Cross-Linking Biomechanical Effect in Human Corneas by Same Energy, Different UV-A Fluence: An Enzymatic Digestion Comparative Evaluation

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Purpose: To evaluate ex vivo the possible difference in corneal cross-linking (CXL) biomechanical effect of different ultraviolet-A (UV-A) irradiances.

Methods: The study involved 25 human donor corneas, randomly allocated to 5 groups (n = 5 each). CXL was applied with UV-A irradiances of 3, 9, 18, 30, and 45 mW/cm^2, maintaining equal cumulative energy dose of 5.4 J/cm^2. UV-A was delivered on half of the cornea. The nonirradiated halves served as controls. Specimens were subjected to collagenase-A enzymatic digestion. The time to complete dissolution in each specimen was recorded.

Results: Time to dissolution in group-A (3 mW/cm^2 for 30 minutes) was 321 ± 13.4 minutes (range: 300–330) compared with 171 ± 8.2 (range: 165–180) for their control. In group-B (9 mW/cm^2 for 10 minutes), it was 282 ± 19.6 minutes (range: 270–315) compared with 177 ± 6.7 (165–180) for their control. In group-C (18 mW/cm^2 for 5 minutes), it was 267 ± 19.6 minutes (range: 240–285) compared with 177 ± 7.7 (range: 165–180) for their control. In group-D (30 mW/cm^2 for 3 minutes), it was 252 ± 12.5 minutes (range: 240–270) compared with 180 ± 10.6 minutes (range: 165–195) for their control. In group-E (45 mW/cm^2 for 2 minutes), it was 204 ± 17.1 minutes (range: 180–225) compared with 186 ± 8.2 minutes (range: 180–195) for their control.

Conclusions: The data in this ex vivo human corneal study indicate that the biomechanical effect of CXL studied by resistance to enzymatic digestion in human corneas is comparable between irradiances of 9, 18 and 30 mW/cm^2 and seems to be reduced at a fluence of 45 mW/cm^2.

Received for publication October 26, 2015; revision received December 2, 2015; accepted December 8, 2015. Published online ahead of print February 4, 2016.

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Design and conduct of the study (A.J.K., G.A.); collection (G.A., Y.L.L.), management (A.J.K.), analysis (G.A., Y.L.L.), and interpretation of the data (A.J.K., G.A., Y.L.L.); manuscript preparation (G.A.), manuscript review (A.J.K., G.A., Y.L.L.), and manuscript approval (A.J.K.).

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Key Words: corneal cross-linking, keratoconus, high-irradiance CXL, corneal biomechanics, high-energy CXL, reciprocity law, Bunsen–Roscoe law, collagenase-A, enzymatic digestion, accelerated CXL, rapid CXL

(*Cornea* 2016;35:557–561)

Corneal cross-linking (CXL) has been clinically used for stabilizing progressive keratectasia for more than a decade.[1–3] This photochemical reactive process is induced by peak 370-nm ultraviolet-A (UV-A) radiation absorbed by riboflavin, a photosensitive vitamin B2 molecule, with an absorption maximum at 365 nm.[4] The procedure is broadly accepted to result in corneal biomechanical strengthening not only in advanced keratoconus[5–7] but also in early-stage[8] and iatrogenic keratectasia.[9,10]

Collagenase has been known to contribute to break down of collagen in the corneal stroma.[11] This collagenase-related breakdown is a vigorous biochemical process that has been used as an indirect metric of corneal biomechanical properties.[12,13] The stabilizing biochemical effect of CXL may be thus reflected by an increased amount of resistance to collagenase digestion.[14,15] CXL-treated porcine corneas have demonstrated nearly double the dissolution time after pepsin, trypsin, and collagenase digestion.[16–19]

The original (standard) Dresden CXL protocol introduced epithelial removal and 30-minute corneal soaking with a dextran-based 0.1% riboflavin solution. UV-A illumination settings were 30 minutes with an irradiance of 3 mW/cm^2, corresponding to a dissipated energy of 5.4 J/cm^2.[1,20,21]

We have subsequently introduced higher fluence, same-energy protocols, and many other investigators have subsequently introduced a multitude of CXL protocols currently in use internationally. The rationale of these protocols has been justified by the Bunsen–Roscoe reciprocity law,[22] which states a certain biological effect is directly proportional to the total radiant exposure (energy dose), irrespective of application time.[23] The reported limitations of the reciprocity law may indicate that there exists a range of applicability of how much the clinical UV-A radiation may be increased (and correspondingly, the application time shortened), which may need to be further investigated.

Despite their widespread clinical practice, a thorough clinical comparative validation of these approaches has not yet been published. The quantitative CXL effect between several of these protocols still remains elusive. The enzymatic

degradation resistance modulations achieved via CXL application at different UV-A irradiances have not been studied in human corneas. The purpose of this study is to comparatively evaluate ex vivo these enzymatic degradation resistance differences.

MATERIALS AND METHODS

This laboratory (ex vivo) study received approval by the Ethics Committee of our Institution. The study involved 25 human donor corneas. These corneas had passed the usual suitable dates for clinical corneal grafting because they were 7 to 14 days post-harvesting. The corneas had been donated by 25 different organ donors (11 male, 14 female) of average age 63.4 ± 12.5 (range: 49–79) years. The corneas were stored in OptiSol (Bausch & Lomb, Rochester, NY) solution and were maintained at 4°C.

Sample Preparation

Epithelium was removed mechanically before treatment. Riboflavin soaking was achieved by dextran-free riboflavin 0.1% solution (VibeX; Avedro Inc., Waltham, MA). The specific solution, containing concentration stabilizers such as disodium hydrogen phosphate, sodium phosphate monobasic dihydrate, and sodium chloride, has been our clinical standard riboflavin solution over the last 6 years.[24] Repeated applications of riboflavin drops on the corneas were performed every 1 minute for a total of 30 minutes. At the conclusion of the riboflavin administration, irrigation by balanced salt solution was used to remove residual superficial riboflavin.

The corneas were randomly allocated to 5 investigative groups (n = 5 each). UV-A irradiation corresponding to equal cumulative energy dose (radiant exposure) of 5.4 J/cm^2 was maintained for all groups. The groups were distinguished by UV-A irradiation level, which varied from 3 to 45 mW/cm^2 as follows: group-A 3 mW/cm^2 applied for 30 minutes, group-B 9 mW/cm^2 for 10 minutes, group-C 18 mW/cm^2 for 5 minutes, group-D 30 mW/cm^2 for 3 minutes, and group-E 45 mW/cm^2 for 2 minutes.

In all cases, the KXL II (Avedro Inc.) UV device was used in a pattern that permitted only half of the cornea to be irradiated. Riboflavin solution was instilled every minute during UV-A exposure. The corneal borders corresponding to the delineation line were manually marked. After UV-A irradiation, all corneas were trephined to 8.5 mm and were then cut along the delineation lines to the corresponding halves. The nonirradiated halves from each group served as control specimens.

Enzymatic Degradation

All specimens were weighed to 0.0001-g precision on a laboratory scale. The average mass of the CXL-treated halves was 0.03 ± 0.004 g (range: 0.02–0.03), whereas the average mass of the nonirradiated (control) halves was 0.03 ± 0.004 g (range: 0.02–0.04). Weighed mass had very good consistency among halves and among successive corneas. The difference in weighed mass between the treated and nontreated halves was nonstatistically significant.

A 0.3% collagenase-A solution (active agent, *Clostridium histolyticum*) (Sigma-Aldrich, St Louis, MO) was prepared via dilution in Dulbecco's phosphate-buffered saline (Sigma-Aldrich).[25] The solution was homogenized by a spinning tube. Forty test tubes were prepared by administering 1.5 mL of collagenase-A solution in each using a precision (digital) pipette (Labopette; Hirschmann Laborgeräte GmbH, Eberstadt, Germany). The specimens were then inserted in the tubes, 1 per tube. The test tube rack was placed inside an incubation chamber (Excella E24; New Brunswick Scientific, Enfield, CT). Temperature was set at 37°C, and the plate shaker spinning rate was set at 175 rotations per minute.

The specimens were observed inside the test tubes every 30 minutes for the first 2 hours, then every 15 minutes. The time to complete dissolution in each specimen was recorded. Complete digestion was defined if there was no visible specimen remnant piece in the test tube.

TABLE 1. Descriptive Statistics of Time to Dissolution per Group

	CXL Treated	Controls	Group to Control	
			*P**	Δ (+), %
Group-A (3 mW/cm^2 for 30 min)				
Average	321	171	0.012	88
SD	13.4	8.2		
Minimum	300	165		
Maximum	330	180		
Group-B (9 mW/cm^2 for 10 min)				
Average	282	177	0.012	59
SD	19.6	6.7		
Minimum	270	165		
Maximum	315	180		
Group-C (18 mW/cm^2 for 5 min)				
Average	267	177	0.012	51
SD	19.6	6.7		
Minimum	240	165		
Maximum	285	180		
Group-D (30 mW/cm^2 for 3 min)				
Average	252	180	0.012	40
SD	12.5	10.6		
Minimum	240	165		
Maximum	270	195		
Group-E (45 mW/cm^2 for 2 min)				
Average	204	186	0.116	10
SD	17.1	8.2		
Minimum	180	180		
Maximum	225	195		

All reported times correspond to minutes of the hour (60 seconds).
Δ (+), relative difference between sample and control (expressed as %).
*Two-tailed Mann–Whitney *U* test.

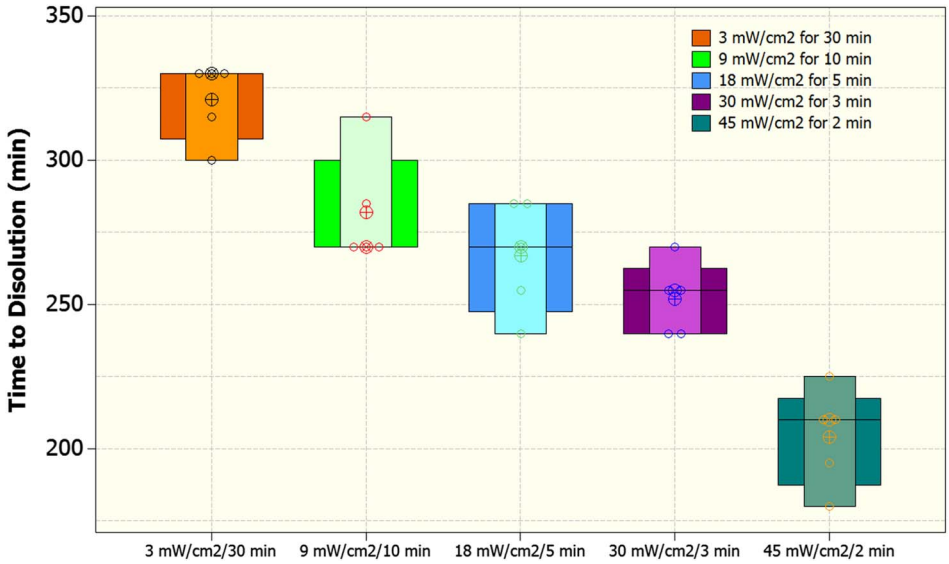

FIGURE 1. Box plot illustrating time to dissolution (in minutes) results from the 5 groups of study.

Statistical analysis was performed by SPSS software version 21.0 (IBM Corporation, New York, NY). *P*-values less than 0.05 were indicative of statistically significant results. Results are reported in the form average ± SD (range: minimum to maximum).

RESULTS

The average time to complete dissolution in the CXL-half specimens was 265 ± 42 minutes, whereas in the non–CXL-half specimens (controls), it was 179 ± 8 minutes. Descriptive statistics of time to dissolution (minutes) per group are presented in Table 1. Results are illustrated in Figure 1.

Time to dissolution results showed that as UV-A irradiance increased (from group-A to group-E), the time to dissolution decreased, indicating that the standard-protocol UV-A irradiance of 3 mW/cm^2 achieved the strongest enzymatic digestion set of data. There was a significant global difference between groups according to the analysis of variance (1-way ANOVA) (P = 0.0005). The following 2-tailed *P*-values were recorded when comparing the 5 investigative groups: group-A with group-B 0.0079, group-B with group-C 0.259, group-C with group-D 0.1878, and group-D with group-E 0.00146. In other words, the standard-protocol UV-A irradiance of 3 mW/cm^2 achieved a statistically significant difference in time to dissolution in comparison with the "accelerated" groups (9, 18, and 30 mW/cm^2). The latter 3 groups displayed nonsignificant differences among them. Finally, group-E (45 mW/cm^2) seemed to have less time to dissolution by a statistically significant difference (P = 0.00146) compared with the previous group-D. In addition, except group-E (45 mW/cm^2), in the remaining groups there was a statistically significant difference between CXL-half specimens and non–CXL-half specimens (controls). The *P*-values for the each CXL group compared with its control indicated significant differences between all groups except group-E (45 mW/cm^2), which had a nonstatistically significant difference with a 2-tailed Mann–Whitney U test *P*-value of 0.116 (Table 1). The 2-tailed *P*-values with records of comparison of the 5 cross-linked groups among them are presented in Table 2, which shows the statistically significant difference between the standard-treatment group-A and the "accelerated" groups (B to E).

DISCUSSION

Higher fluence (irradiance) variations have been shown to offer comparable corneal biomechanical stiffening compared with standard irradiance (3 mW/cm^2).[26,27] Clinical results suggesting comparable effectiveness with the conventional protocol in stabilizing keratoconus progression have been reported.[28,29] Equivalence in clinical parameters (including visual rehabilitation and refraction)[30] and Scheimpflug imaging[31,32] and anterior-segment optical coherence tomography imaging–derived parameters have also been demonstrated.[33] Some reports suggest that accelerated CXL seems to be effective in preventing keratectasia progression in advanced keratoconus cases but is not as effective in less progressed stages.[34]

Adding to this skepticism, some limitations of the reciprocity law have been reported in regard to CXL

TABLE 2. Records of Comparison of the 5 Cross-linked Groups Among Them, Indicating 2-Tailed *P*-Values

	Group-A	Group-B	Group-C	Group-D	Group-E
Group-A		0.012	0.004	0.002	0.001
Group-B			0.413	0.047	0.006
Group-C				0.298	0.002
Group-D					0.012
Group-E					

Group-A: 3 mW/cm^2 for 30 minutes; group-B: 9 mW/cm^2 for 10 minutes; group-C: 18 mW/cm^2 for 5 minutes; group-D: 30 mW/cm^2 for 3 minutes; group-E: 45 mW/cm^2 for 2 minutes.

treatment: very high irradiances (eg, 30 and 100 mW/cm^2) seem to have a reduced and more superficial effect.[27,35] Other investigators have suggested a steady decline in the biomechanical response (stiffening) with increasing UV-A irradiance beyond 30 mW/cm^2 by using either ex vivo corneal testing[36] or theoretical models.[37] Whereas the clinical equivalence of irradiances up to 18 mW/cm^2 has been reported,[38] the scientific proof of a biomechanical strengthening effect achieved with very high irradiances (eg, 30 mW/cm^2 or more) is insufficient.[39]

In this study, we attempted to compare the possible differences between a few currently practiced CXL protocols, by a novel ex vivo evaluation of the enzymatic resistance effect attributed to CXL. We evaluated the differences in time to dissolution of several UV-A irradiation levels corresponding to equal total energy (radiant exposure) of 5.4 J/cm^2. The irradiation range extended from the conventional, Dresden protocol (3 mW/cm^2) to levels afforded by current UV-A radiation devices (45 mW/cm^2), such as the KXL II.[40] The intermediate irradiance values were chosen (9, 18, and 30 mW/cm^2) because they correspond to some currently used values in clinical practice.[39,41,42]

The samples were tested against their own controls. Each tested cornea was dissected meridionally in half. The control halves were otherwise treated identically, save for the UV-A illumination part. The longer turnover time (delayed dissolution) until complete degradation has been considered a metric of increased biomechanical effect. This possibly increased effect of each treatment protocol compared with its control is attributed to the CXL effect.

All specimens treated with CXL demonstrated significant increase of enzymatic digestion resistance by nearly a factor of 2 for each one's control except for the 45-mW/cm^2 fluence group. Our study demonstrates that this effect was not statistically significantly different for the following fluence groups: 3, 9, 18, and 30 mW/cm^2. The clinical relevance of this laboratory finding is that it validates earlier single-case series and comparative clinical studies evaluating clinical effects of different CXL protocols.

The 45-mW/cm^2 fluence group, as studied herein, seemed not to have a statistically significant CXL effect compared with all the above and its own control. This finding may suggest that such higher fluence administration may require longer exposure times, resulting in higher amount of dissipated energy, pulsing of the UV-A radiance, and/or CXL facilitators such as supplemental O_2 and D_2O.[43]

Accrued clinical experience and studies such as this validate in our opinion our initiative to accelerate the CXL surgical technique dating back to 2006.[31] We have been "vocal" over the past few years that large multicenter comparative studies between CXL are warranted, despite the fact that CXL regrettably still lacks approval from the US Food and Drug Administration.

Clinically, the CXL effect accomplished has been challenging to measure objectively in vivo. Perhaps, effective clinical application of Brillouin Spectroscopy may fill some of this void.[44] Most clinical CXL studies have been able to only evaluate accurately corneal curvature stability and/or refractive effect to the anterior cornea of CXL as we have suggested and previously reported by other investigators as "disease regression". Higher fluence CXL has gained clinical acceptance because of its clinical facility and studies such as this one validate its efficacy.

CONCLUSIONS

The data in this human corneal study support previous basic science and clinical studies documenting effective CXL with higher UV-A fluences than the standard 3 mW/cm^2 all the way up to 30 mW/cm^2. The 45-mW/cm^2 UV-A fluence group, as studied herein, did not seem to accomplish a CXL effect. A multitude of CXL protocols currently in clinical practice may require similar validation.

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