Repeated Cross-linking After a Short Time Does Not Provide Any Additional Biomechanical Stiffness in the Mouse Cornea In Vivo

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ABSTRACT

PURPOSE: To study whether repeated collagen cross-linking (CXL) performed in vivo in mice shows an additive effect on mechanical corneal stiffness.

METHODS: In this experimental study, epithelium-off CXL was performed in a total of 18 eyes from male C57BL/6 mice, with 0.27%-riboflavin solution applied for 20 minutes, followed by ultraviolet-A (UVA) irradiation (365 nm, 9mW/cm^2) for 2:50 minutes (fluence 1.53 J/cm^2). CXL was performed as either a single (1×CXL) or a repeated (2×CXL) treatment. Un-irradiated corneas served as controls. In the 2×CXL group, the procedure was performed on day 1 and day 4 to ensure complete reepithelialization between sessions. Biomechanical analysis was performed on day 7. Corneas were harvested with a small scleral ring and mounted on a customized two-dimensional flap holder. The biomechanical measurement consisted of three parts: (1) pre-conditioned during three cycles from 0.04 to 0.4 N, (2) stress relaxation during 120 seconds following 0.4 N force application, and (3) stress-strain curve until break.

RESULTS: After the relaxation period of 120 seconds, highly significant differences ($P < .001$) were found between the controls and both 1×CXL corneas and 2×CXL corneas. No significant difference ($P = .70$) was detected between the 1×CXL and 2×CXL groups. The stress remaining after relaxation was 355 ± 25.2 kPa in the control group, 457 ± 34.1 kPa in the 1×CXL group, and 463 ± 22.2 kPa in the 2×CXL group. No significant differences in the stress-strain curves were found between the conditions.

CONCLUSIONS: Repeated CXL 3 days after the first procedure does not further increase corneal stiffness in mice in vivo.

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Corneal cross-linking (CXL) with riboflavin and ultraviolet-A (UVA) is a treatment modality that halts the progression of keratoconus with good long-term results.[1-5] It is also effective in arresting the progression of postoperative ectasia after LASIK and photorefractive keratectomy.[6,7] Currently, photoactivated riboflavin for CXL is under investigation for its effect on infectious keratitis.[8,9]

Typically, CXL for keratoconus and postoperative ectasia shows a high success rate in stabilizing the cornea biomechanically.[1,10] However, in certain cases the ectasia continues to progress even after the CXL procedure. The question arises whether a second CXL procedure may further help in stabilizing the cornea.

Our group recently reported the clinical case of a patient with progressive keratoconus who received a first CXL procedure, but whose cornea showed progression again and only stabilized after a second CXL procedure 4 years after the first treatment.[11] Nevertheless, an experimental study that addressed biomechanical changes after repeated CXL sessions (control, single, double, and triple treatment groups) in post-mortem human corneas did not find any additional stiffening after the second and third CXL procedure.[12] It is unclear whether ex vivo corneal tissue loses its capacity to be cross-linked after a certain post-mortem time.

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TABLE 1
Treatment Protocol, With Timing of the Procedures

Group	Mouse	OS	OD	Day 1 OS/OD	Day 4 OS/OD
1×CXL	1	ribo	1×CXL	ribo/ribo	ribo/CXL
	2	ribo	1×CXL	ribo/ribo	ribo/CXL
	3	ribo	1×CXL	ribo/ribo	ribo/CXL
2×CXL	4	1×CXL	2×CXL	ribo/CXL	CXL/CXL
	5	1×CXL	2×CXL	ribo/CXL	CXL/CXL
	6	1×CXL	2×CXL	ribo/CXL	CXL/CXL
Control	7	ribo	2×CXL	ribo/CXL	ribo/CXL
	8	ribo	2×CXL	ribo/CXL	ribo/CXL
	9	ribo	2×CXL	ribo/CXL	ribo/CXL

OS = left eye; OD = right eye; 1×CXL = single corneal cross-linking treatment; ribo = riboflavin; 2×CXL = repeated corneal cross-linking treatment; control = unirradiated control (deepithelialization and riboflavin administration)

In this study, we aimed to learn more about the dynamic processes related to CXL and repeated treatments. For this purpose, we chose an in vivo mouse model and an observation time that allowed for complete corneal reepithelialization between CXL sessions.

MATERIALS AND METHODS

The CXL procedures were timed to ensure maximal reproducibility. To ensure complete reepithelialization, the interval between the first and the second treatment session was set to 3 days. The experimental set-up is illustrated in **Table 1**. The biomechanical stiffening effect was measured using static and dynamic stress-strain tests.

All procedures concerning animals in this study were conducted after approval by the local ethical committee and in adherence to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Animals

Four-week-old male C57BL/6 wild-type mice (n = 9) were used in the experiments. Corneas (n = 18) were equally divided into three groups: one-time cross-linking (1×CXL group), repeated cross-linking (2×CXL group), and un-irradiated controls (control group).

For each CXL treatment, the mice were anesthetized with an intra-peritoneal dose of ketamine (100 mg/kg; Ketalar Pfizer AG, Zurich, Switzerland) and xylazine (10 mg/kg; Rompun Bayer AG, Zurich, Switzerland). After instillation of local anesthesia drops (Tetracaine SDU Faure 1%; Novartis Pharma AG, Basel, Switzerland) for 20 seconds and 35% ethanol for 180 seconds, the epithelium was removed with a hockey knife. An antibiotic ointment (Floxal; Bausch & Lomb AG, Zug, Switzerland) was applied once after each treatment session on the eye. Mice were monitored for pain between treatments and systemic pain medication was adapted as needed. Complete reepithelialization was assessed under the microscope. For the biomechanical characterization, the mice were killed with an intra-peritoneal dose of pentobarbital (0.5 g/10 mL, 100 µL/animal; Inresa Arzneimittel GmbH, Freiburg, Switzerland).

CXL

We previously established a CXL protocol adapted to mice,[12] where the treatment parameters were modified according to the Lambert-Beer law to account for the 5× thinner corneal thickness. Thereby, the relative UVA absorption (ie, the absorbed UVA energy per corneal cross-section) in the mouse cornea is the same as with the Dresden protocol in the human cornea.[5]

Accordingly, 0.27% riboflavin solution (diluted in phosphate buffered saline) was applied on the deepithelialized corneas for 20 minutes, followed by a UVA irradiation of 9 mW/cm^2 for 170 seconds at 365 nm. This corresponds to a fluence of 1.53 J/cm^2 in the mouse, which should be equivalent to 5.4 J/cm^2 in the human cornea.

Biomechanical Testing

Immediately after death, the corneas were excised with a small scleral rim and mounted on a customized two-dimensional flap holder.[12] The biomechanical analysis consisted of three parts: (1) pre-conditioned during three cycles from 0.04 to 0.4 N, (2) stress relaxation during 120 seconds following 0.4 N force application, and (3) stress-strain curve until break. The stress relaxation curve was then fitted to the Prony series expression:[13]

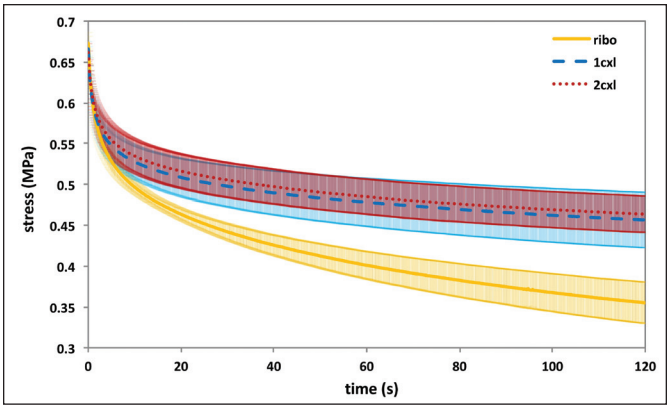

Figure 1. Stress relaxation for single corneal cross-linking treatment (1×CXL), repeated corneal cross-linking treatment (2×CXL), and unirradiated controls (ribo). Highly significant differences ($P < .001$) were found between the controls and 1×CXL and between controls and 2×CXL corneas. No significant differences ($P = .70$) were found between 1×CXL and 2×CXL corneas.

$$E = E_{\infty} + \sum_{i=1}^{N} E_i \cdot e^{-\frac{t}{\tau_i}}$$

where E_{∞} is the long-term elastic modulus (at complete relaxation), E_i are the short-term elastic moduli, and τ_i are the corresponding relaxation times, and

$$E(t) = \frac{\sigma(t)}{\varepsilon_{const}}$$

where σ(t) is the measured stress relaxation curve and ε_{const} the strain, which was kept constant. The slope of the stress-strain curves corresponds to the tangent elastic modulus E (Young's modulus) and was determined at 5% and 10% of strain.

All corneas were measured on the seventh day after starting the experiment. At that time, all corneas had been operated on twice—on the first and fourth days. The 2×CXL group received twice the CXL treatment, the 1×CXL group received the riboflavin instillation on the first day and CXL treatment on the fourth day, and the control group received the riboflavin instillation twice.

RESULTS

Stress Relaxation

After the relaxation period of 120 seconds, we found highly significant differences ($P < .001$) between controls and the 1×CXL group, and between controls and the 2×CXL group, but not between the 1×CXL and 2×CXL groups ($P = .70$) (**Figure 1**). The stress remaining after relaxation was 355 ± 25.2 kPa in controls, 457 ± 34.1 kPa in the 1×CXL group, and 463 ± 22.2 kPa in the 2×CXL group.

Viscoelastic Parameters

The first part of **Table 2** summarizes the results from the numerical fitting procedure. The two time-constants were initially left variable, but were set constant for the final fitting procedure because no significant differences were found between conditions. The retrieved parameters show that both the instantaneous (E_0) and the infinite (E_{∞}) modulus were most affected by CXL. E_0 increased by a factor of 1.6 and E_{∞} by a factor of 2.2 after treatment. Among the viscoelastic components tested, the modulus at 70 seconds decreased (E_2, factor 0.77), whereas the modulus at 6 seconds increased (E_1, factor 1.14).

TABLE 2
Viscoelastic Parameters

Parameter	2×CXL	1×CXL	Ribo	$P_{(ribo\text{-}1\times CXL)}$	$P_{(ribo\text{-}2\times CXL)}$	$P_{(1\times CXL\text{-}2\times CXL)}$
Viscoelastic						
τ_1 (s)	6	6	6	–	–	–
τ_2 (s)	70	70	70	–	–	–
E_1 (kPa)	14.3 ± 1.2	14.8 ± 3.5	20.4 ± 4.7	.044	.012	.71
E_2 (kPa)	19.5 ± 2.73	19.1 ± 5.1	40.2 ± 7.8	< .001	< .001	.86
E_{∞} (kPa)	100 ± 5.2	99.0 ± 8.4	72.7 ± 7.0	< .001	< .001	.74
E_0 (MPa)	1.34	1.33	1.33	–	–	–
Elastic						
$E_{0\_5\%}$ (MPa)	0.862 ± 0.32	0.841 ± 0.19	0.714 ± 0.18	.26	.35	.90
$E_{0\_10\%}$ (MPa)	1.06 ± 0.37	0.916 ± 0.26	0.853 ± 0.17	.72	.60	.46
$E_{\infty\_5\%}$ (MPa)	2.69 ± 0.41	2.38 ± 0.36	2.81 ± 0.38	.76	.61	.19
$E_{\infty\_10\%}$ (MPa)	3.81 ± 0.62	3.29 ± 0.83	3.87 ± 0.36	.91	.84	.24

E_{∞} = infinite modulus; E_i = short-term moduli; τ_i = retardation time constants; E_0 = instantaneous modulus; $E_{0\_5/10\%}$ = tangent elastic moduli during pre-conditioning; $E_{\infty\_5/10\%}$ = tangent elastic moduli after pre-conditioning, both at 5% and 10% of strain

Elastic Parameters

The second part of **Table 2** shows the elastic moduli obtained from the stress-strain extensiometry measurements. On average, corneal stiffness decreased from 0.5% to 1% strain by a factor of 0.81 in the first test (ie, before pre-conditioning), and by a factor of 0.85 in the second test (ie, after stress relaxation). No significant differences in the elastic moduli between conditions were found.

DISCUSSION

We found no significant increase in corneal stiffness with early repeat cross-linking in the mouse cornea. CXL with riboflavin and UVA shows a high success rate of more than 90%.[1,2,10] However, under certain circumstances, treatment failure may occur and/or CXL needs to be repeated. These circumstances include CXL in extremely thin corneas,[13] pregnancy-related changes in estrogen levels and their influence on corneal biomechanics,[14] and CXL in children.[15] The question remains whether the second CXL procedure will provide an additional increase in biomechanical stiffness.

Raiskup et al. reported recurrences of progressive corneal ectasia at 5 and 10 years after the initial treatment in their 10-year follow-up study.[1] Another clinical study[16] showed that repeating CXL (fluence: 5.4 J/cm^2; irradiation: 30 minutes at 3 mW/cm^2) 4 years after the first treatment could again successfully stop the recurrent progression.

In this study, we measured the biomechanical changes after repeated CXL in vivo in mice. Our set-up and study power allowed us to detect a significant biomechanical difference between the groups, if it was higher than 7.6%. Detecting a smaller difference (ie, 1%) would have required a higher power and killing up to 200 mice, which was, in our opinion, not ethically acceptable regarding the aim of this study. We chose this measurement set-up because in a previous study we found that stress relaxation is a more sensitive technique[17] than stress-strain extensometry to measure CXL efficacy, especially in mice.[12] Limitations are that the obtained biomechanical parameters from the two-dimensional testing approach cannot be directly compared to the elastic moduli from one-dimensional testing reported in the literature. We found a large difference in the relaxation behavior between control and 1×CXL corneas. However, 2×CXL did not lead to a significant additional increase in corneal stiffness. These in vivo animal data are in line with the work of Beshtawi et al.[12]

An explanation for the differences between in vivo and ex vivo results could be that the effect of CXL is not only due to immediate biomechanical changes occurring within hours to days after treatment, but also related to secondary mid- to long-term remodeling processes in the cornea. Accordingly, in human eyes, changes are observed even years after the treatment and recurrence of keratoconus can occur up to 10 years after the initial surgery.[1,2] In our experiments, the time between treatments and measurement was limited to 7 days, which does not allow for detection of long-term changes.

Because CXL increases corneal stiffness, the biomechanical properties are typically measured to describe the efficacy of the treatment. A major problem to date is that biomechanical parameters cannot be measured readily in vivo. Most accurate stress-strain tests are performed ex vivo, although even here limitations occur due to changes in tissue hydration and the fact that most testing procedures do not consider the original tissue loading. The state-of-the-art of mechanical characterization is stress-strain extensiometry, which addresses the static material properties.[18] However, we have demonstrated that CXL is also sensitive to the dynamic properties (ie, viscoelastic parameters).[17,19] We were able to confirm these findings in the current study.

Because most biological tissues are viscoelastic, measuring changes in these parameters is extremely interesting, especially because the temporal corneal stiffness is more clinically important than the immediate response to a linearly increasing stress. Stress-strain extensiometry was performed at a speed of 0.5 mm/min (load at 0.5% strain applied within 2.4 seconds and 1% strain within 4.9 seconds). This temporal range comes close to the identified differences between CXL and controls in our viscoelastic testing approach.

Caution should be taken when transferring these conclusions to CXL in human corneas: because the mouse cornea is considerably thinner (factor 5) than a human cornea, its oxygen diffusion is distinctly higher, allowing for a more efficient CXL.[20] Thus, if CXL in humans is actually limited by oxygen diffusion, then a first CXL procedure might not be able to create all potential cross-links between stromal molecules. The same applies to less effective treatment protocols, such as transepithelial CXL. In this case, a second CXL procedure could potentially induce additional stiffening in humans by completing the residual potential cross-links not exploited by the first treatment due to oxygen disposal limitation.[21]

Although the temporal distance between the first and the repeated CXL treatment session was enough to reestablish the physiologic oxygen saturation and to complete the immediate and intermediate CXL and wound healing effects, it was too short to evaluate long-term remodeling effects of the corneal stroma. Therefore, we only could demonstrate that in the short-term,

repeated CXL did not additionally increase the corneal stiffness. Long-term effects may be addressed in future studies by measuring the demarcation line in patients receiving a second CXL treatment years after the first treatment.

A second CXL procedure after a short time did not significantly increase the corneal stiffness in vivo in mice. Although similar results have been reported in human post-mortem corneas,[11] clinical evidence shows that a second CXL procedure performed years after the first CXL procedure may be efficient in stabilizing the cornea in recurrent ectasia. This suggests that in certain patients the biomechanical effect of the first CXL procedure may wear off (eg, due to an increased matrix-metalloproteinase production). In this case, a second CXL procedure can potentially induce additional corneal stiffening and restore the biomechanical resistance that was present immediately after the initial CXL.

AUTHOR CONTRIBUTIONS

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

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