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,1 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

Correspondence: Arie L. Marcovich, Department of Plant Sciences and Environmental Health, the Weizmann Institute of Science, POB 12, Rehovot, 76100 Israel; arie.marcovich@gmail.com.

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PURPOSE. To determine the long-term safety and efficacy of WST-D/near-infrared (NIR) corneal stiffening.

METHODS. One eye of 23 New Zealand White rabbits was de-epithelialized mechanically followed by topical application of 2.5 mg/mL WST11, combined with dextran-500 (WST-D) for 20 minutes. Subsequently, samples were irradiated with a NIR (755 nm) laser at 10 mW/cm² for 30 minutes. Untreated fellow eyes served as controls. One week (n = 4), 1 month (n = 6), 4 months (n = 9), or 8 months (n = 4) after treatment rabbits were euthanized. Corneal strips were cut in superior-inferior direction for extensiometry testing (1, 4, and 8 months), and histologic sections were prepared for evaluation of keratocyte distribution (1 week and 8 months).

RESULTS. Elastic modulus after treatment was significantly higher than in paired controls (16.0 ± 2.3 MPa versus 9.6 ± 3.6 MPa [P = 0.008], 18.1 ± 4.5 MPa versus 12.6 ± 2.3 MPa [P = 0.003], and 18.6 ± 3.6 MPa versus 14.2 ± 3.6 MPa [P = 0.010], at 1, 4, and 8 months, respectively). A significant decrease in keratocyte count at the anterior stroma was observed directly after treatment (1.5 ± 1.7 vs. 19.0 ± 4.1 [P = 0.002]). At 8 months keratocyte repopulation appeared completed, with similar distribution in treated and untreated corneas (15.9 ± 1.1 vs. 14.5 ± 2.5 [P = 0.562]). Corneal thickness was comparable between treated and untreated corneas at all time points.

CONCLUSIONS. WST-D/NIR treatment resulted in significant and persistent long-term increase in corneal stiffness. Initial keratocyte apoptosis in the anterior stroma is followed by repopulation to normal level at 8 months after treatment. The safe nature of NIR light allows treatment of corneas of any thickness without endangering corneal endothelium or deeper ocular structures, potentially benefiting patients deemed unsuitable for riboflavin/UV-A cross-linking.

Keywords: cross-linking reagents, bacteriochlorophylls, photosensitizing agents, keratoconus, corneal ectasia

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 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 (Vitamed, Benyamina, Israel). The epithelium of the left eyes was mechanically removed up to 2 to 3 mm of the limbus with a PRK spatula (Becton Dickinson, Franklin Lakes, NJ, USA). Treated corneas were impregnated for 20 minutes with 2.5 mg/mL WST11 (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 0.9% saline solution to determine the tissue elastic modulus. WST-D/NIR Corneal Stiffening: Long-Term Results

Corneal Pachymetry
Central corneal thickness (CCT) was determined by ultrasound pachymetry (Humphrey ultrasonic pachymeters; Humphrey Instruments, San Leandro, CA, USA) before treatment (all animals) and after treatment 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-construct-ed 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.

FIGURE 1. Flowchart of steps in current study. Left eye of each rabbit was (1) mechanically de-epithelialized, (2) its central corneal thickness was determined with ultrasound pachymetry, (3) WST-D solution was applied topically for 20 minutes, and (4) the cornea was irradiated with NIR for 30 minutes. (5) One week, 1 month, 4 months, or 8 months after treatment the rabbits were euthanized. The cornea’s elastic modulus was determined by using strip extensometry 1 month, 4 months, and 8 months after treatment. Histologic slides were prepared both 1 week and 8 months after treatment.

4 months (n = 9), or 8 months (n = 4) after treatment, rabbits were euthanized by intravenous injection of pentobarbital sodium (CTS Chemical Industries Ltd, Kiryat Malachi, Israel), and corneal samples were prepared for biomechanical or histologic evaluation.
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.

**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

**TABLE 1. Mean Central Corneal Thickness Before Treatment and Before Extensiometry of Treated and Control Eyes, per Follow-up Time**

<table>
<thead>
<tr>
<th></th>
<th>$N$</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>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>4</td>
<td>401.3, 95% CI (375.8, 426.7)</td>
<td>399.5, 95% CI (373.6, 425.4)</td>
<td>0.275</td>
</tr>
<tr>
<td><strong>1 Month</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>6</td>
<td>387.2, 95% CI (354.2, 420.1)</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>578.8, 95% CI (554.6, 603.1)</td>
<td>598.2, 95% CI (554.9, 641.4)</td>
<td>0.378</td>
</tr>
<tr>
<td><strong>4 Months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>9</td>
<td>411.7, 95% CI (394.2, 429.2)</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>426.1, 95% CI (409.9, 442.4)</td>
<td>422.8, 95% CI (400.8, 444.7)</td>
<td>0.645</td>
</tr>
<tr>
<td><strong>8 Months</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>4</td>
<td>355.5, 95% CI (346.8, 364.2)</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>399.8, 95% CI (368.8, 432.7)</td>
<td>422.0, 95% CI (382.9, 461.1)</td>
<td>0.091</td>
</tr>
</tbody>
</table>

Mean and 95% confidence interval in $\mu m$. $P$ value for paired Student’s $t$-test comparing treatment effect between control and treated eyes on the CCT.
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 control eyes, which might have been significant given a larger sample size.
fellow eyes. Keratoconus often presents during adolescence, progressing over time up to the third or fourth decade of life. During this period of approximately 20 years, nonenzymatic glycosylation occurs, stiffening the cornea to a degree such that progression is arrested.\textsuperscript{20,21} To effectively prevent progression, the stiffening effect of cross-linking has to persist until natural corneal stiffening has occurred. The results of this study showed that the stiffening effect of WST-D/NIR persists up to 8 months when natural age-related stiffening reaches its highest values, in an in vivo NZW rabbit animal model.

Currently, RF/UV-A is the only clinically approved CXL technique, with reported success rates between 90% to 100% and follow-up up to 10 years.\textsuperscript{22–24} Despite excellent clinical outcomes, the classic Dresden protocol is unsuitable for corneas thinner than 400 μm, owing to potential damage to the corneal endothelium and deep ocular structures.\textsuperscript{5–9} WST-D has a peak excitation wavelength of approximately 755 nm in the NIR region, which provides deep tissue penetration, induces a low retinal response, and is safe to the eye for extended exposure at an irradiance of 10 mW/cm\textsuperscript{2}.\textsuperscript{25} Even higher irradiances are permitted for exposure times under approximately 16 minutes.\textsuperscript{25} The applied irradiance in this study (10 mW/cm\textsuperscript{2} for 30 minutes) is thus completely safe to the eye. In RF/UV-A CXL a minimal corneal thickness of 400 μm, fully impregnated with RF, is required to provide sufficient absorption. RF/UV-A irradiance thus prevents damage to the endothelium. Alternatively, NIR light is nontoxic and thus does not have to be absorbed by WST-D to prevent endothelial damage, potentially permitting safe corneal stiffening regardless of corneal thickness.

In addition to measuring the biomechanical effect, histology at the last follow-up was compared to that obtained shortly after treatment as a measure for long-term safety of WST-D/NIR cross-linking. We found loss of keratocytes in the anterior stroma throughout, covering an average of 40% of stromal thickness 1 week after treatment, with full repopulation at 8 months. Full keratocyte repopulation suggests WST-D/NIR treatment does not compromise long-term corneal cellularity. The ability of activated keratocytes to migrate within the corneal stroma and repopulate after damage is well known.\textsuperscript{26} Comparable keratocyte counts in the unaffected middle and posterior stroma of control and treated eyes, 1 week and 8 months after treatment, indicate that repopulation of the anterior stroma with keratocytes is independent of keratocyte counts in the middle and posterior stroma. Keratocyte repopulation therefore begins in the anterior direction. Keratocyte repopulation therefore begins from the periphery and follows the direction of the lamellae, rather than in posterior-anterior direction.

The underlying mechanism of keratocyte apoptosis is likely related to formation of ROS, superoxide and hydroxyl radicals, upon irradiation of WST-D by NIR light, similar to the reaction initiated by RF/UV-A, whereby mainly singlet oxygen is generated and initiates CXL. A local excess in ROS, causing oxidative stress to the cornea, could potentially be detrimental to the corneal endothelium. To avoid such toxicity, RF/UV-A applies light attenuation by a layer of RF that covers the cornea through treatment. Notably, RF penetration to the posterior cornea is not prevented by dextran. In contrast, the addition of high-molecular-weight dextran T500 to the WST11 solution limits its penetration depth and speed, and prevents damage to the corneal endothelium from ROS.\textsuperscript{11} This is in contrast to RF, where dextran maintains corneal dehydration, but does not influence RF penetration pattern. Dextran thus provides the possibility to control the penetration depth of WST-D and prevent localized damage to the endothelium by ROS formation, theoretically allowing safe treatment of patients with a thin cornea (CCT below 400 μm).

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\textsuperscript{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.\textsuperscript{28,29} 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.\textsuperscript{30} Clinical data, with a follow-up of up to 10 years, available on RF/UV-A CXL, show persistent stabilization of keratoconus.\textsuperscript{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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