Stiffening of Rabbit Corneas by the Bacteriochlorophyll Derivative WST11 Using Near Infrared Light

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PURPOSE. We evaluated the efficacy and safety of photochemical corneal stiffening by palladium bacteriochlorin 13-(2-sulfoethyl)amide dipotassium salt (WST11) and near infrared (NIR) illumination, using ex vivo and in vivo rabbit eye models.

METHODS. Corneas of post mortem rabbits and living rabbits were pretreated topically with 2.5 mg/mL WST11 in saline or in 20% dextran T500 (WST-D), washed and illuminated with an NIR diode laser (755 nm, 10 mW/cm2). Studies with corneas of untreated fellow eyes served as controls. Tensile strength measurements, histopathology, electron spin resonance, and optical spectroscopy and fluorescence microscopy were used to assess treatment effects. Comparative studies were performed with standard riboflavin/ultraviolet-A light (UVA) treatment.

RESULTS. WST11/NIR treatment significantly increased corneal stiffness following ex vivo or in vivo treatment, compared to untreated contralateral eyes. The incremental ultimate stress and Young’s modulus of treated corneas increased by 45, 113, 115%, and 10, 79, and 174% following 10, 20, and 30 minutes of incubation with WST11, respectively. WST-D/NIR had a similar stiffening effect, but markedly reduced post-treatment edema and shorter time of epithelial healing. WST11/NIR and WST-D/NIR generate hydroxyl and superoxide radicals, but no singlet oxygen in the cornea. Histology demonstrated a reduction in the keratocyte population in the anterior half of the corneal stroma, without damage to the endothelium.

CONCLUSIONS. Treatment of rabbit corneas, with either WST11/NIR or WST-D/NIR, increases their biomechanical strength through a mechanism that does not involve singlet oxygen. The WST-D/NIR treatment showed less adverse effects, demonstrating a new potential for clinical use in keratoconus and corneal ectasia after refractive surgery. (Invest Ophthalmol Vis Sci. 2012;53:6378–6388) DOI:10.1167/iovs.12-9913

Treatment with riboflavin (RF) followed by ultraviolet-A (UVA, 370 nm) illumination, results in corneal stiffening, presumably due to collagen crosslinking (CXL). This method has been used increasingly for halting the progression of keratoconus and post-refractive laser surgery corneal ectasia.1–3 Yet, there are several drawbacks to this treatment, including the prolonged time of RF treatment (30 minutes), the prolonged eye exposure to UVA irradiation (30 minutes), and finally toxicity to keratocytes1–6 and corneal endothelial cells,7–9 making treatment of corneas thinner than 400 µm problematic.10,11 Hence, there is a need for a safer treatment that can stiffen the cornea with less risk to the patient.12,13 One possibility is to use photosensitizers that inflict corneal stiffening upon illumination, at a nonhazardous spectrum, such as near infrared (NIR, 755 nm).

Our group has synthesized chemical derivatives of the photosynthetic pigments (chlorophylls and bacteriochlorophylls) that may overcome the above drawbacks. Upon NIR illumination, these novel photosensitizers generate O2·− and •OH radicals.14–17 These compounds also serve in the treatment of prostate cancer, by tumor ablation via vascular-targeted photodynamic therapy.18–21 In particular, the water-soluble palladium bacteriochlorin 13-(2-sulfoethyl)amide dipotassium salt (WST11)17 is presently in phase III clinical trials.22 Based on the photogeneration of oxygen radicals and the possible role of these radicals in promoting protein crosslinking,23 we hypothesized that topical application of WST11 on the cornea, followed by NIR illumination, should result in corneal stiffening for the treatment of keratoconus and similar pathologies. We also hypothesized that, for safety purposes, WST11 should be formulated with dextran T500 (WST-D). To our knowledge, we provide the first reported evidence in support of these hypotheses, demonstrating that corneal treatment by NIR illumination of the rabbit eye, after topical application of WST-D, consistently resulted in significant and prolonged corneal stiffening with minimal side effects.

METHODS

Photosensitizers

WST11 was supplied by STEBA Laboratories, Rehovot, Israel. Two formulations of WST11 were prepared: (1) in saline only, at a concentration of 2.5 mg/mL and pH adjusted to 7.3 (further referred as WST11), and (2) at 2.5 mg/mL in saline containing 20% dextran T500 (31,392 Fluka, Switzerland) adjusted to pH 7.3 (further referred as WST-D).
Two formulations of RF were used: (1) riboflavin-5'-phosphate 0.1% (Fl5750; Sigma-Aldrich, Rehovot, Israel) in saline, adjusted to pH 7.5 (RF), and (2) Riboflavin-5'-phosphate 0.1% in 20% dextran T-500, at pH 6.8 (Medio Cross, Medio-Haus Medizinprodukte GmbH, Neudorf, Germany; referred to as RF-D).

**Light Sources**

We used a diode laser with tunable output up to 1 W at 755 nm (CeramoOptec, Bonn, Germany) and a self-constructed device consisting of two 3 mW LEDs emitting at 370 nm (Roithner Lasertechnik, Vienna, Austria).

**Animal Models**

New Zealand White (NZW) rabbits were housed and handled with ad libitum access to food and water at the Core Animal Facility of the Weizmann Institute of Science (Rehovot, Israel). All experimental procedures were approved by the Institutional Animal Care and Use Committee, in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Biomechanical Testing of Corneal Stiffness**

**Ex Vivo Studies.** Eyes of euthanized rabbits (15–16 weeks old, 3–4 kg weight) were enucleated immediately postmortem. Before enucleation, for preservation of subsequent cutting orientation, the 12- and 6-hour positions on the sclera were marked. Central corneal thickness was measured by ultrasonic pachymetry (Humphrey ultrasonic pachometer; Humphrey Instruments, San Leandro, CA). The corneas were deepithelialized mechanically using a PRK scraper (Becton Dickinson, Franklin Lakes, NJ) and treated either by WST11/NIR (N = 10) or by RF-D/UV (N = 2).

In the WST11/NIR group, 10 eyes from euthanized rabbits were immersed upside down for 30 minutes in WST11 saline (2.5 mg/mL) solution, followed by NIR illumination (755 nm, 10 mW/cm²) for 30 minutes. The untreated contralateral eyes served as controls. In the RF-D/UV group, RF-D solution was applied topically on 2 eyes of 2 rabbits, every 10 minutes, for 30 minutes, followed by UVA illumination at 370 nm (3 mW/cm²) for 30 minutes. The corneas were deepithelialized mechanically using a PKR scraper. In the RF-D/UV group, RF-D solution was applied topically on 2 eyes of 2 rabbits, every 10 minutes, for 30 minutes, followed by UVA illumination at 370 nm (3 mW/cm²) for 30 minutes. After the treatment, the corneoscleral rings were removed and placed on paraffin hemisphere buttons with a matching shape to assure accurate sectioning without stretching the tissue. Corneal strips, 4 ± 0.2 mm wide, were cut from the epithelial side, as described above in the ex vivo section. The corneal strips were transferred to the biomechanical tester without delay.

**Biomechanical Testing.** The corneal strips were clamped horizontally, at a distance of 6 mm, between the jaws of a microcomputer-controlled biomechatr tester (Minimat; Rheometric Scientific GmbH, Benzheim, Germany) with a 200 Newton force cell. Controlled tightening of screws was performed with a calibrated screwdriver at a preset torque of 9 N.m (Torqueleader, Surrey, UK). The strain was increased linearly at a rate of 1.0 mm/min and was measured up to tissue rupture. Young's modulus was calculated by the testing machine. Ultimate stress and Young's modulus were expressed in MegaPascals (MPa).

**Corneal Drug Accumulation, Penetration, and Photobleaching**

**Accumulation of WST11 in the Rabbit Cornea.** Six eyes of 3 euthanized rabbits were deepithelialized mechanically immediately postmortem. Five eyes were exposed to WST11 for 10, 20, and 30 minutes using an eye cap, and one eye served as an untreated control. The corneas were removed, and the central buttons of 8-mm diameter were punched out with a round trephine and placed onto the outer wall of polyvinylmethacrylate cuvette in the area of light beam passage. Absorption spectra were recorded, and optical density (OD) at 755 nm was measured using V-570 spectrophotometer (Jasco, Tokyo, Japan).

**Depth of WST11 Penetration into the Rabbit Cornea.** A total of 20 rabbit eyes was enucleated and deepithelialized mechanically postmortem. An eye cap was used for pretreatment of the central cornea. The eyes were exposed to WST11 for 10 minutes (2 eyes), and 30 minutes (5 eyes), and to WST-D for 10 minutes (3 eyes) and 30 minutes (5 eyes) in darkness. Controls included untreated (4 eyes) using dextran T-500 only for 30 minutes (1 eye). Following pretreatment the corneas were rinsed briefly with saline, and the central 8-mm buttons were trephined, removed, wrapped in aluminum foil, and frozen on dry ice until further use. Central serial corneal sagittal slices (12 μm) were cut with a cryomicrotome, mounted on a microscope glass slide, and stored frozen in −70°C until use. For measurement of fluorescence, the individual slices were placed on the microscope base and a photographic record was taken immediately. Fluorescence intensity of WST11 or WST-D from 3 serial cryosections was recorded at 760 nm, upon excitation at 740 nm, using a fluorescence microscope (BX61 Olympus, Tokyo, Japan) equipped with CCD camera (Cascade 512B; Roper Scientific, Trenton, NJ) and a long pass filter of above 760 nm. The digital data were analyzed using ImageJ software (NIH, Bethesda, MD).

**Photobleaching of WST11.** The corneas of 10 eyes from five euthanized rabbits were deepithelialized. Eight eyes were pretreated for 20 minutes with WST11, using an eye cap. The solution excess was tipped off carefully with filter paper. The eyes were irradiated for the specified time durations: 0, 10, 30, and 60 minutes (two corneas per time frame). An additional two eyes were used as control. The corneas were removed, and central buttons of 8 mm diameter were punched out with a round trephine. Absorption spectra were recorded using V-570 spectrophotometer (Jasco).

**Histology**

One rabbit underwent treatment with WST11 in saline (20-minute incubation, 30-minute irradiation at 755 nm). Six rabbits underwent treatment with WST-D (N = 4, 20-minute incubation, 30-minute irradiation at 755 nm), or with RF-D (N = 2, 50-minute incubation, 30-minute irradiation at 370 nm) as described above (in vivo studies section). Contralateral eyes were used as control. After 48 hours, 5 rabbits (1 after WST11, 2 after WST-D and 2 after RF-D) were
euthanized, and the eyes were enucleated immediately and fixated in Davidson’s fixative. Two rabbits post WST-D were euthanized after 1 week, and the corneas were removed and fixated in 4% formaldehyde. We prepared 6 μm sections from either the whole eye, or corneas and stained with hematoxylin-eosin (H&E). The pathology of the corneal and retinal sections was examined. The sections were photographed with a digital video camera (Nikon DS-Ri1, Tokyo, Japan), mounted on a light microscope (E-800 Leica, Solms, Germany). Keratocyte and endothelial cell densities were counted in 2 areas of 2 central histologic sections and calculated, using Image-Pro software (MediaCybernetics, Rockville, MD). For apoptosis detection, three rabbits were euthanized one day after WST-D. Their corneas were removed, fixated in 4% formaldehyde and embedded in paraffin. Then, 6-μm central sections were prepared from the corneas, placed on microscope slides, and deparaffinized. Peroxidase-based TUNEL assay was performed according to the manufacturer’s instructions (Apop-Tag assay, Chemicon; Merck Millipore, Darmstadt, Germany).

Four rabbits underwent analysis of corneal endothelial damage 1 day post treatment, using alizarine red S and trypan blue staining. The treatment included either a 20-minute pretreatment with WST-D, followed by 30-minute irradiation at 755 nm (2 rabbits), or only a 30-minute irradiation at 755 nm (2 rabbits). Contralateral nontreated eyes served as controls.

Fluorescence Spectroscopy of the Rabbit Cornea after WST11/NIR and RF/UVA Treatment

Six rabbit eyes were enucleated postmortem. The corneal epithelium was deepithelialized with a scraper. Two eyes were immersed in WST11 and WST-D solutions for 30 minutes, followed by NIR irradiation (30 minutes). RF-D solution was applied on two eyes for 30 minutes, followed by UVA irradiation (30 minutes). Two eyes served as controls. The corneas were removed and the central 8-mm buttons were punched out with a round trephine. The buttons were mounted on a glass slide that was placed obliquely at 45° (10A) in a spectrophluorometer (Varian-Cary Eclipse; Varian Medical Systems, Palo Alto, CA). Excitation beam was set at 295, 315, 320, 328, and 335 nm, and corresponding emission spectra were read at 360–480 nm. Other readings were performed with excitation at 350 nm and emission at 380–480 nm, and excitation at 370 nm and emission at 400–700 nm. Excitation slit was 10 nm, emission slit –10 nm.

RESULTS

WST11 Uptake by Rabbit Corneal Tissue Ex Vivo

The Accumulation of WST11 in the Rabbit Cornea. The accumulation of WST11 in the corneal stroma was measured following incubation for 10, 20, and 30 minutes and determination of the optical absorption (600–900 nm) in the washed corneas. As shown in Figure 1, the optical density of the impregnated corneas increased with the time of incubation, without interference with the native spectrum of WST11.

The Depth of Penetration of WST11 into the Rabbit Cornea. Upon topical application, the penetration of the photosensitizer and depth of the photochemical impact on the corneal tissue were determined. The deeper the penetration, the higher was the probability of endothelial impairment. The penetration depth of WST11 into the de-epithelized cornea, following various incubation times, was determined in the absence or presence of dextran in eyes of euthanized rabbits. Sagittal frozen sections mounted on glass slides were subjected to fluorescence microscopy, as described above. The distribution of WST11 across the cornea was recorded photographically by fluorescence microscopy (excitation/emission 740/760 nm). Following 10 minutes of exposure, WST11 was evident through the entire outer half of the stroma (Fig. 2A). Further exposure (30 minutes) resulted in diffused fluorescence all the way to Descemet’s membrane (Fig. 2B). This treatment increased the stromal thickness from 360–380 μm to 600–730 μm. In contrast, application of WST-D limited the penetration depth to the outer 1/3 (200 of 520 μm) of the corneal stroma, at 10 and 30 minutes exposure times (Figs. 2C, 2D). Importantly, the fluorescence of WST-D in the cornea showed a relatively sharp front of drug migration that did not exceed the center of the stroma. However, at the depth of 200 μm the level of fluorescence still was higher for corneas following the 30-minute exposure to WST-D, suggesting a higher accumulation level.

The Photochemistry of WST11 in the Cornea

Photobleaching of WST11 in Ex Vivo Treated Corneas. As we reported previously, generation of oxygen radicals upon illumination of WST11 in the absence of serum albumin is accompanied by the bleaching of the NIR transition and absorption increase at ~ 645 nm, due to the photochemical generation of a chlorophyll-like molecule. Therefore, such changes are important markers for the photochemical activity of WST11 in situ. Hence, we set out to monitor changes in the optical absorption of WST11 during corneal illumination. It was found that the optical absorption of corneas at 755 nm, following preincubation with WST11 for 20 minutes, and further illumination for 10, 30, and 60 minutes declined by 30%, 50, and 75%, respectively (Fig. 3). In parallel, the absorption increased at 645 nm, typical to the respective chlorin derivative formed by the photochemical interaction of WST11 with molecular oxygen.
Figure 2. Penetration of WST11 into rabbit corneas ex vivo. Rabbit corneas were exposed to WST11 for (A) 10 minutes \( (N=2) \) and (B) 30 minutes \( (N=5) \), and to WST-D for (C) 10 minutes \( (N=3) \) and (D) 30 minutes \( (N=5) \). Subsequently, the corneas were rinsed with saline, and the central 8-mm buttons were trephined, wrapped in aluminum foil, and frozen on dry ice. Central corneal sagittal slices (12 μm), were cut with a cryomicrotome, mounted on a microscope glass slide and stored frozen at \(-70^\circ C\). For measurement of fluorescence, the individual slices were placed on the microscope base and a photographic record was taken immediately. Fluorescence at 760 nm was detected upon excitation at 740 nm. Corneas showed complete stromal penetration when exposed to WST11 for 30 minutes (B), and approximately 50% penetration after 10 minutes (A). Exposure of corneas to WST-D for either 10 or 30 minutes showed fluorescence only in the outer third of the stroma (C, D). All other details are described in the Methods section.
Corneal Stiffening in Response to WST11/NIR Treatment

Stress-Strain Measurements of Ex Vivo Treated Eyes. The stress-strain measurements following WST11/NIR with a 30-minute pre-incubation showed a nearly 3-fold increase in the corneal stiffness, compared to untreated control eyes (Fig. 4). There was a maximal increase of 267% in the ultimate stress (P < 0.0001) from a mean of 1.63 MPa without treatment to 5.98 MPa post-treatment, and an increase of 369% in Young’s modulus (P < 0.0001), from a mean of 3.76 MPa without treatment to 17.65 MPa post-treatment (Table 1).

The stiffening, due to WST11/NIR treatment, appears similar to that observed in the two RF-D/UVA-treated corneas, where the mean ultimate stress increased from 1.44 MPa without treatment to 6.46 MPa after treatment, and in the mean Young’s modulus from 3.28 MPa without treatment to 20.72 MPa after treatment.

In Vivo WST11/NIR Cornea Treatment. Corneas in live rabbits were pretreated for 10, 20, and 30 minutes with WST11 (2.5 mg/mL). NIR illumination (755 nm, 10 mW/cm²) then was delivered for 30 minutes. One month later the ultimate stress of the treated corneas was found to increase by 45, 113, and 126%, respectively, compared to the nontreated eyes. The mean Young’s modulus in WST11/NIR-treated corneas showed a 10, 79, and 173% increase in the same time sequences (Fig. 5; Tables 1, 2).

Corneas treated by WST11/NIR had edema for one week after treatment. The corneal epithelial defect healed gradually after 10–14 days. After epithelial healing, the corneas regained transparency, with some corneas demonstrating epithelial haze.

In Vivo WST-D/NIR Cornea Treatment. To explore the role of dextran, the photochemical treatment with a formulation of WST11 2.5 mg/mL with 20% dextran T-500 was examined. Application of WST-D for 20 minutes before 30-minute illumination did not appear to affect the approximately two-fold increase in the mean ultimate stress and Young’s modulus, compared to untreated corneas of fellow eyes (Fig. 6). However, it significantly reduced the extent and duration of the edema and epithelial defect. Corneal edema cleared after 5 days, and the epithelium healed within 7–9 days without haze development. In the RF and RF-D treated corneas, the epithelial defect healed after 4 days. However, in the RF-D group the edema resolved after 4 days with recovery of transparency, whereas in the RF group the edema persisted for 6 days, followed by 2 days of central epithelial haze.

Endothelial and Keratocyte Response to WST11 or WST-D/NIR Treatment

Histologic examination of the corneas two days after WST11/NIR treatment in saline showed marked edema (cornea swelling to 890 µm), and a reduced number of keratocytes throughout the stroma, more pronounced in the anterior half.
A honeycomb-like lacunar hydration pattern, containing keratocytes or keratocyte debris, was present, as previously reported after RF-D/UVA. However, the endothelial cell layer appeared intact and did not differ from the control. There was no statistical difference in the endothelial counts between treatment and control ($P=0.47$). Corneas treated with WST-D showed minimal corneal edema, absence of the epithelium in the central area, and a statistically significant reduction in the number of keratocytes (481 ± 121 cells/mm$^2$ in the treated corneas, compared to 1060 ± 210 cells/mm$^2$ in controls, $P < 0.0001$) limited to the outer half of the stroma (Fig. 7C). There was no evidence of damage to the endothelium in comparison with the control, both after vital (data not shown) and H&E staining (Fig. 7A). One week after treatment, the histologic sections showed shrinkage of the anterior stroma to 250 μm (in addition to the stromal compaction that took place after formaldehyde fixation of control samples to 340 μm), loss of keratocytes in the anterior 1/3 of the stroma (80 μm) with epithelial healing, but no endothelial damage (Fig. 7D). H&E staining of the retina two days after WST11/NIR treatment did not show any morphologic changes compared to control (data not shown). Apoptosis was examined using TUNEL assay. One day postoperatively, TUNEL-positive keratocytes were detected in the anterior stroma of treated corneas. No staining for TUNEL was observed in the posterior stroma or endothelium as well as in the control corneas (Figs. 8A, 8B).

**Table 1.** Rabbit Corneal Stiffening following WST11/NIR Treatment

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<th>Settings</th>
<th>Sample</th>
<th>Mean Ultimate Stress</th>
<th>Mean Young Modulus</th>
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<td></td>
<td></td>
<td>Intensity, MPa</td>
<td>Increase,* % (P Value)</td>
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<td>Ex vivo</td>
<td>Control: untreated fellow eyes (N = 10)</td>
<td>1.65 ± 0.58</td>
<td>3.76 ± 1.96</td>
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<td>WST11/NIR treated eyes (N = 10)</td>
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<td>In vivo 30 d</td>
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<td>WST11/NIR treated eyes (N = 6)</td>
<td>6.91 ± 0.53</td>
<td>126 (0.0049)</td>
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</table>

* Relative to control.

**Figure 5.** Stress-strain measurements of in vivo treated eyes one month after WST11/NIR treatment. The cornea of one eye of each rabbit was deepithelialized and incubated for 10, 20, or 30 minutes with WST11 (2.5 mg/mL) using an eye cap, followed by laser illumination (755 nm, 10 mW/cm$^2$) for 30 minutes with occasional saline wetting. The eyes were allowed to heal. One month after treatment, the rabbits were euthanized, the corneas were excised, and corneal strips were prepared. Ultimate stress (A) and Young's modulus (B) expressed in MPa were determined and compared to untreated control fellow eyes. N = 16 in three experiments: 10', 20' - 6 and 30' - 6 rabbits. All other details are described in the Methods section.

**Electron Spin Resonance (ESR) Spectroscopy**

(Methods are Described in Supplementary Material S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9913/-/DCSupplemental)

Figure 9A compares the ESR spectra of 4-POBN in aqueous solutions following generation of reactive oxygen species (ROS) by WST11/NIR (black) and RF/UVA (light blue). The quartet pointed by arrows reflects the trapping of singlet oxygen and is observed only in the RF/UVA spectrum, while the sextet that represents superoxide and hydroxyl radical formation (stars) is observed in WST11/NIR- and RF/UVA-treated solutions.

Figure 9B shows the ESR spectra of ROS trapped in the rabbit cornea following WST11/NIR. The signals are similar to those observed in aqueous solutions with no traces of singlet oxygen. The signals generated by RF/UVA were at the noise level.

**Fluorescence Spectroscopy of Rabbit Corneas**

Excitation of RF-D/UVA treated corneas at 320 nm generated emission signal at 405 nm that probably corresponds to the fluorescence of dityrosine, a known signature of collagen crosslinking. Such emission was absent in the WST11-dextran/NIR treated corneas and in the controls (Fig. 10B). Excitation at any other wavelength, as indicated in the Methods...
section, did not provide any significant change in the cornea emission profile.

**DISCUSSION**

WST11/NIR sensitized reactions resulted in consistent corneal stiffening ex vivo and in vivo. Ex vivo incubation with WST11 for 30 minutes before illumination increased the Young's modulus, and the ultimate stress by 369 and 267%, respectively, relative to untreated eyes. Treatment with the same parameters performed in vivo raised the Young's modulus and ultimate stress by 173 and 126%, respectively, compared to the untreated eyes (Table 1).

The water-soluble bacteriochlorophyll WST11 penetrates the deepithelialized cornea fairly quickly and in a time-dependent manner, in the presence and absence of dextran T-500. However, significantly deeper penetration is observed in the first case (Fig. 2). As demonstrated in Figures 2A and 2B, WST11 crosses 1/2 of the corneal stromal depth within 10 minutes of incubation and the entire stromal depth after 30 minutes of incubation, in the absence of dextran. In contrast, application of WST11 with dextran T-500 limits the penetration depth to the outer 1/2 of the corneal stroma after 10 or 30 minutes of incubation (Figs. 2C, 2D). No further penetration was observed at longer incubation times (data not shown).

Therefore, it was concluded that dextran has a critical role in determining the penetration depth of WST11. This also is true for RF, as is evident by its fluorescence distribution, which is limited to the anterior 50 μm of the cornea in the presence of dextran T-500. The accumulated WST11 is active photochemically, as reflected by the continuous bleaching and spectral changes to the oxidized chlorin form during illumination (Fig. 3).

While treatment with WST11 formulated with dextran T-500 did not diminish the corneal stiffening effect, it reduced greatly the adverse effects of the treatment. Clinically, the duration of corneal edema and epithelial healing was shortened significantly. In addition, epithelial haze, as observed in some treated corneas, appeared only in the absence of dextran. Histologic examination two days after WST11/NIR treatment demonstrated marked corneal edema (Fig. 7B). The absence of endothelial cell damage in this case may suggest a different etiology of stromal edema. Indeed, Wollensak and Herbst revealed the pattern of unique honeycomb-like lacunar stromal edema after collagen cross-linking with RF-D/UVA. Edematous fluid around apoptotic keratocyte debris is trapped within the keratocyte space. Histology performed two days after WST-D/NIR presented minimal edema. There was a reduction in the keratocyte population in the anterior half of the cornea without evident damage to the posterior cornea and endothelium compared to the control (Figs. 7A, 7C). Histology performed 1 week after WST-D/NIR demonstrated an absence of keratocytes in the anterior third of the cornea, with compaction of anterior stromal fibers, resulting in corneal thinning (Fig. 7D). Apoptotic keratocytes were detected by TUNEL assay 24 hours after WST-D/NIR treatment. However, no evidence of apoptosis was found in the posterior stroma and endothelium (Fig. 8). Wollensak et al. reported that standard RF-D/UVA treatment could lead to endothelial damage

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**Table 2.** Effect of WST11 Incubation Time on the Stiffening of Rabbit Corneas

<table>
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<tr>
<th>Sample</th>
<th>Mean Ultimate Stress</th>
<th>Mean Young Modulus</th>
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<td>Intensity, MPa</td>
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<td>In vivo</td>
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<td>Control: untreated fellow eyes (N = 10)</td>
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<td>WST11 10-minute treated eyes (N = 4)</td>
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<td>WST11 20-minute treated eyes (N = 6)</td>
<td>6.66 ± 0.67</td>
<td>113 (&lt;0.0001)</td>
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* Relative to control.
FIGURE 7. Histologic sections of rabbit corneas 2 days after WST11/NIR treatment. (A) Control (×20 magnification). (B) After WST11/NIR treatment (×10 magnification). The epithelium was absent in the treated cornea and there was marked edema of the corneal stroma, but no evidence of endothelial damage. (C) After treatment with WST-D/NIR (×20 magnification) showing absence of epithelium, no stromal edema, and a reduced number of keratocytes in the outer 1/2 of the stroma. There was no evidence of damage to the endothelium in comparison with the control. (D) One week after treatment with WST-D/NIR (×20 magnification) showing a significant shrinking and loss of keratocytes in the anterior 1/3 of the stroma, with epithelial healing, but no endothelial damage. Sections stained with H&E. All other details are in Methods section.

FIGURE 8. Histologic sections of rabbit corneas 1 day after WST-D/NIR treatment. (A) Control (×10 magnification). (B) After WST-D/NIR treatment (×10 magnification). The epithelium was absent in the treated cornea and there was edema of the corneal stroma. TUNEL-positive staining for apoptosis of keratocytes was seen in the outer 1/2 of the stroma (arrows). No evidence of apoptotic staining in the inner stroma and the endothelium was detected as well as in the control corneas. Sections stained with TUNEL apop-tag kit. All other details are in Methods section.
FIGURE 9. (A) ESR signal of 4-POBN in solution containing ethanol (8%) and WST11 (black) or RF (light blue) following NIR illumination. **Black arrows** point at signals due to trapped singlet oxygen. **Stars** correspond to the signals of trapped hydroxyl and superoxide radicals. (B) ESR signal of 4-POBN generated by NIR illumination of WST11 (black) or RF (light blue) in corneal strips prepared and examined as described in Methods section.
when applied to corneas thinner than 400 μm.7,8 Recently, severe endothelial damage after RF-D/UVA was reported in rabbits by Hovakimyan et al.28 Histologic sections of the retina two days after WST-D/NIR treatment did not demonstrate any morphologic damage.

The presence of dextran appears to protect the endothelial layer and preserve the keratocytes in the posterior cornea (Figs. 7C, 8D). This protection, as well as the attenuated edema and shortened time of healing, probably are related to the reduced depth of WST11 penetration and, subsequently, the spatially limited effect of the photogenerated radicals. Similar stromal penetration of RF-D limited to the anterior 200 μm even under prolonged exposure time was reported recently.29

The traditional regimen of RF-D/UVA treatment requires prolonged application and irradiation. The optimal applied energy and time of delivery still are under investigation. However, the use of higher irradiation fluence, with the same delivered energy, most probably will shorten treatment duration (Roizenblatt R, et al., IOVS 2010;51:ARVO E-Abstract 1797). Such optimization for the WST-D/NIR treatment currently is under investigation in our labs. The absence of detectable WST11 in the circulation during and after topical application on the cornea (see Supplementary Material S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9913/-/DCSupplemental), and the absence of heat generation during treatment (see Supplementary Material S3, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9913/-/DCSupplemental) further assure the treatment safety.

On the ground of recently obtained data from experiments involving rabbit corneas, it was suggested that RF/UVA stiffening involves singlet oxygen-mediated process of homo- and hetero-covalent crosslinking between collagen and proteoglycan moieties.31,32 The extensive trapping of singlet oxygen in aqueous solution following RF-D/UVA corroborates with this suggested mechanism. However, as we reported previously, WST11 appears to generate only superoxide and hydroxyl radicals with no traces of singlet oxygen following NIR illumination in aqueous solutions,15 and the same is observed when looking at the treated corneas (Fig. 9B). Although the hydroxyl and superoxide radicals can initiate collagen crosslinking, the dityrosine bond fluorescence at 405 nm, a footprint of cornea treated by RF-D/UVA treatment, could not be seen after treatment with WST11/NIR (Fig. 10B). These two observations suggested that the stiffening effect of RF/UVA and WST11/NIR may involve different mechanisms. Alternatively, the generated singlet oxygen and dityrosine formation during RF-D/UVA are not related to the stiffening effect, but rather, byproducts of the in situ photochemical reactions (currently under investigation).

In summary, treatment by WST-D/NIR appears as efficient as RF-D/UVA in the animal models, but with potentially less adverse effects and possibly different mechanisms of action, supporting the potential for clinical use in keratoconus and corneal ectasia after refractive surgery.

Acknowledgments

Bella Finarov, DVM, is gratefully appreciated for her valuable help with the animals.

References


