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Research article

Repeated application of riboflavin during corneal cross-linking does not improve the biomechanical stiffening effect ex vivo

Hormoz Abdshahzadeh [a,b,c,1], Reyhaneh Abrishamchi [a,b,c,1], M. Enes Aydemir [b], Nikki Hafezi [b,d], Mark Hillen [b], Emilio A. Torres-Netto [a,b,c], Nan-Ji Lu [b,d,e], Farhad Hafezi [a,b,c,f,g,*]

[a] Laboratory for Ocular Cell Biology, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland
[b] ELZA Institute, Dietikon, Switzerland
[c] Faculty of Medicine, University of Geneva, Geneva, Switzerland
[d] Faculty of Medicine and Health Sciences, University of Antwerp, Wilrijk, Belgium
[e] Department of Ophthalmology, Antwerp University Hospital, Edegem, Belgium
[f] Department of Ophthalmology, Wenzhou Medical University, Wenzhou, Zhejiang, China
[g] USC Roski Eye Institute, USC Los Angeles, Los Angeles, CA, USA

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ABSTRACT

Purpose: To evaluate whether repeated application of riboflavin during corneal cross-linking (CXL) has an impact on the corneal biomechanical strength in *ex-vivo* porcine corneas.
Design: Laboratory investigation.
Methods: Sixty-six porcine corneas with intact epithelium were divided into three groups and analyzed. All corneas were pre-soaked with an iso-osmolar solution of 0.1% riboflavin in a phosphate-buffered saline (PBS) solution ("riboflavin solution"). Then, the corneas in Groups 1 and 2 were irradiated with a standard epi-off CXL (S-CXL) UV-A irradiation protocol (3 mW/cm^2 for 30 min); while the corneas in Group 3 were not irradiated and served as control. During irradiation, Group 1 (CXL-PBS-Ribo) received repeated riboflavin solution application while corneas in Group 2 (CXL-PBS) received only repeated iso-osmolar PBS solution. Immediately after the procedure, 5-mm wide corneal strips were prepared, and elastic modulus was calculated to characterize biomechanical properties.
Results: Significant differences in stress-strain extensiometry were found between two cross-linked groups with control group ($P = 0.005$ and 0.002, respectively). No significant difference was observed in the normalized stiffening effect between Groups 1 and 2 ($P = 0.715$).
Conclusions: The repeated application of riboflavin solution during UV-A irradiation does not affect the corneal biomechanical properties achieved with standard epi-off CXL. Riboflavin application during CXL may be omitted without altering the biomechanical stiffening induced by the procedure.

1. Introduction

Keratoconus (KC) is a corneal ectatic disease, typically progressive in nature, and disproportionally affects children and adolescents. With disease progression, the cornea adopts a "cone-like" shape and visual impairment increases as irregular astigmatism progresses (Gomes et al., 2015). Published KC prevalences range from 1:21 and 1:2000 (Kennedy et al., 1986; Torres Netto et al., 2018).

KC and related corneal ectasias can be treated by corneal cross-linking (CXL) (Randleman et al., 2015). CXL involves saturating the corneal stroma with riboflavin, then exposing the cornea to ultraviolet (UV)-A light. The interaction between UV-A photons and the riboflavin is oxygen-dependent and results in a photochemical reaction that induces the formation of covalent bonds between the molecules of the stroma, which consists mostly of collagen and proteoglycans (Kling et al., 2015; Richoz et al., 2013; Torres-Netto et al., 2018). This biomechanically strengthens the cornea and arrests disease progression (Randleman et al., 2015).

The current standard-of-care for performing CXL in KC patients is called the Dresden protocol (Belin et al., 2018; Hersh et al., 2017; Raiskup-Wolf et al., 2008). The Dresden protocol involves mechanical

* Corresponding author. University of Zurich, Center for Applied Biotechnology and Molecular Medicine, Winterthurerstrasse 190, Zurich, Switzerland.
E-mail address: farhad@hafezi.ch (F. Hafezi).
[1] HA and RA contributed equally as co-first authors.

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Abbreviations

KC	keratoconus
CXL	corneal cross-linking
HPMC	hydroxypropyl methylcellulose
N	Newton
PBS	phosphate-buffered saline
Ribo	riboflavin
S-CXL	Standard corneal cross-linking
UV	ultraviolet

debridement of the corneal epithelium with a hockey knife to help riboflavin to penetrate the corneal stroma. This is achieved by soaking the cornea with 0.1% riboflavin for approximately 20 min. The stroma is then irradiated with 3 mW/cm^2 UV-A intensity (365 nm) for 30 min in a total fluence of 5.4 J/cm^2. The debrided cornea, nonetheless, is at risk of dehydration through evaporation throughout the procedure. To counterbalance this, and to ensure that a sufficient amount of unbleached riboflavin is present in the cornea, surgeons have traditionally applied the same riboflavin solution approximately every 2 min–5 min during the procedure (Hashemi et al., 2013).

On the other hand, the repeated application of riboflavin could, in theory, be disadvantageous for the CXL process. As the CXL reaction proceeds, riboflavin molecules are continuously bleached, starting in the anterior stroma and proceeding to the deeper stroma. The longer the cross-linking reaction lasts, the deeper its effect in the stroma becomes (Scarcelli et al., 2013). Therefore, the concern is that by applying repeated riboflavin during UV irradiation, riboflavin in the superficial stroma is replenished. This additional riboflavin in turn absorbs photons, inhibiting the UV light to reach the unbleached riboflavin in the deep stroma (Wollensak et al., 2003). This might theoretically compromise the biomechanical strengthening effect of the procedure.

Accordingly, there has been a change in consensus to rinse riboflavin off the cornea using iso-osmolar phosphate-buffered saline (PBS) before commencing UV irradiation, and to hydrate the cornea during the procedure with only PBS (Lombardo et al., 2016). However, there is no experimental evidence base to support this and many surgeons still apply riboflavin during UV irradiation. Here, we assess whether applying riboflavin solution during UV-A irradiation would alter the stiffening effect obtained by the CXL procedure.

2. MATERIALS and METHODS

2.1. Specimens and groups

Sixty-six freshly enucleated porcine eyes were obtained from the local slaughterhouse (Zurich, Switzerland) and used within 8 h. Eyes were collected from young adult pigs aged 6–8 months and had not been steamed. All eyes had intact epithelium and were randomly divided into three experimental groups (n = 31 per group; Table 1).

2.2. Experimental protocols

2.2.1. Mechanical abrasion and riboflavin soaking

All corneas were had the central 11 mm region of the corneal epithelium mechanically debrided with a hockey knife. This was followed by soaking corneas for 20 min with iso-osmolar 0.1% riboflavin (Sterol Pharma, Uznach, Switzerland) prepared in a 400 mOsmol/l PBS solution.

2.2.2. CXL procedures

CXL was performed using a UV-A light irradiation at an intensity of 3 mW/cm^2, with a 11 mm spot irradiation diameter, for 30 min (C-Eye device, EMAGine AG, Zug, Switzerland), to deliver a total fluence of 5.4 J/cm^2. During irradiation, Group 1 corneas received iso-osmolar 0.1% PBS-riboflavin solution (CXL-PBS-Ribo) every 2 min, whereas Group 2 corneas received iso-osmolar PBS without riboflavin (CXL-PBS) every 2 min. Group 3 corneas served as control, and were only de-epithelialized and soaked with iso-osmolar 0.1% riboflavin and did not receive any UV-A irradiation.

Table 1
Corneal cross-linking experimental protocols.

Parameter	Group 1	Group 2	Group 3
Total fluence (J/cm^2)	5.4	5.4	0
Soak Time, interval (minutes)	20 (2)	20 (2)	20 (2)
Intensity (mW/cm^2)	3	3	–
Treatment time (minutes)	30	30	–
Epithelium status	Off	Off	Off
Chromophore	0.1% riboflavin (iso-osmolar)	0.1% riboflavin (iso-osmolar)	0.1% riboflavin (iso-osmolar)
Hydration during treatment	Iso-osmolar PBS + riboflavin	Iso-osmolar PBS	–
Light source	C-Eye	C-Eye	–
Irradiation mode	Continuous	Continuous	No irradiation
Protocol abbreviation	CXL-PBS-Ribo	CXL-PBS	Control

CXL, corneal cross-linking; PBS, phosphate-buffered saline; Ribo, riboflavin.

2.3. Biomechanical stress-strain measurements

Stress-strain extensiometry was performed on the corneas, as described previously (Hammer et al., 2014; Kling et al., 2017). In brief, before the measurements, all the corneas were stored in iso 400 mOsmol/l PBS solution for 10 min in order to standardize the hydration of all corneas. Following corneoscleral button removal, two full-thickness corneoscleral strips of 5 mm width were prepared centrally in the vertical axis from each cornea. Four millimeters of the ends of each strip were dedicated to fixation, leaving approximately 11 mm of central corneal strip length to undergo extensiometry.

A stress-strain extensometer (Z0.5; Zwick GmbH & Co., Ulm, Germany) was used to perform tensile strength measurements, calibrated with a distance accuracy of 2 mm and a tensile sensor with ≤0.21% of measurement uncertainty between 0.25 and 50 N. The extensometer has a linear holder extension arm that moves with a controllable speed, and the instrument is able to measure the real-time force in Newton exerted by the arm on the held specimen. The force to stress conversion was calculated from the width and thickness of the specimen. In the conditioning cycles, the arm speed was 2 mm per minute; during the test phase, the position was controlled at the point where load was applied. The biomechanical characterization included elastic testing up to 4 N standard force.

The stress-strain curve was considered for the present analysis, as the stress-strain curve slope corresponds to the tangent elastic modulus and was determined between 5% and 10% of strain. The stiffening effect was calculated with respect to the control group. Data analysis was performed using the Xpert II-Testing Software (Zwick GmbH & Co., Ulm, Germany).

2.4. Sample size calculation and statistical analysis

The sample size was calculated by a software program (Lenth, 2006). Thirty-one samples per group was calculated as being sufficient to detect a 10% change in elasticity between each group with the detection power of 0.95. Statistical analysis was performed in SPSS (version 24; IBM Corporation, Armonk, New York, USA) and R (version 4.0.4, R Foundation for Statistical Computing, Vienna, Austria; https://www.R-pro

ject.org/) software. The Shapiro-Wilk test was conducted to verify the normality of the data. Descriptive statistics were presented as mean ± standard deviation. For continuous variables, analysis of variance (ANOVA) and Kruskal-Wallis H test were conducted to analyze the differences between the three groups, and post-hoc tests were performed with a Bonferroni correction. For all tests, a P value of 0.05 was considered statistically significant.

3. Results

The mean elastic modulus as a function between 5% and 10% of strain was 4.22 ± 1.38 N/mm, 4.33 ± 1.19 N/mm, and 3.39 ± 0.72 N/mm in Groups 1, 2, and 3, respectively. The distributions and comparisons between groups are shown in Fig. 1. There were no significant differences in the elastic modulus between both cross-linked groups (Group 1 *vs.* Group 2, $P = 0.715$), but highly significant differences were found between both two cross-linked groups with the control group ($P =$ 0.005 and 0.002, respectively).

4. Discussion

The objective of this study was to determine whether there was a difference in corneal biomechanical strength, as measured by stress-strain extensiometry, between corneas that received iso-osmolar PBS without riboflavin, and those that received iso-osmolar riboflavin to keep the cornea hydrated during the UV irradiation phase of standard, Dresden protocol epithelium-off CXL. The results presented here found no significant difference in the ultimate biomechanical strengthening effect achieved by either intraoperative corneal hydration approach.

The Dresden protocol was chosen for use in the current study for two reasons: (1) it represents a recognized standard-of-care CXL protocol; (2) the cornea is irradiated for 30 min, so any potential influence of repeated riboflavin application during irradiation would have a greater impact when compared to accelerated CXL protocols with shorter irradiation times.

Different stromal riboflavin concentrations at different stromal depths can potentially have consequences on demarcation line depth and potentially also the clinical efficacy of CXL (Ehmke et al., 2016; Mazzotta et al., 2019). The hypothesis that applying riboflavin during UV irradiation may result in a more superficial stromal cross-linking effect is sound in principle, it appears not to result in a measurable impact on corneal biomechanics. It is worth noting that our experimental setup is sensitive enough to assess post-CXL biomechanical changes even in the mouse cornea (Hammer et al., 2015; Kling et al., 2017, 2021), so we believe that it is safe to assume that our setup would detect even subtle differences between the porcine corneas used in this study. Our results also align with previously published estimates of riboflavin concentration in the corneal stroma, which state that riboflavin concentration is stable for up to 60 min following the initial soaking time (Salmon et al., 2017).

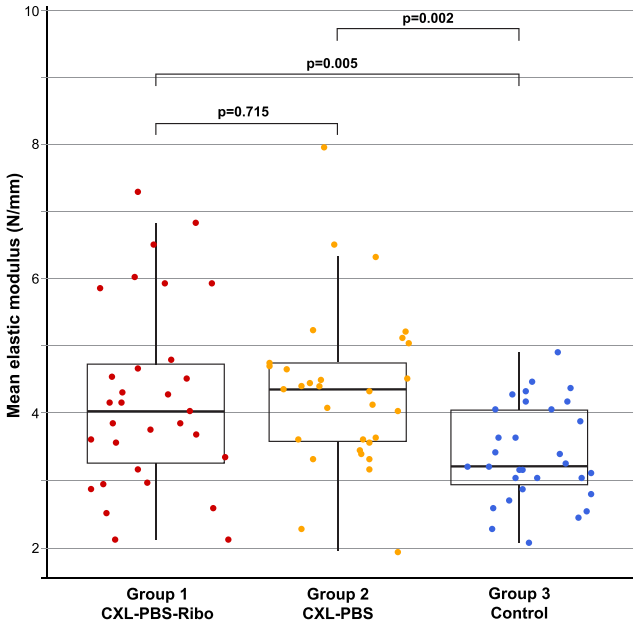

Fig. 1. The distributions and comparisons of patients' mean elastic modulus among three groups. CXL = corneal cross-linking; PBS = phosphate-buffered saline; Ribo = riboflavin.

This study used iso-osmolar riboflavin diluted in PBS. Commercially available riboflavin formulations for clinical use were not examined in this study. These can be divided into three groups. The first is dextran-based riboflavin solutions. These represent the first commercially available riboflavin solutions, but thanks to osmotic effects, thin the cornea, and are no longer used clinically (Ehmke et al., 2016). The second group comprises riboflavin solutions that contain hydroxypropyl methylcellulose (HPMC), that adds viscosity to the solution. HPMC solutions are slightly hypo-osmolar and induce a minor increase in corneal thickness (Ehmke et al., 2016). The third group is composed of slightly hypo-osmolar riboflavin solutions that do not contain dextran or HPMC (Hafezi et al., 2021a; Mazzotta et al., 2018). We believe that our iso-osmolar riboflavin solution represents an appropriate and effective means to model the effect of repetitive riboflavin application during a cross-linking procedure without altering corneal thickness. This study has been performed exclusively using 0.1% iso-osmolar riboflavin without HPMC or dextran.

This study has some limitations. The mechanism by which we perform the extensiometry analyses is well-established and well characterized and involves *ex-vivo* destructive testing of corneal strips (Hammer et al., 2015; Kling et al., 2017). Nevertheless, it is possible that our extensometer findings may not fully equate with the biomechanical response *in-vivo*. Moreover, a potential source of error for studies employing such experimental model system are altered corneal hydration and thickness. To minimize this potential for error, all corneas in our study were stored in iso-osmolar (400 mOsm/l) PBS prior to the experiments and exposed to riboflavin in the same, standardized manner. We did not compare different riboflavin solutions (such as dextran or HPMC-carrier based formulations described above), as we did not believe that the riboflavin carrier would make a significant difference to the results achieved. However, further study evaluating different riboflavin formulations would be required to definitively answer whether the carrier molecule used in riboflavin solutions would make a difference in this context. Furthermore, the method used evaluated the elastic modulus of full-thickness corneal strips, therefore, assumptions about sectorial and depth-dependent differences cannot be made. New technologies such as Brillouin microscopy (Zhang et al., 2019) or quasi-static optical coherence elastography (Kling et al., 2020, 2021; Torres-Netto et al., 2020) may further substantiate our observations.

In conclusion, the biomechanical strengthening effect of CXL was not significantly increased whether PBS-only or PBS-riboflavin were used during a CXL procedure *ex vivo*. Consequently, it is not necessary to apply riboflavin during UV-A irradiation in a CXL procedure: riboflavin application can be discontinued at the end of the riboflavin soaking period. This simplifies the procedure, particularly within the context of newer applications like CXL performed at the slit lamp, where the patient sits upright (Hafezi et al., 2021a, 2021b).

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Commercial relationship disclosure

FH holds a patent on a UV light source (PCT/CH, 2012/000090). NH is CEO of EMAGine AG, a company producing a CXL device. No other authors have no proprietary or commercial interest in any of the materials discussed in this article.

Data availability

Data will be made available on request.

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HA, RA, ET, and FH conceived the study design; HA, RA, MEA and NJL collected the data. All authors analyzed and interpreted the data and drafted the manuscript. MH, ET, NJL, and FH critically revised the manuscript.

References

Belin, M.W., Lim, L., Rajpal, R.K., Hafezi, F., Gomes, J.A.P., Cochener, B., 2018. Corneal cross-linking: current USA status: report from the cornea society. Cornea 37, 1218–1225.

Ehmke, T., Seiler, T.G., Fischinger, I., Ripken, T., Heisterkamp, A., Frueh, B.E., 2016. Comparison of corneal riboflavin gradients using dextran and HPMC solutions. J. Refract. Surg. 32, 798–802.

Gomes, J.A., Tan, D., Rapuano, C.J., Belin, M.W., Ambrosio Jr., R., Guell, J.L., Malecaze, F., Nishida, K., Sangwan, V.S., Group of Panelists for the Global Delphi Panel of, K., Ectatic, D, 2015. Global consensus on keratoconus and ectatic diseases. Cornea 34, 359–369.

Hafezi, F., Richoz, O., Torres-Netto, E.A., Hillen, M., Hafezi, N.L., 2021a. Corneal cross-linking at the slit lamp. J. Refract. Surg. 37, 78–82.

Hafezi, F., Torres Netto, E.A., Randleman, J.B., Hafezi, N.L., Mazzotta, C., Ambrosio, R., Kollros, L., 2021b. Corneal cross-linking for keratoglobus using individualized fluence. J Refr Surg Case Rep 1, e10–e14.

Hammer, A., Kling, S., Boldi, M.O., Richoz, O., Tabibian, D., Randleman, J.B., Hafezi, F., 2015. Establishing corneal cross-linking with riboflavin and UV-A in the mouse cornea in vivo: biomechanical analysis. Invest. Ophthalmol. Vis. Sci. 56, 6581–6590.

Hammer, A., Richoz, O., Arba Mosquera, S., Tabibian, D., Hoogewoud, F., Hafezi, F., 2014. Corneal biomechanical properties at different corneal cross-linking (CXL) irradiances. Invest. Ophthalmol. Vis. Sci. 55, 2881–2884.

Hashemi, H., Seyedian, M.A., Miraftab, M., Fotouhi, A., Asgari, S., 2013. Corneal collagen cross-linking with riboflavin and ultraviolet a irradiation for keratoconus: long-term results. Ophthalmology 120, 1515–1520.

Hersh, P.S., Stulting, R.D., Muller, D., Durrie, D.S., Rajpal, R.K., 2017. United States multicenter clinical trial of corneal collagen crosslinking for keratoconus treatment. Ophthalmology 124, 1259–1270.

Kennedy, R.H., Bourne, W.M., Dyer, J.A., 1986. A 48-year clinical and epidemiologic study of keratoconus. Am. J. Ophthalmol. 101, 267–273.

Kling, S., Hammer, A., Conti, A., Hafezi, F., 2017. Corneal cross-linking with riboflavin and UV-A in the mouse cornea in vivo: morphological, biochemical, and physiological analysis. Translational vision science & technology 6, 7.

Kling, S., Richoz, O., Hammer, A., Tabibian, D., Jacob, S., Agarwal, A., Hafezi, F., 2015. Increased biomechanical efficacy of corneal cross-linking in thin corneas due to higher oxygen availability. J. Refract. Surg. 31, 840–846.

Kling, S., Torres-Netto, E.A., Abdshahzadeh, H., Espana, E.M., Hafezi, F., 2021. Collagen V insufficiency in a mouse model for Ehlers Danlos-syndrome affects viscoelastic biomechanical properties explaining thin and brittle corneas. Sci. Rep. 11, 17362.

Kling, S., Torres-Netto, E.A., Spiru, B., Sekundo, W., Hafezi, F., 2020. Quasi-static optical coherence elastography to characterize human corneal biomechanical properties. Invest. Ophthalmol. Vis. Sci. 61, 29.

Lenth, R.V., 2006. Java Applets for Power and Sample Size [Computer software].

Lombardo, M., Serrao, S., Raffa, P., Rosati, M., Lombardo, G., 2016. Novel technique of transepithelial corneal cross-linking using iontophoresis in progressive keratoconus. J Ophthalmol 2016, 7472542.

Mazzotta, C., Bagaglia, S.A., Vinciguerra, R., Ferrise, M., Vinciguerra, P., 2018. Enhanced-fluence pulsed-light iontophoresis corneal cross-linking: 1-year morphological and clinical results. J. Refract. Surg. 34, 438–444.

Mazzotta, C., Riomani, A., Burroni, A., 2019. Pachymetry-based accelerated cross-linking: the "M nomogram" for standardized treatment of all-thickness progressive ectatic corneas. Int. J. Keratoconus Ectatic Corneal Dis. 7 (2), 137–144.

Raiskup-Wolf, F., Hoyer, A., Spoerl, E., Pillunat, L.E., 2008. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. J. Cataract Refract. Surg. 34, 796–801.

Randleman, J.B., Khandelwal, S.S., Hafezi, F., 2015. Corneal cross-linking. Surv. Ophthalmol. 60, 509–523.

Richoz, O., Hammer, A., Tabibian, D., Gatzioufas, Z., Hafezi, F., 2013. The biomechanical effect of corneal collagen cross-linking (CXL) with riboflavin and UV-A is oxygen dependent. Translational vision science & technology 2, 6.

Salmon, B., Richoz, O., Tabibian, D., Kling, S., Wuarin, R., Hafezi, F., 2017. CXL at the slit lamp: No clinically relevant changes in corneal riboflavin distribution during upright UV irradiation. J. Refract. Surg. 33, 281.

Scarcelli, G., Kling, S., Quijano, E., Pineda, R., Marcos, S., Yun, S.H., 2013. Brillouin microscopy of collagen crosslinking: noncontact depth-dependent analysis of corneal elastic modulus. Invest. Ophthalmol. Vis. Sci. 54, 1418–1425.

Torres Netto, E.A., Al-Otaibi, W.M., Hafezi, N.L., Kling, S., Al-Farhan, H.M., Randleman, J.B., Hafezi, F., 2018. Prevalence of keratoconus in paediatric patients in Riyadh, Saudi Arabia. Br. J. Ophthalmol. 102, 1436–1441.

Torres-Netto, E.A., Kling, S., Hafezi, N., Vinciguerra, P., Randleman, J.B., Hafezi, F., 2018. Oxygen diffusion may limit the biomechanical effectiveness of iontophoresis-assisted transepithelial corneal cross-linking. J. Refract. Surg. 34, 768–774.

Torres-Netto, E.A., Spiru, B., Kling, S., Gilardoni, F., Lazaridis, A., Sekundo, W., Hafezi, F., 2020. Similar biomechanical cross-linking effect after SMILE and PRK in human corneas in an ex vivo model for postoperative ectasia. J. Refract. Surg. 36, 49–54.

Wollensak, G., Spoerl, E., Seiler, T., 2003. Stress-strain measurements of human and porcine corneas after riboflavin–ultraviolet-A-induced cross-linking. J. Cataract Refract. Surg. 29, 1780–1785.

Zhang, H., Roozbahani, M., Piccinini, A.L., Golan, O., Hafezi, F., Scarcelli, G., Randleman, J.B., 2019. Depth-dependent reduction of biomechanical efficacy of contact lens-assisted corneal cross-linking analyzed by Brillouin microscopy. J. Refract. Surg. 35, 721–728.