Long-Term Biomechanical and Histologic Results of WST-D/NIR Corneal Stiffening in Rabbits, Up to 8 Months Follow-up

Jurriaan Brekelmans,1,2 Alexandra Goz,2,3 Mor M. Dickman,1 Alexander Brandis,4 Xiaomeng Sui,5 H. Daniel Wagner,7 Rudy M. M. A. Nuijts,1 Avigdor Scherz,2 and Arie L. Marcovich2,3

1University Eye Clinic Maastricht, Maastricht, The Netherlands
2Department of Plant Sciences and Environmental Health, the Weizmann Institute of Science, Rehovot, Israel
3Department of Ophthalmology Kaplan Medical Center, Rehovot, Israel
4Department of Biological Services, the Weizmann Institute of Science, Rehovot, Israel
5Department of Materials and Interfaces, the Weizmann Institute of Science, Rehovot, Israel

Keratoconus, a bilateral progressive condition characterized by reduced corneal strength, progressive corneal thinning, and decreased visual acuity, is the leading cause of corneal transplantation at an early age, with high personal and societal costs.1,2 Corneal collagen cross-linking (CXL) using riboflavin (RF) and ultraviolet A (UV-A) irradiation is the first, and currently the only, clinically approved treatment capable of effectively arresting keratoconus progression. RF/UV-A CXL provides additional stiffening to the cornea, bridging the gap between disease onset and natural arrest of progression. Indeed, recent reports confirm a decrease in corneal transplantations for keratoconus.

Despite its promising long-term results, RF/UV-A CXL has some disadvantages, mostly related to the toxic nature of UV-A irradiation. Ocular exposure to high doses of UV-A irradiance causes damage to deeper ocular structures, in particular the endothelium and the lens.5,6 It is therefore of the utmost importance that enough UV-A be absorbed by intrastromal RF, which can be achieved only in corneas with a minimal thickness of 400 μm.7–9 Keratoconus, an ectatic disorder, is characterized by progressive corneal thinning, rendering many patients unsuitable for RF/UV-A CXL.10

The cascade of events triggered by RF upon UV-A illumination, like that of many other photosensitizers, is initiated by generation of reactive oxygen species (ROS) that induce cross-links between collagen fibers.7 Our group has recently reported on the safety and efficacy of corneal stiffening using near-infrared (NIR) light to activate the bacteriochlorophyll-derived photosensitizer WST-D.11 NIR irradiation at a wavelength of 755 nm is safe to the eye and causes no
discomfort by itself. The ability to treat corneas of any thickness without endangering corneal endothelium or deeper ocular structures could potentially benefit patients deemed unsuitable for riboflavin/UV-A cross-linking.

Keratoconus progression knows a relatively short lifespan, with a general onset at adolescence and natural regression during the third or fourth decade of life. Accumulation of nonenzymatic glycation end products, causing a stiffening effect of approximately 16% per decade, has been suggested to underlie age-related regression. Therefore, to be successful, keratoconus treatment has to provide sufficient corneal stiffening and maintain stability for an extended period to bridge the period between onset and natural stabilization. We have shown that WST-D/NIR treatment results in significant corneal stiffening 1 month after treatment in an in vivo and ex vivo rabbit animal model without toxic effect to corneal endothelial cells. Safety of systemic WST-D administration has also been established through its use in vascular-targeted photodynamic therapy of prostate cancer. In the current study we set out to determine the long-term outcomes and histologic changes after WST-D/NIR treatment in an in vivo rabbit model, to evaluate the validity of this novel treatment for corneal CXL in a clinical setting.

**Materials and Methods**

**Animal Model**

One eye of twenty-three 3-month-old New Zealand White (NZW) rabbits was treated with WST-D/NIR according to standard protocol (Fig. 1). The rabbits had ad libitum access to food and water, and were housed at the Core Animal Facility of the Weizmann Institute of Science, Rehovot, Israel. All procedures were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research, and following approval of the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

**Treatment Procedure**

Anesthesia was induced by intramuscular injections of 35 mg/kg ketamine (Rhone Merieux, Lyon, France) and 5 mg/kg xylazine (Vetamed, Benyamina, Israel). The epithelium of the left eye was mechanically removed up to 2 to 3 mm of the limbus with a PRK spatula (Becton Dickinson, Franklin Lakes, NJ, USA). Treated eyes were impregnated for 20 minutes with 2.5 mg/mL WST-11 (Steba Biotech, Luxembourg, France), in a 20% dextran 500-kDa (WST-D, *Leuconostoc* spp. Mr 450,000–650,000; Sigma-Aldrich Corp., St. Louis, MO, USA) and 0.9% saline solution corrected to a pH of 7.2 to 7.3. WST-D-impregnated eyes were briefly rinsed with a few milliliters of artificial tears and irradiated for 30 minutes with a 755-nm NIR diode laser (CeramOptec, Bonn, Germany). Laser intensity at the corneal apex was confirmed to be 10 mW/cm² with a power meter (NOVEL; Ophir Optics, Jerusalem, Israel). Artificial tears (Tears Naturale Free; Alcon, Puurs, Belgium) were applied every 5 minutes during irradiation to prevent corneal dehydration. Immediately after treatment, Maxitrol ointment (Alcon) was applied and repeated once or twice daily until full re-epithelialization was confirmed by slit lamp evaluation with a fluorescent dye. The contralateral right eyes served as untouched controls and were kept closed during treatment. The rabbits were examined for corneal pathology by a trained cornea specialist (ALM) with a portable slit lamp, before treatment, regularly during epithelial healing, and directly before killing. Full epithelial regrowth was confirmed by fluorescent dye staining. One week (n = 4), 1 month (n = 6), 4 months (n = 9), or 8 months (n = 4) after treatment, rabbits were euthanized by intravenous injection of pentobarbital sodium (CTS Chemical Industries Ltd, Ktirat Malachi, Israel), and corneal samples were prepared for biomechanical or histologic evaluation.

**Corneal Pachymetry**

Central corneal thickness (CCT) was determined by ultrasound pachymetry ( Humphrey ultrasonic pachymeters; Humphrey Instruments, San Leandro, CA, USA) before treatment (all rabbits) and before euthanasia (in the 1-, 4-, and 8-month follow-up groups). The last pachymetry measurement was used to determine the tissue elastic modulus.

**Biomechanical Measurements**

After enucleation, corneoscleral buttons were prepared in the 1-, 4-, and 8-month follow-up groups, and placed endothelial side down on paraffin hemispheres matching the corneal curvature. Corneas in the 1-week follow-up group were not tested for their biomechanical strength, as they might not have completely returned to physiological conditions. Central strips of 4 ± 0.2-mm width with approximately 2 to 3 mm of sclera on both ends were cut in superior-inferior direction by using a self-constructed double-bladed cutter. Corneoscleral strips were kept on ice in closed humid Eppendorf containers pending biomechanical testing, approximately 2 hours after enucleation. The time between enucleation and biomechanical measurement was similar for all strips. Strips were mounted horizontally between two clamps at a gauge length of 6 mm of a biomaterial tester loaded with a 200 N load cell (Minimat; Rheometric Scientific GmbH, Benzheim, Germany). Clamp tightening was controlled...
to be 9 cN.m by using a calibrated screwdriver (Torqueleader, Surrey, UK). Clamp distance was increased with a constant speed of 1 mm/min, while the applied force and corresponding displacement were measured constantly. Stress was calculated by normalizing the applied force by the cross-sectional area before testing, and strain by normalizing the displacement by the gauge length expressed percentually. Figure 2 shows the derived stress–strain curves of both a WST-D/NIR treated and control cornea, with their respective slopes representing the cornea’s elastic modulus. Furthermore, in Figure 2 three distinguishable areas within the stress–strain curves are indicated. These areas represent different biomechanical properties: (1) a flat initial phase with nonlinear toe region, (2) a linear steep increase in the elastic region, and (3) a shallow increase during the plastic phase. Subsequently, a drop in force is seen, indicating breaking of the sample and end point of the measurement. The modulus was determined in the linear region (2), as this is considered the true elastic modulus in which corneas exhibit only elastic properties under the physiological influence of intraocular pressure.16,17 A blinded examiner (JB) determined the slope of the steep linear increase (i.e., the tissue’s elastic modulus) three times for each sample to assure objective determination of the start and end of the linear region. The average was used in further analyses.17,18

**Histologic Evaluation**

One hemisphere of each cornea in the 1-week and 8-month follow-up groups was fixed in 4% formaldehyde. The fixed hemispheres were mounted in paraffin in anatomic configuration conserving the original corneal curvature, and 4-μm-thick sagittal slices were cut. After staining with hematoxylin and cosin, five images of each sample were captured at ×200 magnification (Eclipse 80i; Nikon, Tokyo, Japan) with a high-resolution charge-coupled device camera (DS-Fi1; Nikon). On each image, a 300-μm-wide area in the center of the cornea was selected in which the coordinates of the stromal borders and keratocytes were manually determined by using the image-processing software Fiji and its Cell Counter plugin (Fig. 3).19 Keratocytes were counted in a random fashion, blinded from treatment procedure or follow-up time, according to a predetermined counting protocol developed together with a certified pathologist (Ori Brenner, PhD, Department of Veterinary Resources, Weizmann Institute of Science, Rehovot, Israel). In accordance with this protocol, a cell was counted if it showed a dark-stained and oval nucleus 10- to 20-μm wide in a horizontal orientation, and was at least 50 μm apart from other keratocytes in the same horizontal plane. All other cellular objects were considered to reside in a plane other than the section under evaluation and were therefore not included in the count. The corneal stroma was divided to anterior, middle, and posterior thirds, and the relative position of keratocytes throughout the stroma was determined by using a self-written MATLAB script (MATLAB R2016b; The MathWorks, Inc., Natick, MA, USA). In the treated eyes 1 week after treatment the depth of CXL was assessed by determining the average depth of keratocyte loss. An average depth of the five most anterior keratocytes was calculated, to limit the influence of single remaining keratocytes in the cross-linked stroma, and expressed as percentage of total stromal thickness (Fig. 3).

**Statistical Analyses**

Elastic moduli and keratocyte counts were analyzed by using a repeated-measures analysis of variance (ANOVA), with treatment (treatment versus control) as within-subject effect and follow-up time as between-subject effect. A dependent...
Student’s t-test was applied to the mean elastic moduli, central corneal pachymetry, and keratocyte counts of control and treated corneas. The level of statistical significance was set at 0.05 for all analyses. Statistical calculations were done with Statistica version 12 (StatSoft, Inc., Tulsa, OK, USA).

**RESULTS**

**Corneal Pachymetry**

Table 1 shows the mean CCT for control and treated corneas, measured just before treatment (all corneas) and before extensiometry (1-, 4-, and 8-month groups). No significant differences were found between control and treated eyes before treatment and before extensiometry at all follow-up moments.

<table>
<thead>
<tr>
<th></th>
<th>Central Corneal Thickness, Control Eyes</th>
<th>Central Corneal Thickness, Treated Eyes</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1 Week</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>4</td>
<td>399.5, 95% CI (373.6, 425.4)</td>
<td>0.275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>401.3, 95% CI (375.8, 426.7)</td>
<td></td>
</tr>
<tr>
<td><strong>1 Month</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>6</td>
<td>395.5, 95% CI (354.4, 436.6)</td>
<td>0.436</td>
</tr>
<tr>
<td>Pre-extensiometry</td>
<td>6</td>
<td>598.2, 95% CI (354.9, 441.4)</td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>387.2, 95% CI (354.2, 420.1)</td>
<td>578.8, 95% CI (354.6, 403.1)</td>
<td></td>
</tr>
<tr>
<td><strong>4 Months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>9</td>
<td>411.9, 95% CI (393.9, 429.9)</td>
<td>0.842</td>
</tr>
<tr>
<td>Pre-extensiometry</td>
<td>9</td>
<td>422.8, 95% CI (400.8, 444.7)</td>
<td>0.645</td>
</tr>
<tr>
<td></td>
<td>411.7, 95% CI (394.2, 429.2)</td>
<td>426.1, 95% CI (409.9, 442.4)</td>
<td></td>
</tr>
<tr>
<td><strong>8 Months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>4</td>
<td>363.8, 95% CI (351.4, 376.1)</td>
<td>0.133</td>
</tr>
<tr>
<td>Pre-extensiometry</td>
<td>4</td>
<td>422.0, 95% CI (382.9, 461.1)</td>
<td>0.091</td>
</tr>
<tr>
<td></td>
<td>355.5, 95% CI (346.8, 364.2)</td>
<td>399.8, 95% CI (366.8, 432.7)</td>
<td></td>
</tr>
</tbody>
</table>

Mean and 95% confidence interval in μm. P value for paired Student’s t-test comparing treatment effect between control and treated eyes on the CCT.

**Elastic Modulus**

Four biomechanical measurements were excluded from analysis owing to accidental prestretching (n = 1, control group, 4 months), software error during measurement (n = 1, treatment group, 4 months), unequal strip preparation (n = 1, treatment group, 1 month), and slippage of the sample from the clamps during measurement (n = 1, treatment group, 8 months). Treatment was effective in all corneas compared to

**FIGURE 3.** Representative histologic evaluation of a 300-μm-wide central area of a control (left) and WST-D/NIR–treated (right) cornea. Per cornea, five areas were analyzed, and the average keratocyte count and CXL depth was used for statistical testing. Lines indicate anterior stromal border (blue), posterior stromal border (green), derived partitioning lines dividing the stroma in three equal parts (white), and CXL depth (red). Keratocytes of 10- to 20-μm width, with a horizontal distance of at least 50 μm, and a clear visible nucleus, are marked (black cross).
their paired untreated controls (Fig. 4). Corneal stiffness was significantly higher in treated eyes than their paired controls by 6.4 MPa (16.0 MPa, 95% CI [13.2, 18.8] vs. 9.6 MPa, 95% CI [5.1, 14.0], n = 5, P = 0.008), 5.5 MPa (18.1 MPa, 95% CI [14.0, 22.3] vs. 12.6 MPa, 95% CI [10.5, 14.7], n = 7, P = 0.005), and 4.4 MPa (18.6 MPa, 95% CI [9.5, 27.6] vs. 14.2, 95% CI [5.3, 23.1], n = 3, P = 0.010) at 1, 4, and 8 months after treatment, respectively (Fig. 5). A nonsignificant trend of gradual increase in corneal stiffness over time was noted in the control eyes, which might have reached significance given a larger sample size (factorial ANOVA; F_{2,12} = 2.620, P = 0.114).

**Keratocyte Count**

One pair (8 months) was excluded from analysis owing to problems during fixation, resulting in respectively four and three pairs, with five different sections per cornea, to be included in histologic examination at 1-week and at 8-month follow-up. Table 2 shows the average number of keratocytes counted per 300-μm-wide region of the corneal stroma, 1 week or 8 months after treatment, for control and treated eyes. Compared with paired untreated eyes, a highly significant decrease in the number of keratocytes was measured in the anterior stroma 1 week after treatment (P = 0.002), with an average CXL depth in treated eyes of 39.3% stromal thickness (95% CI [32.0, 46.5]). Eight months after treatment, the number of keratocytes in the anterior stroma did not differ between treated and control eyes (P = 0.562). Keratocyte count in the middle and posterior thirds of the stroma did not differ between control and treated eyes at 1 week and at 8 months and between treatment groups at 1 week and at 8 months (factorial ANOVA; F_{1,5} = 0.263, P = 0.630 and F_{1,5} = 0.022, P = 0.887, respectively).

**Adverse Effects**

During this study no adverse effects, such as corneal edema, loss of transparency, haze formation, scarring, or corneal thinning, were observed after WST-D/NIR treatment. The applied WST-D slightly stains the cornea red during treatment, but this coloring disappeared within 1 to 2 days. Epithelial regrowth occurred within 5 to 7 days in all rabbits without complications, and no difference in thickness between control and treated eye, as a sign of corneal decompensation, was seen at any time point (Table 1). Although keratocyte loss was found in the anterior stroma 1 week after treatment, no related adverse effects were observed.

**DISCUSSION**

This study showed that WST-D/NIR achieves effective and sustained corneal stiffening over a period up to 8 months in an in vivo rabbit animal model. During this period, a trend toward natural age-related corneal stiffening was seen in the untreated eye. A significant treatment effect was seen in all pairs at all time points.

**FIGURE 4.** Elastic modulus of control relative to paired treated corneas, 1, 4, and 8 months after treatment. Dashed line indicates similar stiffness of control and treated corneas, with values left of the line indicating a stiffening effect after treatment. A significant treatment effect was seen in all pairs at all time points.

**FIGURE 5.** Boxplot of elastic moduli of treated and paired control eyes, per follow-up time. Elastic modulus increased significantly in the treated eyes compared to their paired control from 9.6 ± 3.6 MPa to 16.0 ± 2.3 MPa (P = 0.008), 12.6 ± 2.3 MPa to 18.1 ± 4.5 MPa (P = 0.005), and 14.2 ± 3.6 MPa to 18.6 ± 3.6 MPa (P = 0.010) at 1, 4, and 8 months, respectively. In the control eyes a nonsignificant trend was seen of increasing corneal rigidity over time (F_{2,12} = 2.620, P = 0.114), which might have been significant given a larger sample size.

**TABLE 2. Keratocyte Count 1 Week and 8 Months After Treatment, per Stromal Region for Control and Treated Eyes**

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Control Mean (95% CI)</th>
<th>Treated Mean (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>4×5</td>
<td>12.9, 95% CI (11.4, 14.3)</td>
<td>14.4, 95% CI (9.3, 19.5)</td>
<td>0.463</td>
</tr>
<tr>
<td>Middle</td>
<td>4×5</td>
<td>12.8, 95% CI (10.2, 15.4)</td>
<td>12.1, 95% CI (8.8, 15.5)</td>
<td>0.640</td>
</tr>
<tr>
<td>Anterior</td>
<td>4×5</td>
<td>19.0, 95% CI (12.5, 25.5)</td>
<td>1.5, 95% CI (−1.2, 4.2)</td>
<td>0.002</td>
</tr>
<tr>
<td>8 Months</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior</td>
<td>3×5</td>
<td>11.2, 95% CI (9.0, 13.4)</td>
<td>12.4, 95% CI (8.6, 16.2)</td>
<td>0.355</td>
</tr>
<tr>
<td>Middle</td>
<td>3×5</td>
<td>11.2, 95% CI (6.8, 15.6)</td>
<td>12.1, 95% CI (1.9, 22.3)</td>
<td>0.810</td>
</tr>
<tr>
<td>Anterior</td>
<td>3×5</td>
<td>14.5, 95% CI (8.5, 20.6)</td>
<td>15.9, 95% CI (15.1, 18.6)</td>
<td>0.562</td>
</tr>
</tbody>
</table>

Mean and 95% confidence interval, in keratocytes per 300-μm-wide histologic section. For each corneal sample, the average keratocyte count of five different areas was used. P value for paired Student’s t test comparing control and treated eyes per follow-up time.
WST-D/NIR Corneal Stiffening: Long-Term Results

There is a limited understanding of the turnover of the corneal extracellular matrix and the exact biochemical changes during corneal CXL, limiting the prediction capability of the stiffening effect duration. The term (photosensitizer) “corneal collagen cross-linking,” such as used for RF/UV-A and WST/D/NIR treatment, suggests that the stiffening effect is based on the formation of additional (covalent) bonds between, and within, the corneal extracellular matrix. The photochemical events and their kinetics are however still under debate and there is only limited evidence that covalent bonds are formed directly between collagen fibrils after RF/UV-A CXL. Notably, the nontreated cornea in the aged rabbit approaches the stiffening of the treated cornea in the young rabbit. This finding suggests that 30-minute illumination after WST-D/NIR impregnation saturates the stiffening capability of the treated cornea. Indeed, in a very recent study 27 we show that delivering a lower light dose, by reducing irradiation time with the same light fluency, can result in a similar stiffening effect. A translation of this outcome to the clinical setting may significantly ease the treatment to the average patient.

To our knowledge, no studies have been performed comparing ageing of ocular tissue in rabbits to that in human, which is a limitation in the clinical translation of this study’s results. In NZW rabbits, where sexual maturation occurs at the age of 6 months, corneal parameters such as keratometry and CCT are known to stabilize at approximately 8 months of age. 28 With measurements done at the age of 4, 7, and 11 months, data provided here, concerning corneal biomechanics, complement the preclinical landscape by addressing a relatively wide age range, with a premature, an intermediate, and a mature age included. Furthermore, a similar long-term follow-up study in rabbits treated by RF/UV-A CXL shows results similar to this WST-D/NIR study. 30 Clinical data, with a follow-up of up to 10 years, available on RF/UV-A CXL, show persistent stabilization of keratoconus. 22–24

In summary, WST-D/NIR treatment resulted in significant and long-lasting corneal stiffening that sustains for at least 8 months after treatment, bridging the period until natural age-related stiffening occurs. Treatment affected the anterior 40% of the corneal stroma, based on full keratocyte depletion 1 week after treatment. Eight months after treatment, full keratocyte repopulation was observed. The excellent safety characteristics of nontoxic NIR irradiance and the ability to control WST-D penetration suggest WST-D/NIR treatment could be a safe alternative for patients with advanced corneal thinning unsuited for RF/UV-A CXL.

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