

Increased Biomechanical Efficacy of Corneal Cross-linking in Thin Corneas Due to Higher Oxygen Availability

Sabine Kling, PhD; Olivier Richoz, MD, PhD; Arthur Hammer, MD; David Tabibian, MD; Soosan Jacob, MS, FRCS, DNB; Amar Agarwal, MS, FRCS, FRCOphth; Farhad Hafezi, MD, PhD

ABSTRACT

PURPOSE: To compare the currently available ultraviolet-A (UV-A) corneal cross-linking (CXL) treatment protocols for thin corneas with respect to oxygen, UV fluence, and osmotic pressure.

METHODS: Freshly enucleated murine ($n = 16$) and porcine ($n = 16$) eyes were used. The dependency on oxygen and the amount of UV absorption were evaluated using different CXL protocols, including standard CXL, contact lens-assisted CXL (caCXL), and CXL after corneal swelling. The CXL protocol was adapted from the treatment parameters of the human cornea to fit the thickness of murine and porcine corneas. Immediately after CXL, the corneas were subjected to biomechanical testing, including preconditioning, stress relaxation at 0.6 MPa, and stress-strain extensometry. A two-element Prony series was fitted to the relaxation curves for viscoelastic characterization.

RESULTS: Standard CXL was most efficient; prior corneal swelling reduced the long-term modulus by 6% and caCXL by 15% to 20%. Oxygen reduction decreased the long-term modulus G_{∞} by 14% to 15% and the instantaneous modulus G_0 by 2% to 5%, and increased the short-term modulus G_2 by 22% to 31%. Reducing the amount of absorbed UV energy decreased the long-term modulus G_{∞} by 5% to 34%, the instantaneous modulus G_0 by 7% to 29%, and the short-term modulus G_2 by 17% to 20%. The amount of absorbed UV light was more important in porcine than in murine corneas.

CONCLUSIONS: The higher oxygen availability in thin corneas potentially increases the overall efficacy of riboflavin UV-A CXL compared to corneas of standard thickness. Clinical protocols for thin corneas should be revised to implement these findings.

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Corneal cross-linking (CXL) has been successfully translated into clinical ophthalmology. It shows a high success rate and is considered safe for corneas thicker than 400 μm of stroma.¹ However, corneas with a stromal thickness of less than 400 μm still represent a major therapeutic challenge. They are biomechanically weaker than corneas of normal thickness and need reinforcement, but conventional CXL is not safe in thin corneas because a higher amount of UV energy reaches the posterior corneas and may exceed the threshold for endothelial toxicity. Several modified CXL protocols have been proposed to address this problem.

The first was proposed by Hafezi et al.² and was based on swelling the corneal stroma to a thickness of at least 400 μm using a hypo-osmolar riboflavin and then performing the standard irradiation. However, this treatment option showed reduced clinical efficacy.^{3,4} More recently, contact lens-assisted cross-linking (caCXL) was proposed⁵ for thin corneas. The caCXL procedure uses a riboflavin-soaked contact lens to absorb part of the UV light to ensure the safety of the endothelium from UV-mediated damage. However, this protocol might hinder oxygen diffusing into the corneal tissue.

Kamaev et al.⁶ proposed in 2012 that there may be two oxygen-dependent reactions in the corneal stroma during UV-mediated cross-linking: one favorable at higher oxygen concentration and, presumably, more efficient, and one occurring at low oxygen

From the Laboratory for Ocular Cell Biology, University of Geneva, Geneva, Switzerland (SK, OR, AH, DT, FH), the Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland (SK, FH), the ELZA Institute, Zurich, Switzerland (FH); Dr. Agarwal's Eye Hospital and Eye Research Center, Chennai, India (SJ, AA); and the Department of Ophthalmology, University of Southern California, Los Angeles, California (FH).

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Correspondence: Sabine Kling, PhD, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail: kling.sabine@gmail.com

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concentrations. Our group has shown that the absence of oxygen during CXL prevents the biomechanical increase usually observed after the procedure.⁷

According to Fick's law of diffusion, the diffusion rate is dependent on the distance and concentration gradient. Hence, we speculate that the oxygen availability may be higher in thin corneas when compared to thick corneas and that the biomechanical stiffening effect of CXL may be more efficient.

In this study, we investigated the effect of oxygen availability on the biomechanical changes induced by various clinically applied thin cornea CXL protocols, in both thin (murine) and thick (porcine) corneas.

MATERIALS AND METHODS

Different CXL treatment protocols were investigated and verified for high corneal thicknesses (porcine eyes) representing standard corneas with a stroma greater than 400 μm and low corneal thicknesses (murine eyes) representing keratoconic corneas with a stroma less than 400 μm . For this purpose, 16 freshly enucleated porcine eyes were obtained from a local slaughterhouse, stored at 4°C, and processed within less than 6 hours after enucleation. Sixteen eyes of 8 C57BL/6 mice (Animal Facility, University of Geneva) were obtained after termination of preceding experiments immediately after death and investigated within 1 hour after enucleation. Only ex vivo animal tissue was used for the current study.

We tested six conditions: (1) standard CXL was used as a control for the maximum possible stiffening effect; (2+3) caCXL allowed us to analyze the effect of oxygen restriction and light attenuation; (4) CXL after corneal swelling was used to measure the effect of osmotic pressure/fibril spacing; and (5+6) the riboflavin conditions served as a control for the minimum corneal stiffness without any additional CXL.

STANDARD CXL

The so-called "Dresden protocol" is the original CXL protocol established in 1999.⁸ It includes three steps in porcine corneas: corneal deepithelialization, instillation of 0.1% riboflavin–20% dextran for 30 minutes, and UV-A irradiation ($\lambda = 365 \text{ nm}$) for 30 minutes at 3 mW/cm².

The steps and settings in murine eyes were adapted based on data from Hammer et al.⁹: corneal deepithelialization, instillation of 0.1% riboflavin in phosphate buffered saline for 20 minutes, and UV-A irradiation ($\lambda = 365 \text{ nm}$) for 1 minute at 3 mW/cm².

CACXL

In caCXL, a UV-A-transparent contact lens (SofLens daily disposable, -0.5 diopters (D); Bausch & Lomb,

Zug, Switzerland) presoaked with riboflavin is placed on the cornea to absorb a significant amount of the UV light and hence protect the endothelium in corneas thinner than 400 μm .

In porcine eyes, corneas were deepithelialized and 0.1% riboflavin–20% dextran solution was applied for 30 minutes. The contact lens was soaked separately during the same time. Then the contact lens was placed onto the cornea and UV-A irradiation ($\lambda = 365 \text{ nm}$) for 30 minutes at 3 mW/cm² was initiated.

In murine eyes, corneas were deepithelialized and 0.1% riboflavin in phosphate buffered saline was applied for 20 minutes. The contact lens was soaked separately during the same time before its size was reduced to 5-mm diameter to fit the murine cornea. Then the contact lens was placed onto the cornea and irradiated with UV-A ($\lambda = 365 \text{ nm}$) for 1 minute at 3 mW/cm².

CXL AFTER CORNEAL SWELLING

We performed this procedure in murine corneas. Corneas were deepithelialized and swelling was induced by a hypo-osmolar 0.1% riboflavin in distilled water solution. UV irradiation was then performed according to the standard CXL protocol.

CONTROL CONDITIONS

Two control conditions were analyzed: caCXL control (caCXLc) and riboflavin-only. In caCXLc, only the cornea (but not the contact lens) was soaked in riboflavin to study whether the biomechanical effect in caCXL is limited by oxygen diffusion. Riboflavin-only control involved deepithelialization and riboflavin application for 30 minutes to study the effect of corneal swelling due to riboflavin solvents, such as phosphate buffered saline and distilled water.

BIOMECHANICAL MEASUREMENTS

The biomechanical properties were determined immediately after treatment. Two testing approaches were applied to extract elastic and viscoelastic parameters. For better comparability we applied the same stress in thin and thick corneas, which implies a higher force in thicker corneas. Due to better handling, porcine corneas were tested with standard one-dimensional extensometry, whereas murine corneas were tested in a customized holder for bi-dimensional extensometry.

Corneas were excised immediately after treatment, leaving a small scleral ring, and prepared for mechanical testing using a stress-strain extensometer (ZwickLine, max 50 N; Zwick Roell, Ulm, Germany). Porcine corneas were aligned and two central flaps of 5-mm width were cut in superior-inferior direction. A flap of 4 mm effective length was then mounted within

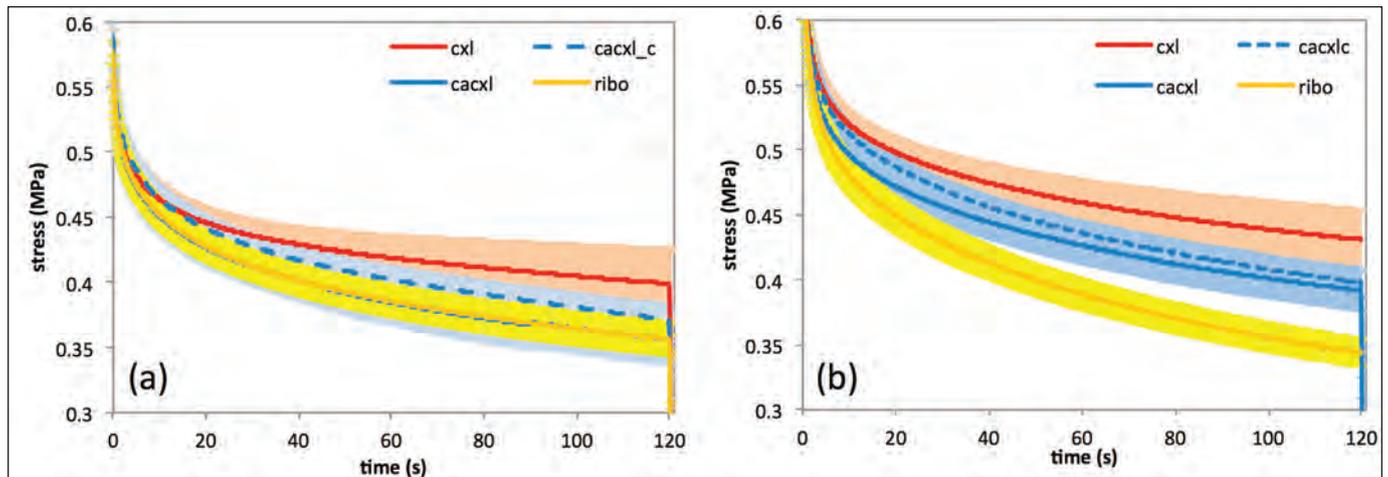


Figure 1. Stress relaxation curves with standard deviation for (A) thick (porcine) and (B) thin (murine) corneas. cxl = standard corneal cross-linking treatment; cacxl = contact lens-assisted CXL treatment; cacxl_c = oxygen-limited CXL treatment; ribo = riboflavin control

the brackets of the extensometer and subjected to one-dimensional mechanical testing between 0 and 2 Newton (N). Murine corneas were fixed in a customized holder and the entire cornea was subjected to two-dimensional mechanical testing between 0 and 0.4 N. The mechanical testing procedure in both species included three steps: (1) preconditioning during three cycles, (2) stress relaxation testing during 120 seconds, and (3) stress-strain curve until break.

Values of the applied force F and corresponding travel Δ (ie, vertical extension) were exported from the extensometer software and converted into stress σ and strain ϵ .

Specific equations used for transformation are listed in **Table A** (available in the online version of this article).

RESULTS

RESTRICTION OF OXYGEN AND ABSORBED UV ENERGY

Separating the effect of oxygen and UV energy is important to better understand by which parameter the CXL treatment is currently limited and how its efficacy might be improved.

STRESS RELAXATION

Figure 1 shows the stress relaxation to a force of 2 N in porcine corneas and 0.40 N in murine corneas, each of which corresponds to an initial stress of approximately 0.60 MPa. As soon as this stress is applied, the strain remains constant and the change in stress is recorded. The higher the remaining stress after a period of 120 seconds, the higher the stress resistance. Riboflavin-treated control corneas presented a significantly lower stress (thick cornea [porcine]: 356 MPa; thin cornea [murine]: 390 MPa) than standard cross-linked corneas (thick cornea: 399 MPa; thin cornea: 431 MPa). The caCXLC corneas (ie, contact lens without soaking

in riboflavin) showed an intermediate stress resistance (thick cornea: 369 MPa; thin cornea: 398 MPa). The caCXL condition (ie, contact lens with prior soaking in riboflavin) showed different results in thick and thin corneas: although caCXL did not show any biomechanical effect (355 MPa) in thick porcine corneas, caCXL in thin murine corneas was similarly effective (392 MPa) as in oxygen-limited cross-linking (caCXLC).

STRESS-STRAIN RELATION

Stress-strain testing revealed significant differences between conditions for thick (porcine) corneas, but not for thin (murine) corneas as shown in **Figure A** (available in the online version of this article). In the riboflavin control condition, thick corneas had a higher Young's modulus than thin corneas and broke at a relatively higher stress.

In thick corneas, the stress-strain relation confirmed the findings from the stress relaxation test. The strongest stiffening effect was obtained with the standard CXL procedure, followed by caCXLC and least with caCXL. **Table 1** provides the corresponding Young's moduli. Although statistical significance was not reached ($P = .098$), we observed the trend that the higher the increase in corneal stiffness, the larger the difference between initial and final Young's modulus, indicating that CXL may predominantly change viscoelastic properties. **Table B** (available in the online version of this article) shows the statistical comparison between Young's moduli at different strains for the different conditions. Although all CXL conditions showed a significant increase compared to riboflavin, no differences were observed between caCXL and caCXLC. The difference between CXL and caCXLC only became significant in the final stress-strain test.

TABLE 1
**Young's Modulus at Different Strains, Obtained From Stress-Strain Testing:
 Comparison Between CXL Protocols in Porcine Corneas**

Porcine Strain (%)	RIBO		CXL		caCXLc		caCXL	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
2.50	4.57	4.54	6.89	8.51	6.06	6.20	5.62	5.60
5.00	6.87	6.90	10.4	14.0	8.92	9.99	8.63	8.70
7.50	–	9.69	–	18.7	–	14.1	–	12.4
10.0	–	12.9	–	22.8	–	18.8	–	16.0

CXL = corneal cross-linking; RIBO = riboflavin; caCXLc = contact lens-assisted CXL control; caCXL = contact lens-assisted CXL

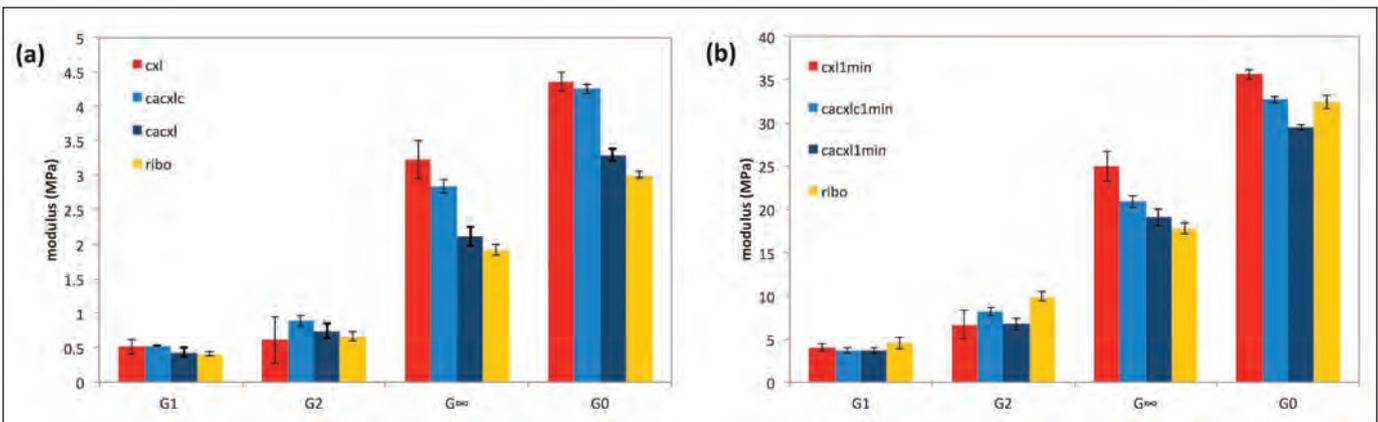


Figure 2. Viscoelastic parameters with standard deviation obtained from fitting to a two-element Prony series: (A) porcine corneas and (B) murine corneas. cxi = standard corneal cross-linking treatment; cacxl = contact lens-assisted CXL treatment; cacxl_c = oxygen-limited CXL treatment; ribo = riboflavin control

VISCOELASTIC PARAMETER FIT

The different moduli obtained from Prony series fitting are shown in **Figure 2**. In both species (ie, in thin and thick corneas), the long-term modulus G_{∞} and the instantaneous modulus G_0 showed a significant ($P \leq .001$) change after CXL. Furthermore, the short-term modulus G_2 showed a significant ($P = .003$) change in thin corneas. Interestingly, the short-term modulus G_2 decreased, whereas the instantaneous modulus G_0 and long-term modulus G_{∞} increased.

By comparing the reduced-oxygen (caCXLc) with the normal-oxygen (CXL) condition, we found that oxygen reduction decreased the long-term modulus G_{∞} by 14% to 15% and the instantaneous modulus G_0 by 2% to 5% and increased the short-term modulus G_2 by 22% to 31%.

By comparing the full-UV (caCXLc) with the reduced-UV (caCXL) condition, we found that reducing the amount of absorbed UV-energy decreased the long-term modulus G_{∞} by 5% to 34%, the instantaneous modulus G_0 by 7% to 29%, and the short-term modulus G_2 by 17% to 20%. Therefore, the amount of absorbed UV light was much more important in thick (porcine) than in thin (murine) corneas.

CORNEAL SWELLING BY HYPO-OSMOLAR SOLUTION

Understanding the effect of osmotic pressure on the efficacy of CXL helps improve our current understanding of where the additional cross-links are formed.

STRESS RELAXATION

Figure 3A shows the stress-relaxation curves for normal (phosphate buffered saline) and swollen (water) CXL corneas and their corresponding controls. A higher water content of the corneal tissue (green vs yellow line) significantly ($P = .002$) increased stress resistance of the control condition by 46 kPa (13%). CXL corneas showed the same trend, but with minor magnitude (16 kPa, 3.3%), which did not reach statistical significance ($P = .45$).

VISCOELASTIC PARAMETER FIT

The parameters obtained from fitting are presented in **Figure 3B**. Riboflavin in water controls showed a decrease in the short-term modulus G_2 (-12%, $P = .003$) and an increase in the long-term modulus G_{∞} (15%, $P = .004$) compared to the riboflavin in phosphate buffered saline, showing that increased tissue hydration increases the long-term stiffness. Comparing the two CXL condi-

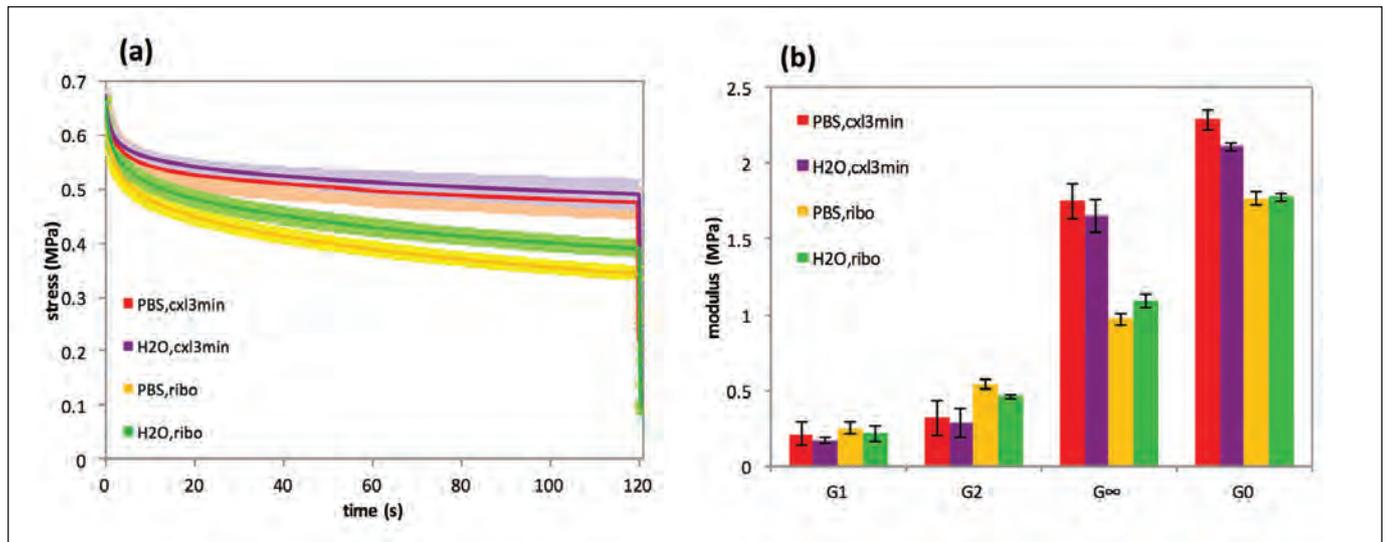


Figure 3. (A) Stress relaxation curves with standard deviation and (B) viscoelastic parameters with standard deviation for normal and swollen murine corneas, before and after standard corneal cross-linking. cxl = standard corneal cross-linking treatment; cacxl = contact lens-assisted CXL treatment; cacxl_c = oxygen-limited CXL treatment; ribo = riboflavin control

tions showed that the instantaneous modulus G_0 was -8.4% lower in swollen than in normal corneas, indicating less CXL efficacy in the latter.

DISCUSSION

By comparing porcine and murine corneas, we found that in both thick and thin corneas the formation of cross-links was equally limited by oxygen. However, differences were observed in the dependency on the UV energy absorbed: thick corneas were more strongly affected by reduced UV energy than thin corneas, indicating that CXL may be more efficient in thin corneas.

The viscoelastic model allowed us to separate the contribution of extracellular matrix and collagen fibrils to the overall corneal stiffness: short-term moduli G_1 and G_2 describe the amount of stress relaxation (ie, the lower the moduli, the less material extension is observed after applying a certain stress). Rigid materials typically have a small viscoelastic component and hence low short-term moduli G_1 and G_2 . Also, viscoelastic properties are mainly related to the extracellular matrix, rather than to the collagen fibrils. In our measurements, we found that UV restriction decreased the short-term modulus G_2 , whereas oxygen restriction did not. These results suggest that oxygen may be required for proteoglycan cross-linking.

The different effect of caCXL in porcine (thick) and murine (thin) corneas is probably related to a higher oxygen presence in the murine cornea. Because the murine cornea is much thinner than the porcine cornea (factor 7), the oxygen diffusion and hence oxygen replenishment during UV irradiation is faster. The higher efficacy of CXL in murine corneas therefore

confirms that the formation of new cross-links is more efficient at higher oxygen concentrations.⁷

Inter-species differences have been reported in the effectiveness of CXL before^{11,12}: human corneas show a larger increase in Young's modulus ($\times 4.5$) than porcine corneas ($\times 1.8$), but a lower increase than rabbit corneas ($\times 5.6$). Because the same (standard) CXL protocol was performed in all three species tested, the difference was ascribed to the UV absorption profile along the cornea that resulted in a different cross-linked depth and hence in a different ratio between cross-linked and remaining corneal tissue. In the current study, however, we adapted the CXL protocol to the thickness of the corneas and hence we had the same UV absorption profile in porcine and murine corneas. Because we still observed a higher CXL efficacy in thin corneas, we suggest that these differences must be due to availability of oxygen. This is further supported by our finding that murine corneas are more sensitive to oxygen than to UV restriction when compared to porcine corneas.

A limitation of this study might be that two different biomechanical testing approaches (one-dimensional¹¹ and bi-dimensional⁹) have been applied to porcine and murine corneas, respectively. This adaptation was necessary because, due to their small size, murine corneas could not be measured with a technique requiring corneal flaps. Generally, bi-dimensional testing provides a slightly lower stress resistance. However, because the purpose of this study was to compare not two species, but rather different conditions within the same species, the different testing approaches should not affect the conclusions taken.

It has been previously suggested that cross-links are

likely to be induced in the protein network surrounding the collagen fibrils, or on the collagen surface itself.¹³ Because stress-strain tests measure the amount of cross-links between collagen lamella (and less within collagen fibers or the extracellular matrix), the effect of CXL is less prominent in stress-strain tests compared to stress relaxation tests.¹⁴ In the current study, the stress-strain diagram showed a stiffening effect in thick (porcine) corneas after CXL, but not in thin (murine) corneas. Murine corneas are thinner and have less lamella than porcine corneas. In consequence, the accumulative (side) effect on the lamellar extensibility after CXL is less prominent.

Although differences in CXL efficiency were found between the various protocols tested, we cannot draw a conclusion as to which is the minimal necessary increase in corneal stiffness to prevent keratoconus progression. According to clinical studies,^{2,5,15} several treatment variations seem to arrest keratoconus clinically, although no data have been published on the stability of the CXL effect for non-standard conditions.

Certainly there is a limit of UV fluence below which the number of generated cross-links will decrease. In this study, the amount of UV light absorbed by the contact lens (central thickness: 90 μm), and thus not contributing to corneal stiffness, was 18% (0.86 J/cm²).

The oxygen transmissibility

$$Dk = 22 \frac{\text{cm}^2 \cdot \text{ml}(\text{O}_2)}{\text{s} \cdot \text{ml} \cdot \text{mmHg}}$$

of the Bausch & Lomb SofLens Daily is similar to the oxygen permeability of the corneal stroma

$$Dk = 25.6 \frac{\text{cm}^2 \cdot \text{ml}(\text{O}_2)}{\text{s} \cdot \text{ml} \cdot \text{mmHg}} .^{16}$$

Thus, regarding oxygen permeability, this contact lens should act similarly to the corneal stroma. The reduction of available oxygen may be estimated with the equivalent oxygen percentage, which is approximately 9.9%¹⁷ for the contact lens used in this study. Compared to the oxygen content in air (approximately 20%), the caCXL protocol reduces the available oxygen by almost 50%. The contact lens in this case reduced oxygen to a higher extent than the UV energy absorbed by the corneal tissue. Because endothelial toxicity is supposed to depend mainly on the amount of absorbed UV energy,¹ the treatment efficacy of caCXL might be improved by using a contact lens with a higher oxygen permeability.

The caCXL procedure in the current state decreased the long-term modulus after CXL by 15% to 20%, whereas osmotic changes (ie, swelling of the cornea) before CXL reduced the long-term modulus by 6%,

both compared to the standard CXL procedure. This may be taken into account when performing caCXL in a clinical setting.

We provide evidence that thin corneas have a higher oxygen availability than corneas of standard thickness. Based on our findings, we propose two alternative clinical treatment modalities for thin corneas that do not decrease the effect of CXL: to decrease the duration of the UV irradiation according to the Lambert–Beer law for the given corneal thickness and/or to increase UV absorption in the anterior cornea by using a higher riboflavin concentration. This is similar to the approach that has been used to successfully transfer CXL from humans to mice.⁹

A better understanding of the working principles of CXL will be key for improving its current efficacy and treatment duration, for adapting the treatment to abnormal corneal thicknesses, and for stepping forward in the development of customized stiffness and curvature changes along the cornea, which might allow for refractive corrections without corneal ablation in the future.

AUTHOR CONTRIBUTIONS

Study concept and design (SK, OR, AH, DT, FH); data collection (SK); analysis and interpretation of data (SK, SJ, AA, FH); writing the manuscript (SK); critical revision of the manuscript (OR, AH, DT, SJ, AA, FH); administrative, technical, or material support (FH); supervision (FH)

REFERENCES

1. Spoerl E, Mrochen M, Sliney D, Trokel S, Seiler T. Safety of UVA-riboflavin cross-linking of the cornea. *Cornea*. 2007;26:385-389.
2. Hafezi F, Mrochen M, Iseli HP, Seiler T. Collagen crosslinking with ultraviolet-A and hypoosmolar riboflavin solution in thin corneas. *J Cataract Refract Surg*. 2009;35:621-624.
3. Hafezi F. Limitation of collagen cross-linking with hypoosmolar riboflavin solution: failure in an extremely thin cornea. *Cornea*. 2011;30:917-919.
4. Hatami-Marbini H, Rahimi A. Interrelation of hydration, collagen cross-linking treatment, and biomechanical properties of the cornea. *Curr Eye Res*. 2015;19:1-7.
5. Jacob S, Kumar DA, Agarwal A, Basu S, Sinha P. Contact lens-assisted collagen cross-linking (CACXL): a new technique for cross-linking thin corneas. *J Refract Surg*. 2014;30:366-372.
6. Kamaev P, Friedman MD, Sherr E, Muller D. Photochemical kinetics of corneal cross-linking with riboflavin. *Invest Ophthalmol Vis Sci*. 2012;53:2360-2367.
7. Richo O, Hammer A, Tabibian D, Gatzoufas Z, Hafezi F. The biomechanical effect of corneal collagen cross-linking (CXL) with riboflavin and UV-A is oxygen dependent. *Transl Vis Sci Technol*. 2013;2:6.
8. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol*. 2003;135:620-627.
9. Hammer AK, Boldi S, Richo O, Tabibian D, Randleman JB, Hafezi F. Establishing corneal cross-linking with riboflavin and

- UV-A in the mouse cornea in vivo: biomechanical analysis. *Invest Ophthalmol Vis Sci.* 2015;56:6581-6590.
10. Wollensak G, Iomdina E, Dittert D-D, Salamatina O, Stoltzenburg G. Cross-linking of scleral collagen in the rabbit using riboflavin and UVA. *Acta Ophthalmol Scand.* 2005;83:477-482.
 11. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg.* 2003;29:1780-1785.
 12. Hayes S, Kamma-Lorger CS, Boote C, et al. The effect of riboflavin/UVA collagen cross-linking therapy on the structure and hydrodynamic behaviour of the ungulate and rabbit corneal stroma. *PLoS One.* 2013;8:e52860.
 13. Richoz O, Kling S, Zandi S, Hammer A, Spoerl E, Hafezi F. A constant-force technique to measure corneal biomechanical changes after collagen cross-linking. *PLoS One.* 2014;9:e105095.
 14. Mazzotta C, Traversi C, Paradiso AL, Latronico ME, Rechichi M. Pulsed light accelerated crosslinking versus continuous light accelerated crosslinking: one-year results. *J Ophthalmol.* 2014;2014:604731.
 15. Harvitt DM, Bonanno JA. Re-evaluation of the oxygen diffusion model for predicting minimum contact lens Dk/t values needed to avoid corneal anoxia. *Optom Vis Sci.* 1999;76:712-719.
 16. Holden BA, Mertz GW. Critical oxygen levels to avoid corneal edema for daily and extended wear contact lenses. *Invest Ophthalmol Vis Sci.* 1984;25:1161-1167.

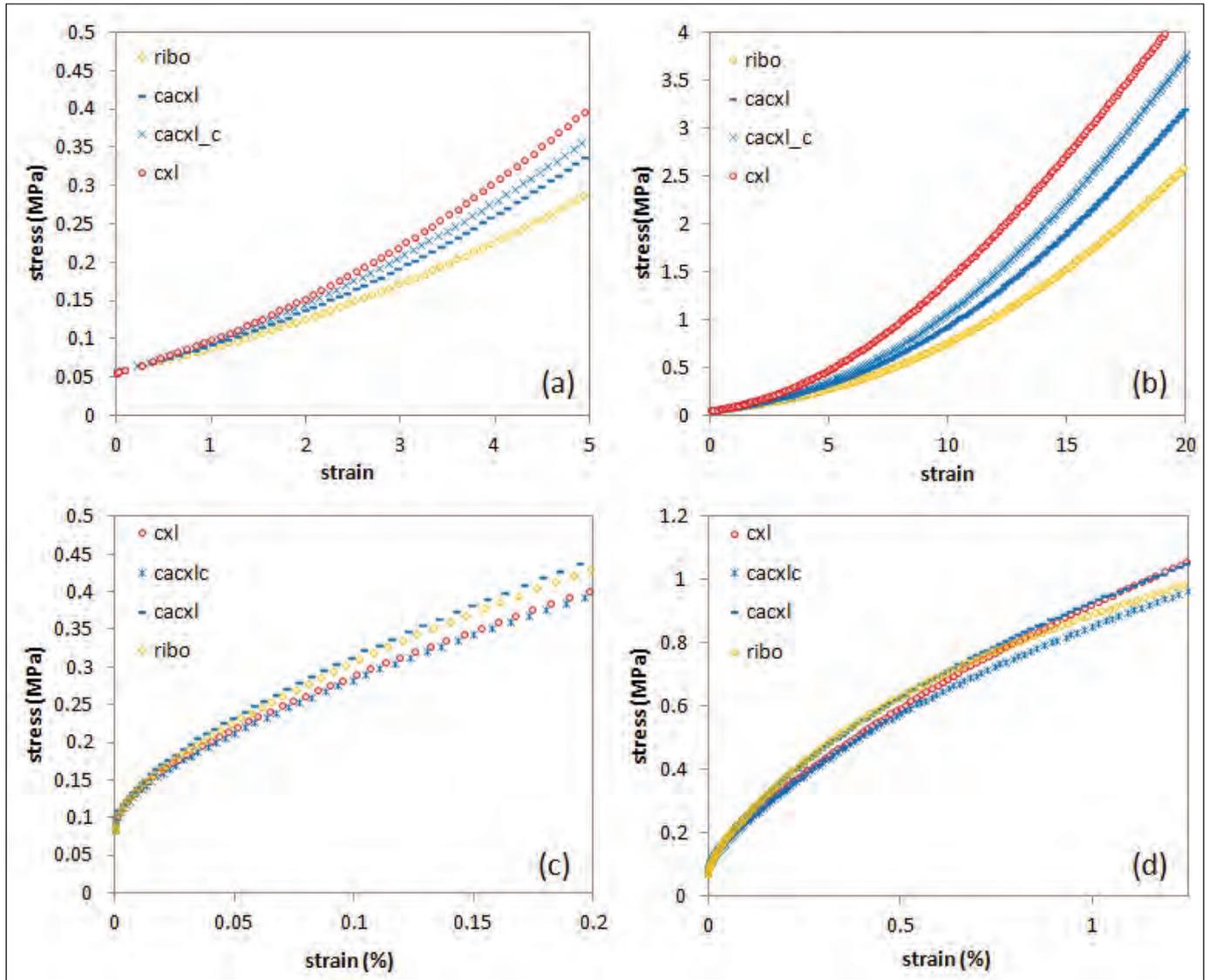


Figure A. Initial stress-strain graphs: (A) thick (porcine) and (C) thin (murine) corneas. Complete stress-strain graphs: (B) thick (porcine) and (D) thin (murine) corneas. Graphs represent average values of individual measurement points. cxi = standard corneal cross-linking treatment; cacxl = contact lens-assisted CXL treatment; cacxl_c = oxygen-limited CXL treatment; ribo = riboflavin control

TABLE A

For porcine corneas the equations for transformation were:

$$\sigma = \frac{F}{W \cdot th} \quad (\text{eq. 1a})$$

$$\varepsilon = \frac{\Delta}{\Delta - L_0} \quad (\text{eq. 1b})$$

where W is the width, th the thickness, and L_0 the initial length of the tested corneal flap (ie, 4 mm).

For murine corneas the equations for transformation were:

$$\sigma = \frac{F}{2 \cdot \pi \cdot R \cdot th} \quad (\text{eq. 2a})$$

$$\varepsilon = \frac{\Delta^2 + R^2}{2\Delta R} \cdot \sin^{-1} \left(\frac{2\Delta R}{\Delta^2 + R^2} \right) - 1 \quad (\text{eq. 2b})$$

where R is the half diameter of the biomechanically tested area and th is mean corneal thickness.

VISCOELASTIC PARAMETERS RETRIEVAL

Relaxation curves were fitted to a Prony series of two spring-dashpot elements using custom programs written in Matlab (MathWorks, Release 2013b; Matlab, Bern, Switzerland).

$$G(t) = G_\infty + \sum_{i=1}^2 G_i \cdot e^{-\frac{t}{\tau_i}} \quad (\text{eq. 3a})$$

where G_∞ is the long-term shear elastic modulus (at complete relaxation), G_i are the short-term shear elastic moduli, and τ_i are the corresponding relaxation times. With

$$\alpha_i = \frac{G_i}{G_0} = \frac{G_i}{G_\infty + \sum_{i=1}^N G_i} \quad (\text{eq. 3b})$$

and

$$G(t) = \frac{\sigma(t)}{\varepsilon_{const}} \quad (\text{eq. 3c})$$

where G_0 is the instantaneous shear elastic modulus, $\sigma(t)$ is the measured stress relaxation curve, and ε_{const} is the strain, which was kept constant during relaxation.

ELASTIC PARAMETERS RETRIEVAL

The slope of the stress-strain curves corresponds to the elastic modulus E (Young's modulus) and was determined at 2.5%, 5%, 7.5%, and 10% of strain. The conversion from elastic modulus to shear modulus is given by the following equation:

$$G = \frac{E}{2(1 + \nu)} \quad (\text{eq. 4})$$

where ν is the Poisson's ratio representing the compressibility of a material, which is typically close to 0.5 for the corneal tissue.

TABLE B
**Statistical Comparison of Stress-Strain Tests
in Porcine Corneas With Different Treatment Conditions**

Strain (%)	RIBO-CXL	RIBO-caCXLc	RIBO-caCXL	caCXL-CXL	caCXL-caCXLc	CXL-caCXLc
Initial						
2.5	< .001	0.014	0.068	0.014	0.280	0.223
5.0	< .001	0.014	0.035	0.024	0.427	0.170
Final						
2.5	< .001	0.027	0.047	0.001	0.457	0.018
5.0	< .001	0.015	0.036	0.001	0.341	0.028
7.5	< .001	0.008	0.033	0.001	0.260	0.039
10.0	< .001	0.004	0.035	0.002	0.198	0.056

RIBO = riboflavin; CXL = corneal cross-linking; caCXLc = contact lens-assisted CXL control; caCXL = contact lens-assisted CXL