Published in final edited form as:

Cornea. 2018 March ; 37(3): 362–368. doi:10.1097/ICO.0000000000001473.

Cornea Crosslinking with Verteporfin and Non-Thermal Laser Therapy

Saleh A. Alageel, MD, FRCS(G)¹, Samer N. Arafat, MD¹, Borja Salvador-Culla, MD¹, Paraskevi E. Kolovou, BSc, MD¹, Khadijeh Jahanseir, MD¹, Adam Kozak, BS², Gavin J.C. Braithwaite, BSc, PhD², Joseph B. Ciolino, MD¹

¹Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, MA, USA

²Cambridge Polymer Group, Boston, MA, USA

Abstract

Purpose—To test that Verteporfin with non-thermal laser increases corneal mechanical stiffness and resistance to enzymatic degradation *ex vivo*.

Methods—Thirty human corneas (n=5 per group) were either treated with Verteporfin alone (V), irradiated with non-thermal laser therapy (689 nm) alone (NTL), or received combined treatment of Verteporfin with non-thermal laser therapy for one sequence (V+NTL1) or six sequences (V+NTL6) of 1 minute of NTL. Positive controls were pre-treated with 0.1% riboflavin/20% dextran every 3 to 5 minutes for 30 minutes, and irradiated with ultraviolet light type A ($\lambda=370\text{nm}$, irradiance=3mW/cm²) for 30 minutes using the Dresden protocol (R+UVA). Untreated corneas were used as negative controls. The corneal biomechanical properties were measured with enzymatic digestion, compression, creep, and tensile strength testing.

Results—V+NTL6 and R+UVA treated corneas acquired higher rigidity and more pronounced curvature than untreated corneas. The stress strain tests showed that V+NTL6 and R+UVA corneas became significantly stiffer than controls ($p<0.005$). The V+NTL6 group appeared to be slightly stiffer than the R+UVA group, although the differences were not statistically significant. V+NTL6 corneas were found to have a significantly lower absolute creep rate (-1.87 vs. -3.46 , $p<0.05$), and significantly higher maximum stress values (7.67 vs. 3.02 $p<0.05$) when compared to untreated corneas.

Conclusion—Verteporfin-NTL (V+NTL6) increases corneal mechanical stiffness and resistance to enzymatic collagenase degradation. Although a clinical study is needed, our results suggest that V+NTL6 induces corneal crosslinking and corneal biomechanical changes that are similar to those induced by standard corneal collagen crosslinking.

Corresponding Author: Joseph B. Ciolino, M.D., Massachusetts Eye and Ear Infirmary, Harvard Medical School, 243 Charles Street, 02114 Boston, MA, USA, Telephone: (401) 935-9662, joseph_ciolino@meei.harvard.edu.

Financial Disclosure(s): JBC and SAA are listed as inventors on a patent application. The other author(s) have no proprietary or commercial interest in any materials discussed in this article.

Contributions to Authors: Design and conduct of the study (SAA, SNA, BSC, PEK, KJ); collection (SAA, SNA, BSC, PEK, KJ), analysis (SAA, SNA, BSC, PEK, JBC), interpretation of the data (SAA, SNA, BSC, PEK, JBC); manuscript preparation (SAA, BSC, PEK), manuscript review (BSC, PEK, JBC), manuscript approval (SAA, SNA, BSC, PEK, KJ, AZ, GJCB, JBC).

Keywords

Corneal Collagen Crosslinking; Photodynamic Therapy; Verteporfin; Non-Thermal Laser; Corneal Enzymatic Digestion

INTRODUCTION

Corneal crosslinking (CXL) has gained considerable interest in recent years as a means of halting the progression of keratoconus. The procedure involves initiation of a photochemical reaction in the corneal stroma that increases binding of collagen fibrils, resulting in increased corneal stiffness.^{1,2} By stabilizing the corneal biomechanics, CXL aims to prevent or postpone the need for corneal transplant.^{3,4} Conventional CXL uses the “Dresden protocol”^{1,2} that involves corneal debridement, riboflavin pretreatment followed by 6 five-minute sessions of ultraviolet-A (UVA) irradiation.^{1,2}

Photoactivation of riboflavin induces the formation of free radicals that interact with corneal proteins leading to covalent bonding between collagen fibrils.⁵ Since generation of free radicals also causes cell death, riboflavin concentration, UVA irradiance, and treatment duration have been specifically chosen to minimize cellular damage and protect the corneal endothelium.⁴ These parameters effectively limit CXL to the anterior 300 µm of the corneal stroma and also restrict UVA-CXL therapy to corneas thicker than 400 µm, since treatment of thinner corneas would risk damage to the endothelium.⁶ Therefore, researchers have sought to develop methods of strengthening the cornea in a manner that would provide greater safety, improved efficacy, and the opportunity to expand the treatment to include thin corneas.

Photodynamic therapy (PDT) utilizes a benzoporphyrin derivative monoacid ring photosensitizing agent, Verteporfin (C₄₁H₄₂N₄O₈), which is activated by a non-thermal laser (698 nm) light through a photochemical reaction that generates oxygen-free radicals that cause tissue changes, without the production of heat.^{7–9} PDT with Verteporfin is commercially available, is usually administered intravenously to induce focal ablation of pathologic blood vessels, and is FDA-approved for the treatment of choriorretinal diseases, such as subfoveal choroidal neovascularization in age-related macular degeneration.⁷ PDT has also been used off-label for the treatment of cornea neovascularization and lipid keratopathy.^{10,11} In general, PDT is thought to cause focal damage to living cells without affecting non-living tissue, such as collagen, a molecule that makes up most of the cornea stroma.¹² However, there have been several reports of PDT used to induce collagen-to-collagen crosslinking in non-ocular structures such as blood vessels, skin, bone, and others.^{8,13–15} Given these reports, we hypothesize that PDT with topical Verteporfin can be used as an alternative to CXL with riboflavin and UVA to crosslink cornea collagen and subsequently increase its biomechanical strength.

MATERIAL AND METHODS

Tissue preparation

Human research corneas were obtained from Tissue Bank International (Baltimore, MD) and North Carolina Eye Bank (Winston-Salem, NC) and stored in Optisol® solution at 4°C until use. All experiments were performed within 2 weeks from the date of death of the donor. All corneas were fit into a Barron® artificial anterior chamber (Katena Eye Instruments, Denville, NJ) filled with normal balanced salt solution (BSS), followed by mechanical removal of 8 mm of the central corneal epithelium with a blunt blade.

Riboflavin and UVA Treatment

Conventional riboflavin-UVA (R+UVA) crosslinking was performed in an *ex vivo* fashion according to the “Dresden protocol”.^{1,2} Fifty mg of riboflavin 5'-phosphate sodium salt hydrate (Sigma Aldrich, St. Louis, MO) was added to 50 ml of 20% (w/w) dextran solution (from *Leuconostoc mesenteroides*; Sigma Aldrich, St. Louis, MO) and mixed thoroughly to make 0.1% riboflavin (1 mg/ml) solution. The solution was covered with aluminum foil for protection from room UV light and stored at 4°C until use. Riboflavin solution was topically administered every 5 minutes onto the surface of the de-epithelialized cornea for a total instillation period of 30 minutes. Then, a LED-based UVA light emitter (370nm; VEGA CBM-X-Linker, CSO, Firenze, Italy) was focused onto the surface of the cornea at an irradiance of 3 mW/cm² (5.4 J/cm² fluence rate) for 5 minutes. The five-minute UVA exposure sequence was repeated 5 more times for a total UVA exposure time of 30 minutes (total of 6 sessions). Between each 5-minute UVA treatment sequence, 1–2 drops of riboflavin were administered onto the surface of the cornea.

Verteporfin and Non-Thermal Laser Treatment

Each de-epithelialized cornea was placed into a Barron® artificial anterior chamber and underwent a treatment protocol (see below) that included either topical Verteporfin solution (Visudyne®; Bausch & Lomb, Inc., Rochester, NY) alone, non-thermal laser alone, or a combination of both topical Verteporfin and non-thermal laser. Verteporfin solution was applied topically using the same formulation that is administered intravenously for the treatment of choroidal neovascularization (6 mg/ml).⁷ A 689 nm wavelength non-thermal laser (Opal Photoactivator; Coherent Inc., Santa Clara, CA) was applied using a spot size of 7 mm at a power intensity of 600 mW/cm². Following the treatment protocol described below, corneas from all treatment groups were placed in Optisol® and refrigerated at 4 °C for approximately 24 hours until the time of testing. The treatment protocol for each subgroup was as follows (table 1; n = 5 per subgroup):

1. **Topical Verteporfin (V) without laser exposure:** One drop of Verteporfin solution was topically applied onto the surface of a de-epithelialized cornea every minute for 15 minutes.
2. **Verteporfin with non-thermal laser, single one-minute irradiation (V+NTL₁):** One drop of Verteporfin solution was applied onto the de-epithelialized cornea every minute for 15 minutes, after which the tissue was exposed to a one-minute NTL irradiation.

3. **Verteporfin with non-thermal laser, six 1-minute irradiations (V+NTL₆):**
One drop of Verteporfin solution was topically applied onto the de-epithelialized cornea every minute for 15 minutes, after which six 1-minute NTL irradiations were performed for a total exposure time of 6 minutes. 1–2 drops of Verteporfin solution were administered between each irradiation.
4. **Non-thermal laser, six 1-minute irradiations without Verteporfin (NTL₆):**
The de-epithelialized cornea was exposed to six 1-minute NTL irradiation sessions for a total exposure time of 6 minutes. Between each irradiation, 1–2 drops of BSS were administered onto the surface of the cornea to maintain hydration.
5. Untreated de-epithelialized corneas served as negative controls.

Enzymatic Digestion

To assess resistance to collagenase digestion, cornea buttons were fully submerged in collagenase A (from *Clostridium histolyticum*, E.C. 3.4.24.3; Sigma Aldrich, St. Louis, MO), and the time until complete digestion was recorded.^{16,17} Briefly, all corneas, which had been de-epithelialized as noted above, were removed from Optisol®, rinsed with BSS, trephined into 8.5-mm buttons, placed in clear glass vials, and incubated in 1 ml of 0.3% collagenase A solution (3 mg/ml in phosphate-buffered solution) at 37°C rotating at 150 rotations-per minute (New Brunswick™ Excella® E24 incubator shaker; New Brunswick Scientific, Enfield, CT). Using the same technique that has been previously described^{17,18}, the samples were observed hourly for the first 12 hours, and then every 30 minutes until the corneal buttons were completely dissolved. All groups were compared to untreated corneas.

Biomechanical Testing

The biomechanical properties of untreated and treated corneas were measured using compression, creep and tensile strength testing. All the specimens were stored at 4 °C in Optisol® for approximately 24 hours until time of testing. Based on the results of the enzymatic digestion study, R+UVA and V+NTL₆ treatment groups were chosen for biomechanical testing and were compared to untreated corneas.

Compression and creep testing—The compressive elasticity of any tissue can be determined by measuring the stress (force on a cross-sectional area) and its relative strain (proportional deformation perpendicular to that area). The Young's modulus is defined as the slope of the linear region of the stress-strain curve, which is essentially the stress divided by the strain, and is measured in Pascal units (1 Pa = 1 Newton/m²). This parameter quantifies the elasticity of the tissue, where a lower Young's modulus implies that a material deforms more easily for a given force. Compression testing was performed using a Q800 DMA (Dynamic Mechanical Analyzer, TA instruments, New Castle, DE). The tissue was placed within a submersion cell and centered beneath a custom 8 mm diameter compression plate (supplemental figure 1). The submersion clamp was filled with 1× phosphate buffered saline solution (PBS) to prevent the sample from drying out during the test. The compression plate was brought into contact with the central corneal tissue without compressing the sclera. The sample thickness was measured by the amount of plate

separation at a preload force of 0.1 N, and then compressed at a rate of 7.5 N/min, up to a force of 15 N. Since the custom compression plate was undersized relative to the tissue, the contact pressure scaled linearly with the force to a maximum of ~100 kPa per sample. This endpoint was selected based on previous work that suggests that this force would still be well below the point at which permanent damage is caused to the corneal tissue.² At the end of the compression test, a short-duration creep test was performed.

Creep testing has been used to evaluate the biomechanical strength and viscoelastic properties of tissues, such as the cornea, by applying a constant stress and monitoring the rate of deformation.^{19,20} Strictly speaking, creep is usually performed after an “instantaneous” application of stress, but in this study creep was measured after the linear force ramp described above, which means that some portion of the creep may have occurred during the initial compression step. Here, corneal tissue was kept under a constant stress (~100 kPa) using a compression force of 15 N. The linear slope of the strain-time curve was calculated between 0 and 4.25 minutes to characterize the rate of tissue deformation. It is worth noting that the compression curve in complex viscoelastic solids will never be simply linear, and in fact the creep testing results in our study indicate an exponential decay in strain, a result of a strongly viscoelastic tissue (supplemental figure 2). This calculation choice allows a simple ranking of the materials and should be sensitive to crosslinking. If the sample is purely elastic this test should eventually reach a strain plateau, whereas a viscous material would continue to creep.

Tensile strength testing—Following compression testing, the same specimens underwent tensile strength testing. Stress-strain measurements using strip-extensometry have demonstrated increased stiffness of the cornea following collagen crosslinking with riboflavin-UVA light.²¹ In this study, strip-extensometry was performed using rectangular strips of corneal tissue (3 mm wide and 14 mm long), and the maximum stress observed prior to failure, the maximum modulus values, and the instantaneous modulus values at specific strain percentages were calculated. The maximum modulus value was also calculated since it corresponds approximately to the slope of the steepest part of the curve in a stress-strain plot, provides a straightforward method for comparing samples, and describes the peak stiffness of the material. Note that the instantaneous modulus values were calculated from the material engineering stress, which uses the initial cross-sectional area of the specimen.

Tensile testing was performed using an AGS-X (Shimadzu, Inc.) electromechanical load frame equipped with a 50 N load cell and pneumatic grips lined with adhesive sandpaper to prevent tissue slippage. Prior to starting the testing, the pneumatic grips were pressurized at ~150 kPa (35 psi), which allowed compensation of sample thinning that occurs as the tissue was stretched. The contact area of the tissue generally held within the grip was approximately 3 × 4 mm. This would imply that the gripping pressure exerted directly on the tissue was of the order of 17 MPa. The initial grip separation was set to 4.5 mm and tested at a rate of 0.025 mm/sec until failure. The strain (ϵ) was therefore increased linearly at a velocity of 0.56 %/sec, and the relative stress (σ) was measured.

Data analysis

Statistical analysis was performed using Graphpad Instat 3.10. Normality was determined using the Kolmogorov-Smirnov test, and non-parametric tests were used when indicated. One-way analysis of variance (ANOVA) and Kruskal-Wallis tests were used to compare times to total corneal enzymatic dissolution between all groups. Mann-Whitney U-statistics was used for comparing non-parametric non-matched groups. The tests were performed using a 2-tailed p-value of 0.05. The results were reported as mean $\pm$ standard deviation. Statistical analysis of mechanical testing results was performed by two-tailed Student's t-test assuming unequal variance (Microsoft® Excel®, © 2010 Microsoft Corporation).

RESULTS

Resistance to enzymatic digestion

Crosslinking of corneal tissue has been shown to increase the resistance of the tissue to enzymatic degradation.^{16,17} The results for all the groups in our study are shown in figure 1. Corneas treated by riboflavin and UV light (R+UVA) demonstrated a significantly slower rate of dissolution (20.1 ± 1.2 hours, $p=0.0001$) than untreated corneas (5.5 ± 0.2 hours), which are both consistent results from a prior publication from the authors.¹⁷ Corneas treated with topical Verteporfin (V) without non-thermal laser and corneas treated with six 1-minute sessions of non-thermal laser without Verteporfin (NTL₆) exhibited an enzymatic digestion time similar to untreated corneas (6.0 ± 0.3 hours and 5.7 ± 0.4 hours, respectively). Corneas that underwent Verteporfin pretreatment for 15 minutes followed by one non-thermal laser irradiation (V+NTL₁) exhibited a digestion time that was comparable to that of untreated corneas (6.0 ± 0.4 hours). However, corneas that were pretreated with Verteporfin for 15 minutes followed by six non-thermal laser irradiation phases (V+NTL₆) demonstrated a significantly slower rate of dissolution compared to untreated corneas (19.8 ± 1.0 hours, $p=0.0001$), with values that are similar to the R+UVA group, indicating an enhanced resistance to enzymatic digestion.

Gross Examination

Corneal tissue that has been crosslinked by riboflavin and UV light has previously been found to be more rigid on gross examination.² In this study, corneas treated by V+NTL₆ and R+UVA were observed to have an increased preservation of corneal curvature, and did not sag as readily when freely hanging, than the untreated control group (figure 2).

Biomechanical Testing

Based on the results of the enzymatic study, the V+NTL₆ and R+UVA groups were chosen for biomechanical testing and compared to untreated corneas (table 2).

Compression Testing—Young's modulus was calculated as a linear fit of the final portion of the stress-strain curve, between stress values of 0.08 and 0.10 MPa (figure 2). The observed slope of the stress-strain data was statistically steeper for R+UVA (0.70 ± 0.14 MPa) and V+NTL₆ (0.80 ± 0.14 MPa) corneas as compared to untreated corneas (0.43 ± 0.06 MPa; $p=0.01$ and $p=0.002$, respectively). These results suggest that both treatments confer increased stiffness compared to untreated corneas.

Creep Testing—As shown in table 2, corneas treated by V+NTL₆ were found to have a significantly lower absolute creep rate (-1.87 ± 0.87 %/min) than untreated corneas (-3.46 ± 0.63 %/min; $p=0.012$). This suggests that the corneas treated by V+NTL₆ appear to have a higher effective viscosity than the untreated corneas. This lower creep rate at moderate deformations is consistent with tighter crosslinking preventing immediate expulsion of the water in a “crosslinked network”. Note that because of the way in which viscous and elastic properties both contribute to the time constant of the creep behavior, a long-term creep experiment (taken to steady state) would be necessary to exactly separate the viscous and elastic components.

Tensile Testing—Corneas treated by V+NTL₆ and R+UVA were observed to reach statistically higher maximum tensile stress values prior to failure (7.67 ± 2.59 MPa and 6.57 ± 1.79 MPa, respectively), relative to untreated corneas (3.02 ± 0.96 MPa; $p=0.013$ and $p=0.058$ respectively, table 2). In addition, we found that V+NTL₆ and R+UVA corneas had statistically larger maximum modulus values than untreated corneas (15.17 ± 4.47 MPa and 14.97 ± 2.18 MPa vs. 7.71 ± 1.50 MPa; $p=0.017$ and $p=0.013$ respectively; table 2).

Finally, the initial toe region of the tensile testing of each sample group was compared (figure 3). The stress-strain data in all samples (figure 4) was offset such that the sample length at a stress of 0.02 MPa was defined as 0% strain (this offset is equivalent to defining a pre-stress of 0.02 MPa at the start of the test). V+NTL₆ corneas were found to have statistically larger modulus values at 4%, 6% and 8% strain compared to untreated corneas ($p=0.027$, $p=0.017$, and $p=0.012$ respectively). However, R+UVA corneas were found to have a substantial amount of variability in the toe region, and no statistically significant difference was observed ($p=0.594$, $p=0.586$, and $p=0.582$ respectively).

DISCUSSION

Photodynamic therapy (PDT) is typically used to treat diseases of the retina, such as choroidal neovascularization.⁷ In this study, we demonstrated that PDT confers changes to the corneal stroma that are consistent with those of corneal collagen crosslinking with riboflavin and UV light. Corneas treated with Verteporfin and 6 phases of non-thermal laser (V+NTL₆) and corneas treated with riboflavin-UVA (R+UVA) demonstrated more resistance to enzymatic degradation and greater biomechanical strength than untreated corneas. These findings were statistically significant and suggest that V+NTL₆ treatment may be strengthening corneal tissue in a similar manner observed with R+UVA corneal crosslinking, since R+UVA and V+NTL₆ corneas demonstrated increased resistance with longer dissolution times (20.1 ± 1.2 hours and 19.8 ± 1.0 hours, respectively) compared to untreated corneas (5.47 ± 0.2 hours). In contrast, corneas treated with only one phase of NTL irradiation (V+NTL₁), topical Verteporfin without PDT (V), or PDT without Verteporfin (NTL₆) demonstrated similar dissolution times as untreated corneas.

Our results suggest that PDT induces changes to the cornea that are consistent with those corneas that have been crosslinked with R+UVA. Similar observations have been made when PDT was used to treat other tissues comprised of collagen.^{13,22} The mechanism of action is unclear, although it has been suggested that the interaction between the oxygen-free

radicals generated during PDT and the photo-oxidizing amino acids present in the tissue produce reactive molecules that form a crosslink with the free amino acids of a nearby molecule.¹³ This interaction seems to induce conformational and chemical changes that biologically activate specific binding sites of proteins within the treated tissue.¹³ Collagen type I is the predominant type of collagen in corneal stroma.²³ Distinct collagen-to-collagen crosslinks have been reported after photodynamic laser therapy of matrix gel solutions containing collagen alone.^{13,22} Moreover, vascular studies demonstrated that photodynamic laser therapy of collagen type I generated high molecular weight complexes, suggesting crosslinking with increased thermal stability, mechanical strength, and increased resistance to protease digestion.¹³

PDT has been reported to be an effective treatment for cornea neovascularization, which can cause leakage of fluid, lipid deposits, and corneal scarring.^{10,11} In these cases, neovascularised corneas were treated with one phase of PDT for 15 minutes after intravenous Verteporfin administration. Interestingly, one report noted that the corneas became thinner in the area of PDT laser treatment.²⁴ The authors hypothesized that the thinning may have resulted from resolution of exudate formation or have been an artifact of the imaging technique. Given that similar thinning of the cornea has also been observed as a result of corneal lamellar compaction from collagen crosslinking with R+UVA,^{25,26} it is possible that there is an alternative explanation: that PDT resulted in focal crosslinking that caused localized corneal thinning. In such cases, the intravenously injected Verteporfin may have leaked from the corneal vessels in a manner similar to that of other molecules, such as lipids, which can easily leak from neovascular corneal vessels and cause lipid keratopathy. If these vessels did indeed leak, then Verteporfin would have been available to crosslink the corneal collagen after the application of the non-thermal laser. This is consistent with our observations of corneal thinning presented herein.

There are several important differences between the prior studies that used PDT to treat cornea neovascularization and this study. Here, Verteporfin was applied topically whereas in the previous studies it was applied intravenously. Moreover, we applied six one-minute phases of NTL irradiation because we previously found that one phase alone exhibited no added benefit compared to untreated tissue. Corneas treated with V+NTL₆ demonstrated results similar to that of R+UVA, suggesting that crosslinking did occur.

Treatment with Verteporfin-NTL may have several advantages over UVA-CXL. Because the PDT laser can be precisely focused to treat specific areas and depths of the cornea, Verteporfin-NTL could be used to treat corneas thinner than 400 µm, currently a contraindication for UVA-CXL due to the potential of damage to the corneal endothelium.^{4,27} Further studies would be needed to determine if PDT is safe for corneas thinner than 400 µm and if it has an adverse effect on endothelial cells. PDT laser could also potentially be used to crosslink the residual stromal bed of corneas that have developed post-LASIK ectasia or be performed focally to address corneal ectasia exacerbated by radial keratotomies, which has been managed in the past by UVA-CXL crosslinking with unpredictable results.²⁸

In conclusion, PDT with Verteporfin increased the corneal mechanical stiffness and resistance to enzymatic collagenase degradation in our study. Although safety could in fact still be a concern given the novel topical application and a clinical study of this methodology in human patients is still needed, our results suggest that PDT with Verteporfin and NTL induces corneal biomechanical changes that are similar to those induced by corneal collagen crosslinking using UVA irradiation combined with riboflavin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: Dr. Ciolino receives financial support from a Career Development Award from Research to Prevent Blindness, Inc., and the National Eye Institute 1K08EY019686-01.

References

1. Spörl E, Huhle M, Kasper M, et al. Increased rigidity of the cornea caused by intrastromal cross-linking. *Ophthalmologe*. 1997; 94 :902–6. [PubMed: 9487761]
2. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin–ultraviolet-A-induced cross-linking. *J Cataract Refract Surg*. 2003; 29 :1780–5. [PubMed: 14522301]
3. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol*. 2003; 135 :620–27. [PubMed: 12719068]
4. Raiskup-Wolf F, Hoyer A, Spoerl E, et al. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. *J Cataract Refract Surg*. 2008; 34 :796–801. [PubMed: 18471635]
5. Kamaev P, Friedman MD, Sherr E, et al. Photochemical kinetics of corneal cross-linking with riboflavin. *Invest Ophthalmol Vis Sci*. 2012; 53 :2360–67. [PubMed: 22427580]
6. Chen X, Stojanovic A, Eidet JR, et al. Corneal collagen cross-linking (CXL) in thin corneas. *Eye Vis (Lond)*. 2015; 2 :15. [PubMed: 26605368]
7. Bressler NM. Treatment of Age-Related Macular Degeneration with Photodynamic Therapy (TAP) Study Group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with Verteporfin: two-year results of 2 randomized clinical trials-tap report 2. *Arch Ophthalmol*. 2001; 119 :198–207. [PubMed: 11176980]
8. LaMuraglia GM, ChandraSekar NR, Flotte TJ, et al. Photodynamic therapy inhibition of experimental intimal hyperplasia: acute and chronic effects. *J Vasc Surg*. 1994; 19 :321–31. [PubMed: 8114192]
9. Brooks BJ, Ambati BK, Marcus DM, et al. Photodynamic therapy for corneal neovascularization and lipid degeneration. *Br J Ophthalmol*. 2004; 88 :840.
10. Fossarello M, Peiretti E, Zucca I, et al. Photodynamic therapy of corneal neovascularization with Verteporfin. *Cornea*. 2003; 22 :485–8. [PubMed: 12827058]
11. Bown SG. Photodynamic therapy for photochemists. *Philos Trans A Math Phys Eng Sci*. 2013; 371 (1995) :20120371. [PubMed: 23776302]
12. Overhaus M, Heckenkamp J, Kossodo S, et al. Photodynamic Therapy Generates a Matrix Barrier to Invasive Vascular Cell Migration. *Circ Res*. 2000; 86 :334–40. [PubMed: 10679486]
13. Pazos, Md; Nader, HB. Effect of photodynamic therapy on the extracellular matrix and associated components. *Braz J Med Biol Res*. 2007; 40 :1025–35. [PubMed: 17665038]
14. Zhang JC, Liu W, Yi ZZ, et al. The molecular mechanism of photodynamic therapy to fibrosis: Regulation on the pyridinoline cross-link formation in collagen. *Chinese Science Bulletin*. 2009; 54 :2230–34.

15. Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Curr Eye Res.* 2004; 29 :35–40. [PubMed: 15370365]
16. Wollensak G, Spoerl E, Seiler T. Stress-strain measurements of human and porcine corneas after riboflavin-ultraviolet-A-induced cross-linking. *J Cataract Refract Surg.* 2003; 29 :1780–5. [PubMed: 14522301]
17. Kohlhaas M, Spoerl E, Schilde T, et al. Biomechanical evidence of the distribution of cross-links in corneas treated with riboflavin and ultraviolet A light. *J Cataract Refract Surg.* 2006; 32 :279–83. [PubMed: 16565005]
18. Lanchares E, del Buey MA, Cristóbal JA, et al. Biomechanical property analysis after corneal collagen cross-linking in relation to ultraviolet A irradiation time. *Graefes Arch Clin Exp Ophthalmol.* 2011; 249 :1223–7. [PubMed: 21494876]
19. Arafat SN, Robert MC, Shukla AN, et al. UV Cross-linking of Donor Corneas Confers Resistance to Keratolysis. *Cornea.* 2014; 33 :955–9. [PubMed: 25014151]
20. Kanellopoulos AJ, Asimellis G, Salvador-Culla B, et al. High-irradiance CXL combined with myopic LASIK: flap and residual stroma biomechanical properties studied ex-vivo. *Br J Ophthalmol.* 2015; 99 :870–4. [PubMed: 25795914]
21. Elsheikh A, Anderson K. Comparative study of corneal strip extensometry and inflation tests. *J R Soc Interface.* 2005; 2 :177–185. [PubMed: 16849178]
22. Henderson BW, Dougherty TJ. How does photodynamic therapy work? *Photochem Photobiol.* 1991; 55 :145–57.
23. Shen HR, Spikes JD, Kopecková P, et al. Photodynamic crosslinking of proteins. I. Model studies using histidine- and lysine-containing N-(2-hydroxypropyl)methacrylamide copolymers. *J Photochem Photobiol.* 1996; 34 :203–10.
24. Nakayasu K, Tanaka M, Konomi H, et al. Distribution of types I, II, III, IV and V collagen in normal and keratoconus corneas. *Ophthalmic Res.* 1986; 18 :1–10. [PubMed: 3513078]
25. Igarashi T, Yaguchi C, Inage M, et al. Topographical alteration in the cornea after photodynamic therapy for neovascularization in lipid keratopathy. *Jpn J Ophthalmol.* 2009; 53 :655–7. [PubMed: 20020250]
26. Bottós KM, Dreyfuss JL, Regatieri CV, et al. Immunofluorescence confocal microscopy of porcine corneas following collagen cross-linking treatment with riboflavin and ultraviolet A. *J Refract Surg.* 2008; 24 :S715–9. [PubMed: 18811117]
27. Kymionis GD, Portaliou DM, Diakonis VF, et al. Corneal collagen cross-linking with riboflavin and ultraviolet-A irradiation in patients with thin corneas. *Am J Ophthalmol.* 2012; 153 :24–8. [PubMed: 21861976]
28. Mazzotta C, Baiocchi S, Denaro R, et al. Corneal collagen cross-linking to stop corneal ectasia exacerbated by radial keratotomy. *Cornea.* 2011; 30 :225–8. [PubMed: 20861723]

Figure 1. Enzymatic digestion

Corneas treated with verteporfin/six 1-minute phases of irradiation by non-thermal laser (V+NLT₆) and riboflavin/ultraviolet A light (R+UVA) showed significantly more resistance to collagenase degradation than untreated corneas or corneas treated by verteporfin alone (V), non-thermal laser alone (NLT₆), or verteporfin/one 1-minute phase of irradiation with non-thermal laser therapy (V+NLT₁). N = 5 per group.

Figure 2.

Left, photographs demonstrating the qualitative increase in stiffness of corneas treated with verteporfin/non-thermal laser (V+NTL₆) and riboflavin/UVA (R+UVA) compared to untreated corneas. **Right**, stress-strain compression testing. The slope of the stress-strain data is observed to be steeper for corneas treated with V+NTL₆ and R+UVA, suggesting that both treatments confer increased stiffness compared to untreated corneal tissue. Negative strain indicates compression in to the sample. Each color within the group represents one cornea tested (n = 5 per group). MPa = megapascals.

Figure 3. Tensile testing

Instantaneous modulus values calculated at 4%, 6%, and 8% strain for all samples tested ($n = 5$). Corneas treated by verteporfin/non-thermal laser (V+NTL₆) exhibited larger instantaneous modulus values than untreated corneas. MPa = megapascals.

Figure 4. Representative plot showing one sample for each group for **A)** tensile testing of the toe region and **B)** stress-strain creep testing.

Table 1

Study arm treatment parameters.

Study Arm	Method of Administration
Untreated	NA
NTL ₆	6 NTL Irradiations
V	Topical verteporfin
V+NTL ₁	Topical verteporfin + 1 minute of NTL Irradiation
V+NTL ₆	Topical verteporfin + 6 one-minute NTL Irradiations
R+UVA	Topical riboflavin + UVA

NA = Not applicable; NTL = non-thermal laser; V = verteporfin; R = riboflavin; UVA = 6 sequences of ultraviolet A of 1 minute each.

Table 2

Biomechanical Testing

Test (Measure)	V+NTL ₆	R+UVA	Untreated
Compression (Young's Modulus, MPa)	0.8 ± 0.14 p = 0.002	0.7 ± 0.14 p = 0.011	0.43 ± 0.06
Creep (Creep Rate, %/min)	-1.87 ± 0.87 p = 0.012	-2.80 ± 0.99 p = 0.248	-3.46 ± 0.63
Maximum Tensile Modulus (MPa)	15.17 ± 4.47 p = 0.017	14.97 ± 2.18 p = 0.013	7.71 ± 1.50
Maximum Tensile Stress (MPa)	7.67 ± 2.59 p = 0.013	6.57 ± 1.79 p = 0.058	2.03 ± 0.96