXL just under the flap.

INTRODUCTION

Corneal collagen cross-linking (CXL) has been clinically employed for stabilising progressive keratectasia.[1 2] This photochemical reactive process is induced by peak 365 nm ultraviolet (UV-A) radiation absorbed by riboflavin, a photosensitive vitamin B2 molecule. The procedure is broadly known as corneal cross-linking—despite the fact that there are some reports suggesting that the mechanism responsible for biomechanical strengthening[3 4] within the stroma is related not to interlamellar cohesion increase, but to inter-fibrillar and intra-fibrillar cohesion.[5] In addition, increased collagen resistance against enzymatic degradation has been associated with CXL.[6–8]

We have introduced an alternative CXL application, adjuvant to myopic laser in-situ keratomileusis (LASIK+CXL). The application aims to improve long-term keratometric stability[9] and to reduce regression likelihood following moderate and high myopic LASIK[10] by proactively restoring corneal biomechanical strength.[11] Riboflavin solution is briefly applied on the exposed stromal bed at the completion of the excimer ablation; the flap is then repositioned, followed by superficial UV-A irradiation.[12 13]

To the best of our knowledge, the biomechanical and/or enzymatic degradation resistance modulations achieved via CXL application concurrent with LASIK have not been studied in human corneas. The purpose of this study is to evaluate ex-vivo biomechanical and enzymatic degradation resistance differences in such application.

MATERIALS AND METHODS

Eight human donor corneas were involved, obtained by the Eye Bank for Sight Restoration Inc (New York, USA), an accredited member of the Eye-Bank Association of America. The corneas were donated by eight different donors (four men, four women) of average age 62.0±9.5 (43–72) years, stored in 4°C OptiSol solution (Bausch +Lomb, Rochester, New York, USA).

Surgical technique

All corneas were subjected to femtosecond-laser assisted myopic treatment. The corneas were mounted on an artificial anterior chamber (Baron, Katena Products, Inc, Denville, New Jersey, USA). Flaps (120 µm thick, 8.5 mm diameter) were created with the WaveLight FS200 femtosecond laser (Alcon Surgical, Ft Worth, Texas, USA), observing standard docking, applanation and vacuum-suction procedures (figure 1A). After flap lifting (figure 1B), the WaveLight EX500 excimer laser (Alcon) was employed to create a −8.00 D myopic ablation over a 6.5 mm diameter optical zone (figure 1C). During the procedure, interferometric pachymetry embedded in the EX500 provided corneal thickness data.

Isotonic saline 0.1% riboflavin solution (Vibex Rapid, Avedro Inc, Waltham, Massachusetts, USA) was instilled on the exposed stromal bed afforded by the lifted flap (figure 1C). Soaking time was 1 min (figure 1E); then excess riboflavin was wiped from the cornea surface. Special care was taken to minimise potential riboflavin soaking to the folded LASIK flap.

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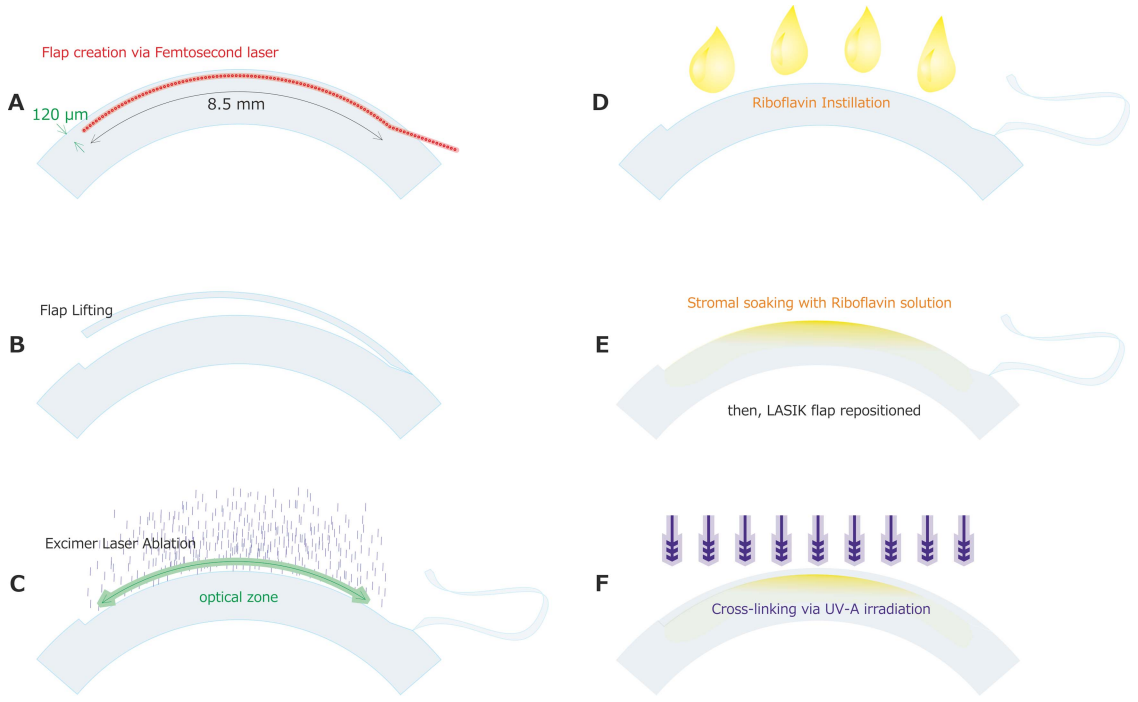

Figure 1 Schematic of the surgical procedure LASIK combined with prophylactic high-irradiance corneal cross-linking (LASIK+CXL).

The corneas were then randomly formed into two groups, four in each. Control group A received no further treatment. Group B (LASIK+CXL) was subjected to cross-linking: with the flap repositioned, the cornea was UV-A irradiated at 30 mW/cm^2 for 80 s (total fluence 2.4 J/cm^2), employing the KXL device (Avedro) (figure 1F).

The flaps were then amputated from all corneas; stroma and flap specimens were stored back to OptiSol (4°C) until testing.

Biomechanical strength testing

The stroma specimens were prepared by razor-blade manual dissection to approximately 12×12 mm. The amputated flaps were tested without additional preparation. Transverse biaxial load-cell resistance measurements were accomplished by tangential shear-force employing the Biotester 5000 (CellScale Biomaterials Testing, Waterloo, Canada). The device records the simultaneous x-axis and y-axis displacement, applied force, and time. An integrated camera captures still, 1280×960-pixel images, which provide precise x-displacement and y-displacement measurements, analysed by custom software (LabJoy V.9.05).[14]

The specimens were fixed (via random orientation) on a 4×5-tine rake arrangement clamped on their centre 3.5×3.5 mm section. The tines, of 250 µm diameter, were spaced by 0.7 mm (figure 2). The specimens were then submerged into an isotonic saline bath, temperature controlled at 37°C, for 5 min before (for temperature stabilisation) as well as during testing (to eliminate temperature-related variability).[15] Shear rate was fixed to 4.16 µm/s. Time (s), x and y displacement (µm), and x and y force (mN) were recorded every second.

Enzymatic degradation

The stroma specimens were trephined into 8.5 mm diameter round buttons; no further processing on the amputated flaps. A 0.3% collagenase-A solution (active agent: Clostridium histolyticum) (Sigma-Aldrich, St Louis, Missouri, USA) was prepared via dilution in Dubecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich). Stroma and flap specimens were incubated within 1.5 mL of collagenase-A solution; the test tube racks were placed at 37°C on a plate shaker at 175 rotations per min.

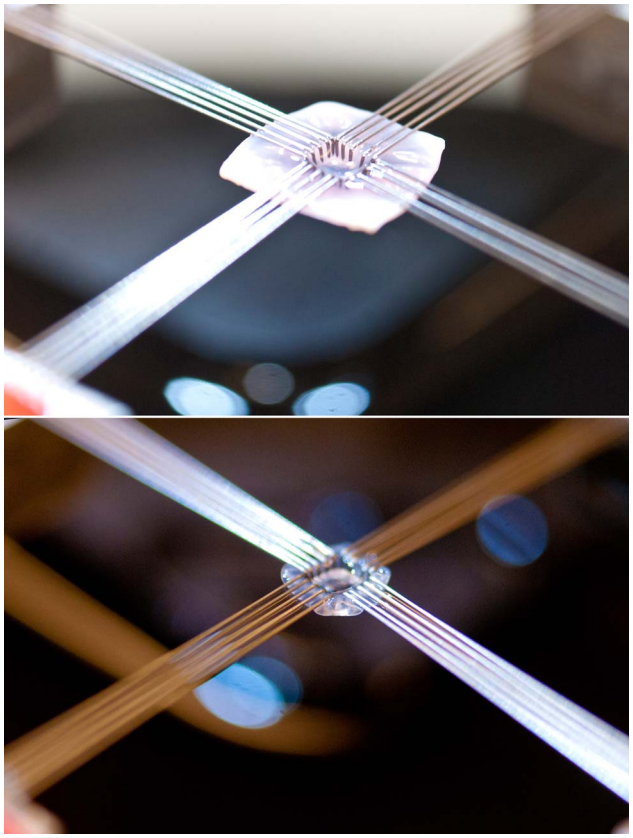

Figure 2 Fitting of the corneal specimens (top) and the flap (bottom) on the BioTester device.

Table 1 Biomechanical comparative measurements between the two groups

	Stress	Units: kPa			Young's shear modulus	Units: MPa		
	@ 10% strain		@ 20% strain		@ 10% strain		@ 20% strain	
Group A (control)	128.0	±11.3	804.1	±30.9	3.7	±2.5	9.5	±1.9
Group B (CXL)	293.4	±19.9	1349.5	±46.1	6.6	±2.4	14.0	±5.2
Δ	129%		68%		79%		48%	
p	0.015		0.025		0.019		0.035	
t	−3.33		−2.96		−3.18		−4.62	

Δ, relative (%) difference between metrics; p, Student t test p value; t, Student t test t value. Results from corneal stroma specimens.

Specimens were observed every 30 min until complete dissolution was achieved. The time to complete dissolution of the stromal and flap specimens was recorded.

Data analysis

Stress, a pressure metric, is the applied force (F) divided by the cross section (A) of the tested area (F/A, units=mN/mm^2=kPa=10^3 Pa; MPa=10^6 Pa; Pa=Pascal). Strain, expressed as percentage, is the unitless relative elongation Δx/lx (or Δy/ly, respectively), where l is the initial length. Cross-section test area was defined by the 3.5 mm sample length multiplied by the respective stroma/flap thickness, respectively. Statistical analysis and graphs of stress (expressed in kPa) in the vertical, versus strain in the horizontal axis, were constructed using the SPSS software V.21.0 (IBM Corporation, New York, USA). The exponential fitting for stress calculation was conducted at the 10% and 20% strain. Linear regression fitting was performed to calculate, by means of the slope function (gradient), the stress/strain ratio, an expression of the shear (Young's) modulus. The shear modulus was calculated as the gradient at 10% and 20% strain. To ensure proper linear fit, we sought a minimum of 0.98 for the trend-line determination coefficient (r^2).

Statistical significance was assessed employing Student's t test. p Values less than 0.05 were indicative of statistically significant results in this study. Results are reported in the form: average ±SD (minimum to maximum).

RESULTS

In group A the donor age at the time of cornea harvest was 63.3 ±13.7 years (48–72) and in group B, 60.8±4.5 years (43–69).

Biomechanical strength results

The distinct datasets during shear-strength measurements on stroma specimens (all stroma specimens) were on average 290 ±37 (240–340), while average displacement was 1205±155 µm (1000–1416). Mean maximum applied force was 3996 ±597 mN (3469–5247). Average value of maximum strain was 30±3% (26–34%).

The distinct datasets on flap specimens (all flap specimens) were on average 222±18 (192–242), while average displacement was 925±74 µm (805–1008). Mean maximum applied force was 1505±197 mN (1.242–1868). Average value of maximum strain was 23.5±2% (21–25%).

The average number for all specimens of distinct data pairs (vertical axis, stress and horizontal axis, strain) within the linear phase of analysis of the stress–strain curves was 119 (95–129). The average coefficient of determination (r^2) of the trend-line was 0.997 (0.994–0.999).

For the stroma specimens in control group A, average stress at 10% strain was 128±11 kPa and at 20% strain 804±31 kPa; Young's modulus at 10% strain was 3.7±2.5 MPa and at 20% strain 9.5±1.8 MPa. In the LASIK+CXL group B, stress at 10% strain was 293±20 kPa and at 20% strain 1350±46 kPa; Young's modulus at 10% strain was 6.6±2.4 MPa and at 20% strain 14.0±5.2 MPa. The relative increase of these biomechanical properties in LASIK+CXL group B in comparison to control group A was stress +129% and +68% for 10% and 20% strain, and Young's modulus +79% and +48%, respectively. All differences were statistically significant (table 1).

For the flap specimens in control group A: stress at 10% strain was 158±17 kPa and at 20% strain 1566±46 kPa; Young's modulus at 10% strain was 5.9±3.9 MPa and at 20% strain 13.6±6.2 MPa. In the LASIK+CXL group B, stress at 10% strain was 182±21 kPa and at 20% strain 1534±134 kPa; Young's modulus at 10% strain was 6.5±3.2 MPa and at 20% strain 11.9±4.1 MPa. The relative changes of these biomechanical properties in LASIK+CXL group B in comparison to control group A were not statistically significant (table 2).

Enzymatic digestion results

Regarding the stroma specimens, the mean time to complete dissolution in control group A was 157±15 min, while in group B (LASIK+CXL) it was 186.2±7.5 min. The relative difference

Table 2 Biomechanical comparative measurements between the two groups

	Stress	Units: kPa			Young's shear modulus	Units: MPa		
	@ 10% strain		@ 20% strain		@ 10% strain		@ 20% strain	
Group A (control)	158.3	±16.8	1566.6	±45.9	5.9	±3.9	13.6	±6.2
Group B (CXL)	182.8	±20.8	1534.5	±134.8	6.5	±3.2	11.9	±4.1
Δ	15%		−2%		11%		−12%	
p	0.084		0.140		0.095		0.089	
t	−2.07		+1.7		−1.98		+2.02	

Δ, relative (%) difference between metrics; p, Student t test p value; t, Student t test t value. Results from flap specimens.

Δ was +18% for group B, a statistically significant difference (p=0.014). Regarding the flaps, the mean time to complete dissolution in group A was 68.75±17.3 min, while in group B it was 80.0±8.1 min. The relative difference Δ was +16% for group B, a difference which, however, was not statistically significant (table 3).

DISCUSSION

Epithelium-on, in-situ CXL is reported in the peer review literature[16 17] with a significantly weaker biomechanical effect in comparison to epi-off CXL.[18] This is partially attributed to reduced riboflavin permeability through the intact epithelium, mainly due to its large molecular weight.[19] This leads to insufficient and inhomogeneous riboflavin stromal diffusion and thus affects UV-A transmission to deeper layers, due to increased UV-A absorption by the superficially concentrated riboflavin.

The second issue that affects epithelium-on CXL relates to UV-A absorption/filtering by the intact epithelium.[20] Some studies suggest absorption by the epithelium of about one-third,[19] leading to less energy reaching the riboflavin-saturated stroma. However, there are studies suggesting that human corneal epithelium and the underlying basement membrane absorb strongly only at wavelengths smaller than 310 nm,[21 22] which compares to the peak 365 nm of the CXL-employed UV-A.

The effect of refractive surgery on corneal biomechanical properties may be evaluated in-vivo by the corneal hysteresis and corneal resistance factor. These indices can be assessed by dynamic tonometry (visualisation of fast deformation of the cornea), employing the Corvis ST (Oculus Optikgeräte GmbH, Wetzlar, Germany) and the Ocular Response Analyser (Reichert, Buffalo, New York, USA). Several studies have evaluated the reduction in corneal biomechanical strength following LASIK,[23–26] and following ReLEx flex and ReLEx smile procedures, suggesting similar corneal biomechanical reduction among these laser refractive procedures.[27 28] However, there is inconclusive evidence in the peer review literature on the specificity of these techniques in the in-vivo evaluation of the effect of corneal cross-linking.[29 30]

The in-situ riboflavin application investigated in this study naturally overcomes the above-mentioned restriction of riboflavin penetration through the intact epithelium, since it is applied directly on the exposed stromal bed. In addition, the ex-vivo nature of this study overcomes the potential shortcomings presented in relation to in-vivo evaluation techniques.

Our team has investigated clinically in-situ (ie, intrastromal) CXL applications as a means to offer proactive cornea stabilisation in high-myopic LASIK.[31] The long-term investigation of epithelial thickness increase was investigated as an indirect measurement of overall corneal stability, supported by a difference in epithelial remodelling, which may indicate increased biomechanical stability in myopic LASIK combined with adjuvant CXL.

In this study we conducted a human ex-vivo investigation of the biomechanical corneal strengthening occurring during in-situ CXL along myopic LASIK. We evaluated changes in the stroma (and also, for the first time reported separately, the LASIK flap) employing biaxial stress–strain measurements, which may be considered superior to the one-dimensional corneal strip extensiometry[32] taking into account the non-uniform topographic distribution of the corneal strength profile.[33 34] We provided 10% and 20% strain values for two reasons. First, these are the ones typically reported by other researchers,[14] in an effort to provide some form of comparison. In addition, the stress–strain plot had adequate linearity in these areas to enable data collection.

The depth dependence of transverse shear modulus of the cornea, stronger at the anterior third,[35] indicates that tissue removal from this upper third may affect corneal rigidity the most. In our study we demonstrated that the effective CXL corneal rigidity in comparison to the non-CXL corneas that received the same treatment was of the order of +129% stress at 10% strain, by a statistically significant margin. Considering that the aim of the in-situ CXL application is to provide prophylactic corneal strengthening to counter weakening due to tissue removal, we view this very significant biomechanical effect as a very important finding. These findings are confirmatory to our previous clinical effects reported on myopic and hyperopic LASIK+CXL applications.[36] CXL maybe therefore be used as a biomechanical modulator offering refractive stability following LASIK.

There was no indication that the flaps had any statistically significant biomechanical difference by any metric employed. This finding is a very important indicator that no actual cross-linking of the flap occurs during our LASIK+CXL technique. There are two main reasons that we target to avoid any cross-linking effect on the flap. First is that the flap does not contribute to the biomechanical properties of the underlying stroma.[37 38] Therefore, there is no benefit from a potential cross-linking of the flap. Second, and perhaps even more important, is that cross-linking such a thin (the LASIK flap consists of ~ 50 µm of epithelium and ~ 60 µm of stroma) stromal content may lead to undesirable stromal shrinking of the flap.

Additionally, the findings in the collagen-enzymatic digestion part of this work provide corroborative evidence of the differential effects of cross-linking on the stroma and not on the flap. This is, to the best of our knowledge, the first manuscript presenting both techniques, bi-axial stress measurements and enzymatic digestion, of ex-vivo human assessment of cross-linking effects.

Additional studies with larger sample size, differentiation of UV-A irradiance, as well as extending the testing period to longer time intervals following treatment are warranted to validate and further investigate the findings in this preliminary study.

Table 3 Enzymatic digestion time to complete dissolution

	Corneas		Flaps	
	Time	Units: min	Time	Units: min
Group A (control)	157.50	±15.00	68.75	±17.32
Group B (CXL)	186.25	±7.50	80.00	±8.16
Δ	18%		16%	
p	0.014		0.0804	
t	−3.45		−2.1	

Δ, relative (%) difference between metrics; p, Student t test p value; t, Student t test t value.

CONCLUSIONS

Adjuvant intrastromal in-situ CXL combined with myopic LASIK appears in ex vivo human study to be a significant biomechanical modulator.

Contributors Design and conduct of the study (AJK, GA); collection (AJK, GA, BS-C), management (AJK, JBC, JC), analysis (AJK, GA, JBC, BS-C, JC), interpretation of the data (AJK, GA, JBC, BS-C, JC); manuscript preparation (GA), manuscript review (AJK, GA, JBC, BS-C, JC), manuscript approval (AJK, GA, JBC, BS-C, JC).

Competing interests Consultant/advisory positions: AJK: Alcon/WaveLight, Avedro, i-Optics, Allegran; Keramed.

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