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Corneal biomechanical properties at different corneal collagen cross-linking (CXL) Irradiances

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ABSTRACT

PURPOSE: New corneal collagen cross-linking (CXL) devices are capable of using higher UV-A light irradiances than used in original CXL protocols. The Bunsen-Roscoe law states that a photochemical reaction should stay constant if the delivered total energy is kept constant; however, little clinical data are available to support this hypothesis.

METHODS: We investigated the biomechanical properties of 4 groups (n = 50 each) of porcine corneas. Three groups were exposed to riboflavin 0.1 % and UV-A irradiation of equal total energy (3 mW/cm2 for 30 minutes, 9 mW/cm2 for 10 minutes, and 18 mW/cm2 for 5 minutes). Controls were exposed to riboflavin 0.1% without irradiation. Young's modulus of 5 mm wide corneal strips was used as an indicator of corneal stiffness.

RESULTS: We observed a decreased stiffening effect with increasing UV-A intensity. Young's modulus at 10% strain showed significant differences between 3 mW/cm^2 and 9 mW/cm^2 ($p=0.002$), 3 mW/cm^2 and 18 mW/cm^2 ($p=0.0002$), 3 mW/cm^2 and the control group ($p<0.0001$), 9 mW/cm^2 and the control group ($p=0.015$). There was no difference between 18 mW/cm^2 and the control group ($p = 0.064$) and between 9 mW/cm^2 and 18 mW/cm^2 ($p=0.503$).

CONCLUSIONS: The biomechanical effect of CXL decreased significantly when using high irradiance/short irradiation time settings. Intrastromal oxygen diffusion capacity and increased oxygen consumption associated with higher irradiances may be a limiting factor leading to reduced treatment efficiency.

Key words: corneal collagen cross-linking, high irradiance, oxygen, efficiency, biomechanics

INTRODUCTION

Corneal collagen cross-linking (CXL) with riboflavin and UV-A is a treatment modality for keratoconus that was first developed in Dresden, Germany in 1998. [1,2] Per the typical cross-linking protocol, 0.1% riboflavin solution with 20% dextran is added to the de-epithelialized cornea and then photoactivated with ultraviolet-A (UV-A) light at 365 nm with irradiance of 3 mW/cm^2 for 30 minutes. The cornea is de-epithelialized to allow adequate penetration of riboflavin into the corneal stroma. Riboflavin acts as photosensitizer; it creates free radicals, forms new molecular crosslinks, and ultimately increases the cornea's mechanical strength. [3-5] The effect of treatment can be assessed postoperatively using the Ocular Response Analyzer (Reichert Inc, Buffalo, NY). The depth of treatment can be measured by the demarcation line, which usually appears at ten to fourteen days after CXL. [6] The success rate of the method at stabilizing keratoconus is higher than 95% and can be monitored using corneal topography. Unfortunately, the method cannot be used in patients with very thin corneas. [7-9]

CXL experienced a rapid transition from laboratory procedure to clinical intervention because of the method's apparent safety and broad array of potential

applications. Early clinical results were reported only 2 years after animal studies. [16] One such clinical application is the treatment of keratoconus. Keratoconus is a degenerative disorder of the eye associated with thinning and subsequent bulging of the cornea, causing poor vision.[10] CXL stops the progression of keratoconus in patients with mild disease, presumably by strengthening the cornea and preventing further bulging.[10] CXL has also been used successfully in the treatment of pellucid marginal degeneration[11], to stabilize early stage keratoconus[12-15], and to treat iatrogenic (postoperative) ectasia.[16,17] CXL is currently in use in over 100 countries..

The Bunsen-Roscoe law indicates that a photochemical reaction will stay constant if the total energy is constant: a shortened irradiation time at higher irradiance should lead to the same increase in biomechanical stiffness as a longer irradiation time at lower irradiance. By applying this theoretical law of photochemistry and in an effort to reduce clinical treatment times, some groups have modified the original method to apply higher irradiances over shorter times though maintaining the same total applied energy. Commercial devices are now available to deliver CXL treatment doses as high as 45 mW/cm^2 shortening the treatment time to as little as 2

minutes. Despite availability of such devices and increased use in the clinic, a thorough validation of this modified approach has not yet been published.

Young's modulus is commonly used to characterize the stiffness of an elastic material. The Young's modulus of the material indicates its stiffness at a given force and related strain . A greater Young's modulus is associated with more resistance to applied forces. It can be determined by measuring the change in length of a material under a tensile load (% strain). Young's modulus is calculated as the ratio of stress (pressure) to strain (dimensionless) applied to the material, and so has units of pressure. For reference, the Young's modulus of the tympanic membrane varies from 34 to 59 Mpa.[18] We evaluated corneal stiffness using Young's modulus measurements. The limits of Bunsen-Roscoe energy reciprocity were evaluated using different CXL irradiance – time settings, with a constant total fluence of 5.4 J/cm^2.

MATERIALS AND METHODS

Corneal collagen cross-linking (CXL)

CXL was performed as described previously. [19] Briefly, freshly enucleated pig eyes with intact epithelium were obtained from a slaughterhouse and randomly assorted

into four different treatment groups (n=50 for each group). Prior to UV-A irradiation, the epithelium was removed using a hockey knife, corneas were saturated with 0.1% riboflavin drops (StreuliPharma AG, Uznach, Switzerland) every minute for 25 minutes and the epi-off CXL procedure was performed using the Schwind CCL-365 Vario system (Schwind eye-tech-solutions GmbH & Co. Kleinostheim, Germany) All corneas were irradiated on a diameter of 11.3 mm using a total energy dose of 5.4 J/cm^2. Group 1 was irradiated with 3 mW/cm^2 for 30 minutes. Group 2 was irradiated with 9 mW/cm^2 for 10 minutes. Group 3 was irradiated with 18 mW/cm^2 for 5 minutes. Un-irradiated corneas served as controls (group 4).

Biomechanical measurements

Corneas from the four groups were allowed to rest in a wet chamber for 30 min after UV or sham UV treatment. The corneas were then excised and a 5 mm x 10 mm nasal-temporal oriented corneal strip was prepared. The Young's modulus at 10% strain was determined using an extensometer (Zwick-Line Testing Machine Z 0.5, Zwick, Ulm, Germany). Data analysis was performed using the Xpert II-Testing Software for Static Testing Systems (Zwick, Ulm, Germany).

135 **Statistical analysis**

136 Data were analyzed with Xlstat 2013 for Windows (Addinsoft, version 2013.4.03). All
137 data are expressed as the mean ± standard deviation (SD). Normal distribution of
138 data was evaluated by the Shapiro-Wilk test. The Young's modulus of all different
139 groups was compared using the non-parametric Kruskal–Wallis one-way analysis of
140 variance (ANOVA). When significant, we proceeded to the non-parametric Mann-
141 Whitney test of the null hypothesis (H0 = populations are the same).. A p value less
142 than 0.05 was considered statistically significant.

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144

145 **RESULTS**

146 The average Young's modulus was determined for each of the four groups and
147 percentage strains (Table 1). Young's modulus of corneas that underwent CXL
148 decreased with increasing UV light irradiance. The average Young's modulus at 10%
149 strain was 11.54 Mpa (+/- 3.02) for the control group, 15.85 Mpa (+/- 3.96) for the 3
150 mW/cm^2 group, 13.48 Mpa (+/- 3.56) for the 9 mW/cm^2 group, and 12.90 Mpa (+/-
151 3.86) for the 18 mW/cm^2 group, respectively (Table 1 and Figure 1).

At 10% strain, Young's modulus showed a significant global difference between groups was found according to the non-parametric Krsukal-Kallis test for the 4 groups ($p < 0.0001$). The p-values for the non-parametric Mann-Withney tests comparing two groups indicated significant differences between 3 mW/cm^2 and 9 mW/cm^2 ($p = 0.002$), 3 mW/cm^2 and 18 mW/cm^2 ($p = 0.0002$), 3 mW/cm^2 and the control group ($p < 0.0001$), 9 mW/cm^2 and the control group ($p = 0.015$) and 18 mW/cm^2 and the control group ($p = 0.064$). There was no difference in the Young's modulus of the 9 mW/cm^2 and 18 mW/cm^2 groups ($p = 0.503$) in the 10% strain group (Table 2).

DISCUSSION

The efficiency of CXL decreased significantly as UV-A light irradiances increased from 3 to 18 mW/cm^2. Indeed, corneas treated with the highest tested irradiance (18 mW/cm^2 for 5 minutes) had stiffness that was indistinguishable from untreated controls (Table 2). Higher light irradiances were associated with lower Young's modulus at each percentage strain tested.

168 Wernli *et al.* evaluated Young's modulus using the same total energy fluence and
169 riboflavin concentration as in our study. [20] They also observed a decrease in Young's
170 modulus for high irradiances, but only at irradiances exceeding 50 mW/cm^2. These
171 differences might be explained by several factors.. First, the groups had different
172 sizes (10 eyes/group versus 50 eyes/group), second, the biomechanical
173 measurements were performed at different times; Wernli and colleagues took
174 measurements at 30 minutes after starting irradiation, regardless of irradiation time.[20]
175 By contrast, we consistently performed measurements at 30 minutes after the end of
176 irradiation. Another difference is that Wernli and colleagues kept corneas immersed
177 in the riboflavin solution.[20] This extended exposure to riboflavin likely increased the
178 amount of riboflavin penetration and subsequent different cross-linking activity.
179 Also, we observed a Young's modulus that was approximately a factor 2 larger
180 than in the Wernli study. Several factors might be responsible for these differences.
181 First, the machines for biomechanical measurements were not the same (Zwick Z 0.5
182 vs MINIMAT; Stretton Shropshire) and second, the methods were slightly different
183 (time before biomechanical testing, length of the corneal strips 10 mm vs 7 mm).

Other, yet unidentified aspects might have further influenced the differences observed.

Lastly, the Wernli study was performed using a beam-optimized device (UV-X 2000, IROC Innocross, Zurich, Switzerland). This device tends to deliver a more homogeneous energy profile to the cornea.[21] In our experiments, a device delivering a less homogeneous distribution of energy with respect to corneal curvature was used (CXL 365 Vario, Schwind eyetech solutions, Kleinostheim, Germany). One might speculate that the differences between the studies might be due to this variation in energy distribution. We do not believe that this is the case: the main interest in both studies was to assess relative differences in the cross-linking effect between the current gold standard (3 mW/cm2 for 30 minutes) and accelerated settings.

In a recent study, Beshtawi et al. analyzed ex vivo human corneas using Scanning Acoustic Microscopy (SAM) to determine stiffness following irradiation at 3 and 9 mW/cm2. Similar to our results, they found a significant increase in stiffness at both settings when compared to controls.[22] In contrast to our findings, they did not see significant differences between both settings. Several factors might explain this

discrepancy: the tissues were different between the Beshtawi study (human corneas) and our experiments (porcine corneas). Also, we performed stress-strain measurements, whereas Beshtawi and colleagues used SAM. Without a doubt, the 9 mW/cm2 for 10 minutes setting provides cross-links to the cornea and clinical validation is needed to better understand the results of both studies.

Oxygen levels in the cornea are related to the oxygen diffusion flux and local oxygen uptake.[23] Corneal oxygen levels decrease during CXL, presumably due to the transformation of oxygen into reactive oxygen species.[24] The reactive species are thought to catalyze the creation of covalent bonds between collagen and proteoglycan molecules, stiffening the cornea.[5] Oxygen seems to be essential to this process and is probably the rate-limiting substrate in the photochemical reaction. We have previously shown that corneas treated in a low oxygen state using an irradiance of 9 mW/cm^2 for 10 minutes exhibit a Young's modulus similar to that of untreated controls. [19] High UV-A irradiances would be expected to have higher oxygen utilization rates. If oxygen conversion to free radicals outpaces oxygen replenishment by diffusion, , the local oxygen levels would fall and collagen cross-linking would be compromised.[24] This would result in lower measured Young's modulus. Our findings

support this hypothesis and are in agreement with previously reported data. [24] Alternatively, other yet unknown mechanisms might also contribute to the biomechanical results observed.

In conclusion, we report a steady and significant decline in the biomechanical response (“ stiffening “) of ex vivo corneas with increasing irradiance and decreased treatment times. This may indicate that the Bunsen-Roscoe law knows limitations in an *in vivo* setup: and cannot be simply applied to the cornea. Whether or not the decline in biomechanical stiffness will be clinically relevant remains to be validated in clinical trials using high-irradiance CXL.

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References

1. Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp Eye Res*, 1998; 66(1): 97-103.
2. Spoerl E, Huhle M, Kasper M, Seiler T. [Increased rigidity of the cornea caused by intrastromal cross-linking]. *Ophthalmologe*, 1997; 94(12): 902-6.
3. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboaflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg*, 2003; 29(9): 1780-5.
4. Spoerl E, Seiler T. Techniques for stiffening the cornea. *J Refract Surg*, 1999; 15(6): 711-3.
5. Brummer G, Littlechild S, McCall S, Zhang Y, Conrad GW. The Role of Non-Enzymatic Glycation and Carbonyls in Collagen Cross-Linking for the Treatment of Keratoconus. *Investigative ophthalmology & visual science*, 2011.
6. Seiler T, Hafezi F. Corneal cross-linking-induced stromal demarcation line. *Cornea*, 2006; 25(9): 1057-9.
7. Hafezi F. Limitation of collagen cross-linking with hypoosmolar riboflavin solution: failure in an extremely thin cornea. *Cornea*, 2011; 30(8): 917-9.
8. Kolli S, Aslanides IM. Safety and efficacy of collagen crosslinking for the treatment of keratoconus. *Expert opinion on drug safety*, 2010; 9(6): 949-57.
9. Spoerl E, Mrochen M, Sliney D, Trokel S, Seiler T. Safety of UVA-riboflavin cross-linking of the cornea. *Cornea*, 2007; 26(4): 385-9.
10. Raiskup F, Spoerl E. Corneal crosslinking with riboflavin and ultraviolet A. I. Principles. *Ocul Surf*, 2013; 11(2): 65-74.
11. Schnitzler E, Sporl E, Seiler T. [Irradiation of cornea with ultraviolet light and riboflavin administration as a new treatment for erosive corneal processes, preliminary results in four patients]. *Klin Monbl Augenheilkd*, 2000; 217(3): 190-3.
12. Caporossi A, Mazzotta C, Baiocchi S, Caporossi T. Long-term results of riboflavin ultraviolet a corneal collagen cross-linking for keratoconus in Italy: the Siena eye cross study. *Am J Ophthalmol*, 2010; 149(4): 585-93.
13. Hashemi H, Seyedian MA, Miraftab M, Fotouhi A, Asgari S. Corneal collagen cross-linking with riboflavin and ultraviolet a irradiation for keratoconus: long-term results. *Ophthalmology*, 2013; 120(8): 1515-20.
14. Raiskup-Wolf F, Hoyer A, Spoerl E, Pillunat LE. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. *J Cataract Refract Surg*, 2008; 34(5): 796-801.
15. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol*, 2003; 135(5): 620-7.
16. Hafezi F, Kanellopoulos J, Wiltfang R, Seiler T. Corneal collagen crosslinking with riboflavin and ultraviolet A to treat induced keratectasia after laser in situ keratomileusis. *J Cataract Refract Surg*, 2007; 33(12): 2035-40.
17. Richoz O, Mavrakanas N, Pajic B, Hafezi F. Corneal Collagen Cross-Linking for Ectasia after LASIK and Photorefractive Keratectomy: Long-Term Results. *Ophthalmology*, 2013.
18. Luo H, Dai C, Gan RZ, Lu H. Measurement of young's modulus of human tympanic membrane at high strain rates. *J Biomech Eng*, 2009; 131(6): 064501.
19. Richoz O, Hammer A, Tabibian D, Gatzioufas Z, Hafezi F. Corneal collagen cross-linking (CXL) with riboflavin and UV-A is oxygen-dependent. *TVST*, (in press).
20. Wernli J, Schumacher S, Spoerl E, Mrochen M. The efficacy of corneal cross-linking shows a sudden decrease with very high intensity UV light and short treatment time. *Invest Ophthalmol Vis Sci*, 2013; 54(2): 1176-80.

21. Koller T, Schumacher S, Fankhauser F, 2nd, Seiler T. Riboflavin/ultraviolet a crosslinking of the paracentral cornea. *Cornea*, 2013; 32(2): 165-8.

22. Beshtawi IM, Akhtar R, Hillarby MC *et al.* Biomechanical properties of human corneas following low- and high-intensity collagen cross-linking determined with scanning acoustic microscopy. *Invest Ophthalmol Vis Sci*, 2013; 54(8): 5273-80.

23. Freeman RD. Oxygen consumption by the component layers of the cornea. *J Physiol*, 1972; 225(1): 15-32.

24. Kamaev P, Friedman MD, Sherr E, Muller D. Photochemical kinetics of corneal cross-linking with riboflavin. *Invest Ophthalmol Vis Sci*, 2012; 53(4): 2360-7.

311 **Table 1.** Young's Modulus at various UV-A light irradiances

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	Young's Modulus (MPa)			
	UV-A light irradiance			
	Untreated Control	3 mW/cm^2 for 30 min	9 mW/cm^2 for 10 min	18 mW/cm^2 for 5 min
% strain 10	11.54	15.85	13.49	12.89
STDEV 10	3.02	3.96	3.56	3.86
Kruskal-Wallis P-value	< 0.0001			

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316 **Table 2:** P-values resulting from individual Mann-Withney tests between Young's
317 Modulus at various UV-A light irradiances (* = Significant)
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P value	Untreated Control	3 mW/cm^2 for 30 min	9 mW/cm^2 for 10 min	18 mW/cm^2 for 5 min
Untreated Control	--	< 0.0001*	0.015*	0.064
3 mW/cm^2 for 30 min	< 0.0001*		0.002*	0.0002*
9 mW/cm^2 for 10 min	0.015*	0.002*		0.503
18 mW/cm^2 for 5 min	0.064	0.0002*	0.503	

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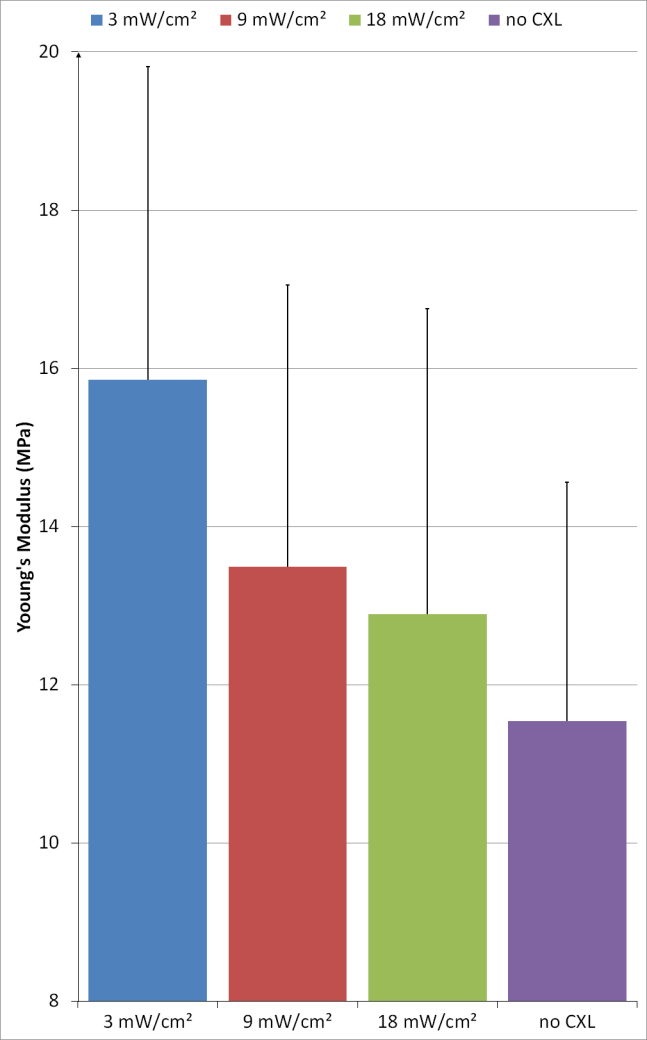
FIGURE 1. Young's Modulus by 10 % of strain at different UV-A light irradiances.

Legend: : Control group (blue), 3 mW/cm^2 of irradiance (red), 9 mW/cm^2 of irradiance (green) and 18 mW/cm^2 of irradiance (purple).


3 mW/cm²
9 mW/cm²
18 mW/cm²
no CXL
20
18
16
14
12
10
8
Yooung's Modulus (MPa)
3 mW/cm²
9 mW/cm²
18 mW/cm²
no CXL