Corneal cross-linking (CXL) with riboflavin and UV-A is a treatment modality for keratoconus that was first developed in Dresden, Germany in 1998. Per the typical cross-linking protocol, 0.1% riboflavin solution with 20% dextran is added to the de-epithelialized cornea and then photoactivated with UV-A light at 365 nm with irradiance of 3 mW/cm² 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. The effect of treatment can be assessed postoperatively using the Ocular Response Analyzer (Reichert Inc., Buffalo, NY, USA). The depth of treatment can be measured by the demarcation line, which usually appears at 10 to 14 days after CXL. 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.

Collagen cross-linking experienced a rapid transition from laboratory procedure to clinical intervention because of the method’s apparent safety and broad array of potential applications. 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. Collagen cross-linking stops the progression of keratoconus in patients with mild disease, presumably by strengthening the cornea and preventing further bulging. Collagen cross-linking has also been used successfully in the treatment of pellucid marginal degeneration to stabilize early stage keratoconus and to treat iatrogenic (postoperative) ectasia. Collagen cross-linking 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 irradiances 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/cm² 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/cm².

### Materials and Methods

#### Corneal Cross-Linking (CXL)

Collagen cross-linking was performed as described previously.19 Briefly, freshly enucleated pig eyes with intact epithelium were obtained from a local slaughterhouse in Geneva and randomly sorted 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 epithelial-off (epi-off) CXL procedure was performed using the Schwind CCL-365 Vario system (SCHWIND eye-tech-solutions GmbH & Co., Kleinotheim, Germany) All corneas were irradiated on a diameter of 11.3 mm using a total energy dose of 5.4 J/cm². Group 1 was irradiated with 3 mW/cm² for 30 minutes. Group 2 was irradiated with 9 mW/cm² for 10 minutes. Group 3 was irradiated with 3 mW/cm² for 30 minutes. Group 2 was irradiated with 18 mW/cm² for 5 minutes. Unirradiated corneas served as controls (group 4).

#### Biomechanical Measurements

Corneas from the four groups were allowed to rest in a wet chamber for 30 minutes 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).

### Statistical Analysis

Data were analyzed with Xlstat 2013 for Windows (Addinsoft, version 2013.4.03; Addinsoft, Paris, France). All data are expressed as the mean ± SD. Normal distribution of data was evaluated by the Shapiro-Wilk test. The Young’s modulus of all different groups was compared using the nonparametric Kruskal-Wallis one-way ANOVA. When significant, we proceeded to the nonparametric Mann-Whitney U test of the null hypothesis (H0 = populations are the same). A P value less than 0.05 was considered statistically significant.

### Results

The average Young’s modulus was determined for each of the four groups and percentage strains (Table 1). Young’s modulus of corneas that underwent CXL decreased with increasing UV light irradiance. The average Young’s modulus at 10% strain was 11.54 Mpa (±3.02) for the control group, 15.85 Mpa (±3.96) for the 3 mW/cm² group, 13.48 Mpa (±3.56) for the 9 mW/cm² group, and 12.90 Mpa (±3.86) for the 18 mW/cm² group, respectively (Table 1, Fig.).

At 10% strain, Young’s modulus showed a significant global difference between groups was found according to the nonparametric Kruskal-Wallis test for the four groups (P < 0.0001). The P values for the nonparametric Mann-Whitney U tests comparing two groups indicated significant differences between 3 mW/cm² and 9 mW/cm² (P = 0.002), 9 mW/cm² and 18 mW/cm² (P = 0.0002), 3 mW/cm² and the control group (P < 0.0001), 9 mW/cm² and the control group (P = 0.015), and 18 mW/cm² and the control group (P = 0.064). There was no difference in the Young’s modulus of the 9 mW/cm² and 18 mW/cm² groups (P = 0.503) in the 10% strain group (Table 2).

### Table 1. Young’s Modulus at Various UV-A Light Irradiances

<table>
<thead>
<tr>
<th>UV-A Light Irradiance</th>
<th>% Strain 10</th>
<th>Standard deviation 10</th>
<th>Kruskal-Wallis P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Control</td>
<td>11.54</td>
<td>3.02</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3 mW/cm² for 30 min</td>
<td>15.85</td>
<td>3.96</td>
<td></td>
</tr>
<tr>
<td>9 mW/cm² for 10 min</td>
<td>13.49</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>18 mW/cm² for 5 min</td>
<td>12.89</td>
<td>3.86</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. P Values Resulting From Individual Mann-Whitney U Tests Between Young’s Modulus at Various UV-A Light Irradiances

<table>
<thead>
<tr>
<th>P Value</th>
<th>Untreated Control</th>
<th>3 mW/cm² for 30 min</th>
<th>9 mW/cm² for 10 min</th>
<th>18 mW/cm² for 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>&lt;0.0001*</td>
<td>0.015*</td>
<td>0.002*</td>
<td>0.064</td>
</tr>
<tr>
<td>3 mW/cm² for 30 min</td>
<td>&lt;0.0001*</td>
<td>0.015*</td>
<td>0.002*</td>
<td>0.0002*</td>
</tr>
<tr>
<td>9 mW/cm² for 10 min</td>
<td>0.015*</td>
<td>0.002*</td>
<td>0.503</td>
<td></td>
</tr>
<tr>
<td>18 mW/cm² for 5 min</td>
<td>0.064</td>
<td>0.0002*</td>
<td>0.503</td>
<td></td>
</tr>
</tbody>
</table>

* Significant.
DISCUSSION

The efficiency of CXL decreased significantly as UV-A light irradiances increased from 3 to 18 mW/cm². Indeed, corneas treated with the highest tested irradiance (18 mW/cm² 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.

Wernli et al.²⁰ evaluated Young’s modulus using the same total energy fluence and riboflavin concentration as in our study. They also observed a decrease in Young’s modulus for high irradiances, but only at irradiances exceeding 50 mW/cm². These differences might be explained by several factors. First, the groups had different sizes (10 vs. 50 eyes/group), second, the biomechanical measurements were performed at different times: Wernli and colleagues²⁰ took measurements at 30 minutes after starting irradiation, regardless of irradiation time. By contrast, we consistently performed measurements at 30 minutes after the end of irradiation. Another difference is that Wernli and colleagues²⁰ kept corneas immersed in the riboflavin solution. This extended exposure to riboflavin likely increased the amount of riboflavin penetration and subsequent different cross-linking activity.

Also, we observed a Young’s modulus that was approximately a factor 2 larger than in the Wernli study. Several factors might be responsible for these differences. First, the machines for biomechanical measurements were not the same (Zwick Z 0.5 versus MINIMAT; Stretton Shropshire) and second, the methods were slightly different (time before biomechanical testing, length of the corneal strips 10 vs. 7 mm). Other, yet unidentified aspects might have further influenced the differences observed.

Lastly, the Wernli study²⁰ 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 eye-tech-solutions GmbH & Co., 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.²² 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 with controls. 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 colleagues²² used SAM. Without a doubt, the 9 mW/cm² for 10 minutes setting provides cross-links to the cornea.²² Another difference is that Wernli and colleagues²⁰ kept corneas immersed in the riboflavin solution. This extended exposure to riboflavin likely increased the amount of riboflavin penetration and subsequent different cross-linking activity.

In conclusion, we report a steady and significant decline in 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.

Acknowledgments

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FIGURE. Young’s Modulus by 10% of strain at different UV-A light irradiances. 3 mW/cm² of irradiance (blue), 9 mW/cm² of irradiance (red), and 18 mW/cm² of irradiance (green), control group (purple).
Disclosures:

A. Hammer, SCHWIND eye-tech-solutions (F); O. Richoz, SCHWIND eye-tech-solutions (F); P. S.A. Mosquera, None; D. Tabibian, SCHWIND eye-tech-solutions (F); F. Hoogewoud, SCHWIND eye-tech-solutions (F); F. Hafezi, SCHWIND eye-tech-solutions (F), P

References