

# High-Fluence Accelerated Epithelium-Off Corneal Cross-Linking Protocol Provides Dresden Protocol-Like Corneal Strengthening

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**Received:** May 20, 2021

**Accepted:** July 23, 2021

**Published:** September 20, 2021

**Keywords:** cross-linking; CXL; accelerated cross-linking; keratoconus; dresden protocol; corneal biomechanics

**Citation:** Abrishamchi R, Abdshahzadeh H, Hillen M, Hafezi N, Torres-Netto EA, Aslanides IM, Chen S, Randleman JB, Hafezi F. High-fluence accelerated epithelium-off corneal cross-linking protocol provides dresden protocol-like corneal strengthening. *Transl Vis Sci Technol.* 2021;10(5):10. <https://doi.org/10.1167/tvst.10.5.10>

**Purpose:** To assess whether optimized technical settings for accelerated epithelium-off corneal cross-linking may lead to increases in biomechanical stiffness similar to the benchmark 30-minute epithelium-off Dresden protocol.

**Methods:** Three-hundred porcine eyes were divided equally into six groups for analysis. All samples underwent epithelial debridement and soaking with 0.1% iso-osmolar riboflavin solution for 20 minutes. Corneal cross-linking (CXL) was performed using epithelium-off protocols varying in acceleration and total fluence (intensity in mW/cm<sup>2</sup> \* time in minutes, total fluence in J/cm<sup>2</sup>): standard (S)-CXL (3\*30, 5.4), accelerated (A)-CXL (9\*10, 5.4), A-CXL (9\*13'20", 7.2), A-CXL (18\*6'40", 7.2), and A-CXL (18\*9'15", 10). Control corneas were not irradiated. The elastic modulus of 5-mm wide corneal strips was measured as an indicator of corneal stiffness.

**Results:** All irradiated groups had significantly higher elastic modulus than controls ( $P < 0.05$ ), with a stiffening effect of 133% S-CXL (3\*30, 5.4), 122% A-CXL (9\*10, 5.4), 120% A-CXL (9\*13'20", 7.2), 114% A-CXL (18\*6'40", 7.2) and 149% A-CXL (18\*9'15", 10). The high-fluence accelerated epithelium-off protocol (18\*9'15", 10) showed the highest stiffening effect. Elastic modulus at 5% strain (1%–5% strain) showed significant differences between A-CXL (18\*9'15", 7.2) and three other accelerated protocols: A-CXL (9\*10, 5.4;  $P = 0.01$ ), A-CXL (9\*13'20", 7.2;  $P = 0.003$ ), and A-CXL (18\*6'40", 10;  $P = 0.0001$ ).

**Conclusions:** An accelerated high-fluence epithelium-off CXL protocol (18 mW/cm<sup>2</sup> for 9'15") was identified to provide a significantly greater stiffening effect than any other accelerated protocols and is indistinguishable from the Dresden protocol, with accelerating irradiation times ranging from 30 to 9 minutes; by combining gentle acceleration with higher fluence, such a protocol does not require supplemental oxygen.

**Translational Relevance:** This A-CXL (18\*9'15", 10) protocol has the potential to become a new standard in epithelium-off CXL, delivering Dresden protocol-like strengthening over a shorter period.

## Introduction

Keratoconus (KC) is the most common corneal ectasia and is characterized by progressive corneal thinning and bulging outward into a cone-like shape. A result of reduced corneal biomechanical strength, KC can severely impact patients' vision.<sup>1</sup> Modern diagnostic equipment and studies in different parts of the world have revealed that the prevalence of keratoconus is considerably higher than what was previously reported and that the classification of KC as a rare disease (prevalence less than 1:2000) must be reconsidered.<sup>2-7</sup>

The normal human cornea shows a considerable degree of structural anisotropy. It is characterized by two preferred collagen fibril orientations orthogonal to each other. Alteration of the regular orthogonal arrangement of the fibrils in keratoconus may be related to the biomechanical instability of the tissue.<sup>8</sup> Reduction of collagen cross-links and a reduction of molecular bonds between neighboring stromal proteoglycans are thought to be relevant to decreased stiffness of the keratoconus corneas.<sup>9</sup>

Collagen is shown to contain several different lysyl oxidase-related (enzymatic) and lysin-derived (nonenzymatic) covalent cross-links. Non-enzymatic cross-linking of collagen, also referred to as glycation, is age related, and there is strong evidence that the total cross-link content of collagen is increased in diabetes. This leads to changes in the physicochemical properties, such as strength, viscosity, stiffening, and thickening.<sup>9</sup>

Corneal cross-linking (CXL) with riboflavin and ultraviolet-A (UVA) light was developed and first introduced into clinical practice in 1998 by a group based in Dresden, Germany.<sup>10</sup> The standard Dresden protocol (S-CXL) involves abrasion of the corneal epithelium (epi-off) and the application of 3 mW/cm<sup>2</sup> UVA irradiation for 30 minutes (3\*30), to deliver a total fluence of 5.4 J/cm<sup>2</sup> in corneas no thinner than 400 μm.<sup>11,12</sup> These technical parameters were chosen to protect the corneal endothelium and deeper structures, such as the lens and retina, from UV-induced damage.<sup>12</sup> The Dresden epi-off protocol provides a success rate of 93% to 97%,<sup>13</sup> and in Europe the introduction of CXL into clinical practice has led to a significant decrease in the number of corneal transplantations in KC.<sup>14,15</sup>

In CXL with riboflavin-UVA treatment, lysin-based cross-links have been hypothesized.<sup>16</sup> Exposing riboflavin to UVA light promotes its photomediator properties and extends the effect of the irradiation to the surrounding tissue. Following exposure, riboflavin is excited into a triplet state, thereby generating reactive oxygen species, singlet oxygen, and superoxide anions.

These then act to induce the formation of new covalent bonds and cross-links among the amino acids of neighboring collagen fibers.<sup>17</sup>

Photochemical reactions can be described by the Bunsen-Roscoe law of reciprocity. In effect, this law states that if all reagents of a reaction (riboflavin, oxygen, and UVA light for CXL) are in excess, the amount of photochemical reaction that occurs is determined by the total amount of light delivered (in this case, the total UVA fluence), irrespective of whether the total energy is delivered as 3 mW/cm<sup>2</sup> for 30 minutes or as 30 mW/cm<sup>2</sup> for 3 minutes—both deliver a total fluence of 5.4 J/cm<sup>2</sup>. This was the rationale for developing accelerated CXL protocols, as decreasing the procedure time benefits both patients and physicians.<sup>18</sup> However, this law of reciprocity is not applicable to the stiffening effect of CXL, as accelerated protocols providing more intensity in less time result in reduced stiffness and shallower demarcation lines.<sup>18-20</sup> In these cases, the essential reagent oxygen is depleted at a faster rate than replenishment occurs, making oxygen diffusion into the cornea the rate-limiting step.<sup>21</sup>

The aim of this study was to identify epi-off protocols under laboratory settings that would allow for acceleration of the CXL reaction while maintaining the excellent stiffening effect of the standard 30-minute Dresden cross-linking (S-CXL) protocol.

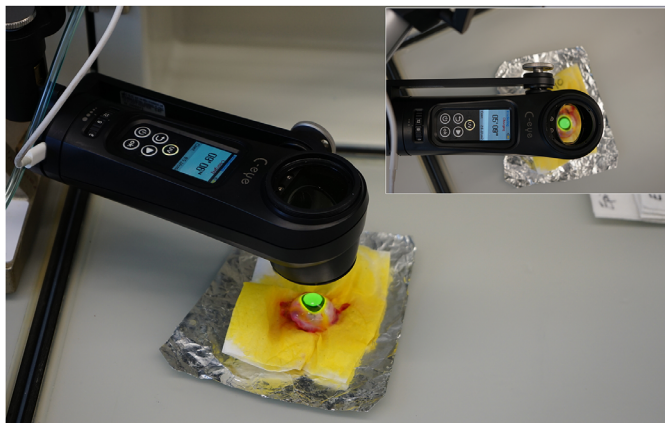
## Materials and Methods

### Specimens

Three hundred freshly enucleated porcine eyes were obtained from the local slaughterhouse (Zurich, Switzerland) and were used within 12 hours. Eyes with an intact epithelium were randomly assorted into six different groups ( $n = 50$  for each group).

### Experimental Protocols

The CXL procedure was performed as described previously. In brief, all corneas were de-epithelialized using surgical blades, followed by the application of an iso-osmolar riboflavin 0.1% solution to the corneal surface every 2 minutes for 20 minutes; all corneas (other than controls) were then irradiated with UVA light (365 nm) using a cross-linking device (C-eye; EMAGine AG, Zug, Switzerland) (Fig. 1). CXL was performed using five different epi-off protocols: S-CXL (3\*30, 5.4 J/cm<sup>2</sup>); accelerated with equivalent total fluence, A-CXL (9\*10, 5.4 J/cm<sup>2</sup>); and three accelerated protocols with increased total fluence—A-CXL (9\*13'20", 7.2 J/cm<sup>2</sup>), A-CXL (18\*6'40", 7.2 J/cm<sup>2</sup>),



**Figure 1.** The C-eye CXL device performing a CXL procedure in an ex vivo porcine cornea.

and A-CXL (18\*9'15", 10 J/cm<sup>2</sup>). Control corneas were prepared similarly but not irradiated. All corneoscleral buttons were excised right after cross-linking and were kept in a 400-mOsm/L phosphate-buffered saline (PBS) solution 10 minutes prior to the biomechanical measurements to standardize the hydration state of all the samples. Details are summarized in [Table 1](#).

### Biomechanical Measurements

The biomechanical properties, including elastic and viscoelastic testing of all corneas within the center area, were determined using a stress–strain extensometer as described previously.<sup>19,22,23</sup> The corneoscleral buttons were excised, and two central corneal strips (5 mm wide) were prepared in the horizontal axis of each corneoscleral button and were mounted vertically between the two arms of the stress–strain extensometer. Four millimeters of the end of each strip were dedicated to fixation, leaving approximately 11 mm of central corneal strip length for biomechanical measurement. Each corneal strip was mounted within the device and preloaded with 0.2 N and elongated by increasing the load from 0.2 to 4 N at a velocity of 2 mm/min. Tensile strength was measured using a stress–strain extensometer (zwickiLine Z0.5; Zwick-

Roell, Ulm, Germany), calibrated with a distance accuracy of 2 mm and a tensile sensor with no more than 0.21% of measurement uncertainty ([Fig. 2](#)). The stress–strain curve was recorded. The slope of the stress–strain curve corresponds to the elastic modulus and was determined between 1% and 5%. Data analysis was performed using the testXpert II software (Zwick-Roell).

### Statistical Analysis

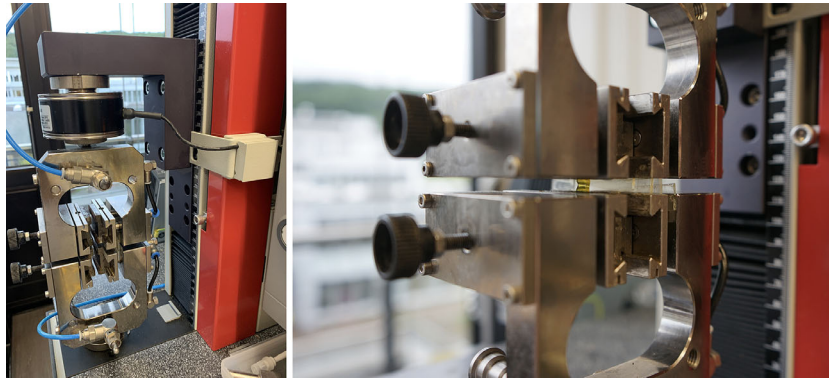
Statistical analysis was performed using Excel 11 for Mac (Microsoft Corporation, Redmond, WA) and Prism 8.0 (GraphPad Software, San Diego, CA). The Shapiro–Wilk test and Kolmogorov–Smirnov test were used as normality tests. As some of the groups were not normally distributed, a Kruskal–Wallis test was used to verify statistically significant differences with a confidence interval of 95% between groups.  $P \leq 0.05$  was considered significant.

## Results

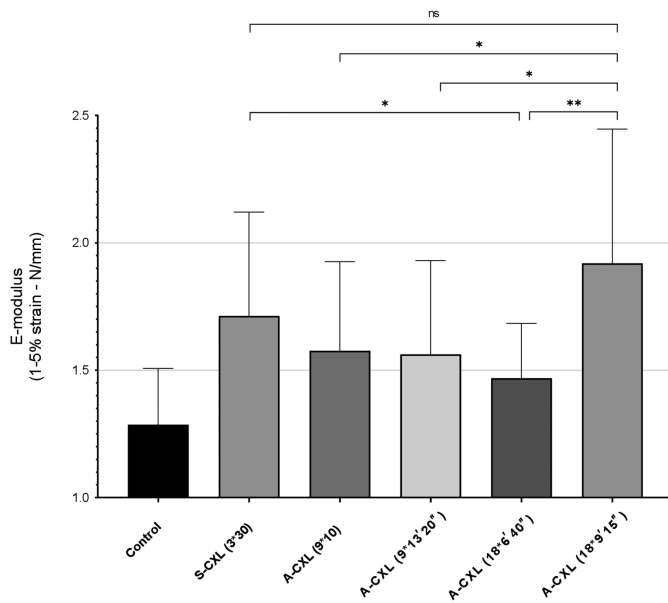
[Figure 3](#) provides a comprehensive overview of the results achieved using the different protocols. [Table 1](#) provides an overview of the various CXL protocols used. All five irradiated groups produced a significant increase in stiffness compared with nonirradiated controls ( $P < 0.05$ ). The normalized stiffening effects were as followed: 133% in the S-CXL (3\*30) group, 122% in the A-CXL (9\*10, 5.4) group, 120% in the A-CXL (9\*13'20", 7.2) group, 114% in the A-CXL (18\*6'4", 7.2) group, and 149% in the A-CXL (18\*9'15", 10) group. Accordingly, the new 10-J/cm<sup>2</sup> accelerated high-fluence CXL group showed the highest stiffening effect among all experimental groups. A comparison of the mechanical outcomes of the varying CXL protocols by quantifying the percentage change in elastic modulus at 1% to 5% strain with respect to each control found that there were significant differences between A-CXL (18\*9'15", 10) and

**Table 1.** Experimental Protocols

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Total fluence (J/cm <sup>2</sup> )	5.4	5.4	7.2	7.2	10	0
Soak time (min); interval	20; q2	20; q2	20; q2	20; q2	20; q2	20; q2
Intensity (mW/cm <sup>2</sup> )	3	9	9	18	18	—
Treatment time	30 min	10 min	13 min 20 s	6 min 40 s	9 min 15 s	—
Epithelium status	Off	Off	Off	Off	Off	Off
Chromophore	Riboflavin, 0.1%	Riboflavin, 0.1%	Riboflavin, 0.1%	Riboflavin, 0.1%	Riboflavin, 0.1%	Riboflavin, 0.1%
Light source	C-eye device	C-eye device	C-eye device	C-eye device	C-eye device	—
Irradiation mode	Continuous	Continuous	Continuous	Continuous	Continuous	No irradiation
Protocol abbreviation in article	S-CXL (3*30)	A-CXL (9*10)	A-CXL (9*13'20")	A-CXL (18*6'40")	A-CXL (18*9'15")	Control



**Figure 2.** The zwickiLine Z0.5 stress-strain extensometer during the stress-strain measurement process.



**Figure 3.** Elastic modulus (E-modulus) at 1% to 5% strain of various cross-linking protocols. There was a significant difference between the control group and all other irradiated groups ( $P \leq 0.05$ ). \* $P = 0.05$  to 0.005; \*\* $P \leq 0.005$ . Irradiation settings are expressed as intensity in  $mW/cm^2$  \* time (minutes, seconds).

three other accelerated protocols: A-CXL (9\*10, 5.4;  $P = 0.03$ ), A-CXL (9\*13'20", 7.2;  $P = 0.008$ ), and A-CXL (18\*6'40", 7.2;  $P = 0.0001$ ). There were no significant differences between Dresden protocol S-CXL (3\*30, 5.4)-treated corneas and those in the A-CXL (9\*10, 5.4), A-CXL (9\*13'20", 7.2), or A-CXL (18\*9'15", 7.2) groups, but there was a significantly higher percentage difference in elastic modulus between Dresden protocol S-CXL (3\*30, 5.4) and A-CXL (18\*6'40", 10;  $P = 0.04$ ). Table 2 shows the mean values with standard deviations and the stiffening effect of each group, and Table 3 shows the  $P$  values found between each condition tested.

## Discussion

The Dresden protocol has remained the gold standard approach for cross-linking ectatic corneas since its introduction over 20 years ago. Part of the reason why the slow, low-intensity approach of  $3\text{-}mW/cm^2$  UVA irradiation for 30 minutes was chosen was simply because of technical limitations at the time. Higher intensity UV light-emitting diodes were not readily available in 1998 (E. Spoerl, personal communication). Although the time-saving benefits to patients and doctors of faster, high-intensity UV irradiation are evident, the trade-off until now was efficacy. Another limitation of the initial Dresden protocol was the total fluence of  $5.4\text{ J/cm}^2$ . This fluence was chosen along with a minimal corneal stromal thickness of  $400\text{ }\mu\text{m}$  to ensure that the published UV damage threshold level of  $0.36\text{ J/cm}^2$  for corneal endothelial cells was not reached.

Newer studies suggest that the irradiation damage threshold for the corneal endothelium is far higher than previously assumed and that the total fluence that could be delivered safely to the cornea during a CXL procedure may be substantially higher than the limits specified in the Dresden protocol.<sup>24</sup> This opened the floodgates for high-fluence accelerated CXL protocols. Now, the challenge is to identify an epi-off protocol that puts UVA intensity, irradiation time, and total fluence into a working relationship to accelerate the CXL procedure, all while maintaining sufficient oxygen supply to the cornea to ensure the excellent biomechanical results achieved with the original Dresden protocol.

In 2018, we published the proof-of-principle to combine all factors involved by demonstrating that even transepithelial cross-linking can achieve a stronger stiffening effect in the absence of supplemental oxygen by slowing down or de-accelerating the CXL process from 30 minutes to 60 minutes.<sup>25</sup> Accordingly, Matthys

**Table 2.** Mean  $\pm$  SD of Elastic Modulus and Stiffening Effect at 5% Strain

Group	Protocol	UVA Irradiation (Intensity, Time)	Total Fluence (J/cm <sup>2</sup> )	Elastic Modulus (Pascal)	Normalized Stiffening (%)
1	S-CXL (3*30)	3 mW/cm, 30 min	5.4	1.71E $\pm$ 0.40E	133
2	A-CXL (9*10)	9 mW/cm <sup>2</sup> , 10 min	5.4	1.57E $\pm$ 0.34E	122
3	A-CXL (9*13'20'')	9 mW/cm <sup>2</sup> , 13 min 20 s	7.2	1.56E $\pm$ 0.36E	120
4	A-CXL (18*6'40'')	18 mW/cm <sup>2</sup> , 6 min 40 s	7.2	1.46E $\pm$ 0.21E	114
5	A-CXL (18*9'15'')	18mW/cm <sup>2</sup> , 9 min 15 s	10	1.92E $\pm$ 0.52E	149
6	Control	—	—	1.28E $\pm$ 0.22E	100

**Table 3.** P Values From the Kruskal–Wallis Test Comparisons Between Each Combination of Treatment Groups (Elastic Modulus at 1%–5% Strain)

Comparison	P
Control vs. S-CXL (3*30)	<0.0001 <sup>a</sup>
Control vs. A-CXL (9*10)	0.0002 <sup>a</sup>
Control vs. A-CXL (9*13'20'')	0.0009 <sup>a</sup>
Control vs. A-CXL (18*6'40'')	0.0365 <sup>a</sup>
Control vs. A-CXL (18*9'15'')	<0.0001 <sup>a</sup>
S-CXL (3*30) vs. A-CXL (9*10)	>0.999
S-CXL (3*30) vs. A-CXL (9*13'20'')	0.5508
S-CXL (3*30) vs. A-CXL (18*6'40'')	0.0325 <sup>a</sup>
S-CXL (3*30) vs. A-CXL (18*9'15'')	>0.999
A-CXL (9*10) vs. A-CXL (9*13'20'')	>0.999
A-CXL (9*10) vs. A-CXL (18*6'40'')	>0.999
A-CXL (9*10) vs. A-CXL (18*9'15'')	0.014 <sup>a</sup>
A-CXL (9*13'20'')	>0.999
A-CXL (9*13'20'')	0.0036 <sup>a</sup>
A-CXL (18*6'40'')	<0.0001 <sup>a</sup>

<sup>a</sup>Significant (calculated with Kruskal–Wallis test).

et al.<sup>26</sup> showed recently that the introduction of supplemental oxygen and pulsed high-fluence settings allows for acceleration of transepithelial CXL.

In our experiments, the ideal settings were achieved when using an accelerated high-fluence (10 J/cm<sup>2</sup>) setting of 18 mW/cm<sup>2</sup> of intensity for a duration of 9 minutes and 15 seconds, which provided a significantly greater stiffening effect than any other accelerated protocols evaluated. When compared with the benchmark epi-off Dresden protocol with a stiffening effect of 133%, our protocol was indistinguishable with a 149% increase in the elastic modulus, despite accelerating treatment from 30 minutes to just over 9 minutes. Supplemental oxygen was not necessary in this protocol; apparently, the choice of the various parameters allowed for sufficient oxygen diffusion to maintain the cross-linking process without limiting oxygen supply.

Clinically, studies have compared functional outcomes between high-fluence accelerated protocols and the traditional Dresden protocol. By comparing 1-year postoperative clinical outcomes of standard,

accelerated, and high-fluence accelerated protocols, Lang et al.<sup>27</sup> observed that the improvement in maximal keratometry and corrected visual acuity was similar in the groups that used irradiations of 3 mW/cm<sup>2</sup> for 30 minutes, 9 mW/cm<sup>2</sup> for 10 minutes, or 30 mW/cm<sup>2</sup> for 4 minutes. Despite this similarity, the standard Dresden protocol still results in greater changes in keratoconus and regularization corneal indexes.<sup>27</sup> Moreover, CXL often induces a hyperopic shift in corneas, as strengthening the cornea increases its resistance to the intraocular pressure that causes the cone-like protrusions in weakened areas of the cornea. There have been reports in the literature of topography-guided CXL being used with fluences ranging from 5.4 to 10 J/cm<sup>2</sup> to induce selective flattening effects to reduce the steepness of keratoconic corneas, inducing a hyperopic shift in these highly myopic corneas.<sup>28</sup> It is therefore possible that clinical use of CXL that employs the parameters used in this experiment will also induce a hyperopic shift. Accordingly, careful consideration of the current refractive state is mandatory when the appropriate CXL protocol is chosen.

Our study has some limitations. Because this was an ex vivo study, despite its approach being widely established methodologically, our extensometry findings may not be fully equivalent to the biomechanical response in vivo. Also, an ex vivo study does not allow corneal remodeling to be estimated. In 2017, our group identified several target genes that might be related to the biomechanical stability and shape of the cornea, and we were able to observe distinct changes in gene transcription in accelerated CXL protocols.<sup>29</sup> Ex vivo studies cannot predict the long-term biological changes that will occur in patients after the application of cross-linking protocols. Interestingly, Seiler and colleagues<sup>28</sup> did not observe excessive amounts of haze clinically when using a 10-J/cm<sup>2</sup> high-fluence protocol.

In conclusion, if the results of this study translate into similar effects in clinical practice, this new accelerated, high-fluence approach holds the potential of becoming a new standard in epi-off CXL, delivering Dresden protocol–like levels of biomechanical stiffening in a fraction of the time, reducing the overall costs

in operating room settings and opening the procedure to slit-lamp-based approaches.<sup>30</sup>

## Acknowledgments

Supported in part by the Light for Sight Foundation (Zurich, Switzerland) and Velux Stiftung (Zurich, Switzerland).

Disclosure: **R. Abrishamchi**, None; **H. Abdshahzadeh**, None; **M. Hillen**, None; **N. Hafezi**, EMAGine AG (E); **E.A. Torres-Netto**, None; **I.M. Aslanides**, None; **S. Chen**, None; **J.B. Randleman**, None; **F. Hafezi**, Apparatus for the treatment and/or prevention of corneal diseases (P), EMAGine AG (I)

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