Mechanisms of Corneal Tissue Cross-linking in Response to Treatment with Topical Riboflavin and Long-Wavelength Ultraviolet Radiation (UVA)

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Purpose. Treatment of de-epithelialized human corneas with riboflavin (RF) + long-wavelength ultraviolet light (UVA; RFUVA) increases corneal stroma tensile strength significantly. RFUVA treatment retards the progression of keratoconus, perhaps by cross-linking of collagen molecules, but exact molecular mechanisms remain unknown. Research described here tested possible chemical mechanisms of cross-linking.

Methods. Corneas of rabbits and spiny dogfish sharks were de-epithelialized mechanically, subjected to various chemical pretreatments, exposed to RFUVA, and then subjected to destructive tensile stress measurements. Tensile strength was quantified with a digital force gauge to measure degree of tissue cross-linking.

Results. For both rabbit and shark corneas, RFUVA treatment causes significant cross-linking by mechanism(s) that can be blocked by the presence of sodium azide. Conversely, such cross-linking is greatly enhanced in the presence of deuterium oxide (D_2O), even when RF is present at only one tenth the currently used clinical concentrations. Blocking carbonyl groups preexisting in the stroma with 2,4-dinitrophenylhydrazide or hydroxylamine blocks essentially all corneal cross-linking. In contrast, blocking free amine groups preexisting in the stroma with acetic anhydride or ethyl acetimidate does not affect RFUVA corneal cross-linking. When both carbonyl groups are blocked and singlet oxygen is quenched, no RFUVA cross-linking occurs, indicating the absence of other cross-linking mechanisms.

Conclusions. RFUVA catalyzes cross-linking reactions that require production of singlet oxygen ($^1O_2$), whose half-life is extended by D_2O. Carbonyl-based cross-linking reactions dominate in the corneal stroma, but other possible reaction schemes are proposed. The use of D_2O as solution media for RF would enable concentration decreases or significant strength enhancement in treated corneas. (*Invest Ophthalmol Vis Sci.* 2010;51:129–138) DOI:10.1167/iovs.09-3738

Clinical cross-linking treatment of keratoconus corneas involves scraping away the corneal epithelium, topically dripping a saline solution containing riboflavin (RF; vitamin B_2) onto the exposed corneal stroma, and then, while continuing the RF drip, exposing the surface of the stroma to long-wavelength ultraviolet light (UVA).[1–3] As a result, the corneal stroma undergoes cross-linking reactions[4] to a depth of approximately 200 to 300 μm[5,6] and prevents further corneal stroma thinning associated with keratoconus[7–14] by increasing corneal rigidity.[4,13,14] The molecular mechanisms that account for the stromal changes may involve cross-linking of collagen fibrils,[15] but the precise steps required have not been determined, nor has the possible involvement of noncollagen molecules been assessed. Lysine-based cross-links have been hypothesized in corneal responses to RFUVA treatment,[15] but they have not been demonstrated chemically.

Riboflavin-catalyzed photosensitization, photo-oxidation, and photopolymerization classically involve the production of singlet oxygen,[16–19] which then reacts with available groups nearby (in the corneal stroma, these could involve collagens and proteoglycans). These reactions may involve tyrosine residues,[20] advanced glycation end products,[21] or changes in secondary or tertiary structure.[22,23] Tyrosine residues in collagen can form pi-pi complexes[17] leading to dityrosine cross-links.[24–27] Histidine residues in collagen may produce cross-links in the presence of singlet oxygen.[28] If the cross-linking reactions occurring in RFUVA-treated corneas involve singlet oxygen, the reaction can be predicted to be inhibited by sodium azide.[17,29] Conversely, reactions mediated by reactive oxygen species ought to be enhanced if the RF is dissolved in D_2O, a solution in which the half-life of singlet oxygen is increased, giving it more time to create the reactive oxygen species reactants for cross-links.[29] The presence of biological molecules has been reported to influence the half-life of singlet oxygen ($^1O_2$) because of differing microenvironments. It was found that the half-life enhancement of $^1O_2$ in D_2O in biologically simulated environments was in the range of 6.8× to 12×, depending on the hydrophobic content of the media.[30]

Treatment of corneas with RFUVA has been shown to increase the diameters of the irradiated collagen fibrils.[31] Such a morphologic change may be a manifestation of simple precip-

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itation of molecules, such as proteoglycans, on the surfaces of the fibrils,[22] independent of any covalent cross-linking involving collagen by itself. The corneal stroma is the epitome of a precisely ordered, dense extracellular matrix because it is a *para*-crystalline array of very small-diameter collagen fibrils surrounded by a special array of four types of proteoglycans: three core proteins bearing glycosaminoglycan (GAG) chains of keratan sulfate (lumican, keratocan, and mimecan) and one bearing GAG chains of chondroitin/dermatan sulfate (decorin). These core proteins belong to a class of proteins known as small leucine-rich repeat proteins. Current molecular models of the corneal stroma suggest that the proteoglycan core proteins wrap themselves laterally around the collagen fibrils in a manner that folds their hydrophobic domains inside, against the collagen fibrils.[32] In contrast, the highly sulfated GAG chains are thought to stick out laterally away from the sides of the collagen fibrils, forming an exterior hydrophilic shell. The thickness of that shell matches the thickness of the shell surrounding adjoining fibrils, producing precise center-to-center spacing between the collagen fibrils that is characteristic of corneal stromas and necessary for their transparency.[33,34]

Considering this structure, the molecular candidates that might be involved in RFUVA-catalyzed corneal cross-linking appear to be of three classes. *Intrafibrillar bonds* could form inside individual collagen fibrils if the ε-amino groups of the lysine residues in one α-chain of tropocollagen[35] were to react with a residue in an adjacent α-chain of another tropocollagen molecule, as occurs during normal aging in an enzymically driven process that produces aldehyde groups,[36] once one of those residues has been activated by RFUVA. *Fibrillar-ECM bonds*, or at least coprecipitations, seem likely for collagen fibrils. They have been seen to increase in diameter as a direct result of the 30-minute RFUVA treatment.[31] In that case, any covalent bonding would be likely to occur between activated residues along tropocollagen molecules and appropriate residues in a proteoglycan core protein because of their molecular proximity.[32] *Interlamellar bonds* would seem to be the most likely to increase the mechanical strength of the cornea because they could physically link entire adjacent lamellae of the corneal stroma, as do the sutural fibers of elasmobranch corneas.[37,38] Physical interactions might occur between separate collagen fibrils within an individual ply and between collagen fibrils in adjacent plies. In both cases, such interactions could occur via the terminal domains of the GAG chains that extend from the sides of all collagen fibrils like the bristles of a bottle brush, with adjacent proteoglycan-coated collagen fibrils interacting only via the tip domains of the GAG chains and with GAG-GAG covalent bonding occurring if the RFUVA treatment caused terminal domains to become photoactivated.

The purpose of this study was to use a variety of chemical treatments to block specific functional moieties on extracellular matrix molecules to determine their specific contribution and mechanistic involvement in RFUVA-catalyzed cross-linking in the rabbit and shark corneal stroma.

Methods

Tissue Preparation

At the Emory University Department of Ophthalmology, fresh rabbit eyes were received from Pel-Freez Biologicals (Rogers, AR) and stored for no more than 2 days at 0°C to 4°C. Corneas were de-epithelialized by gentle scraping of the outer surface with a scalpel (Fig. 1A). Then corneas were removed from eyes, together with a scleral rim, and snap-frozen in plastic bags without liquid on dry ice before storage at −20°C. In preparation for RFUVA experiments and measurement of tissue tensile strength, frozen corneas were thawed by total immersion in a succession of two dishes of Hanks balanced salt solution (BSS) for 5 minutes each; four radial cuts were made across approximately one-third of the cornea in preparation for topical treatment with RF and other solutions.

At the Mount Desert Island Biological Laboratory (Salisbury Cove, Maine), fresh eyes were dissected from adult spiny dogfish sharks (*Squalus acanthias*; average length, 80.1 cm; average weight, 1.78 kg) within 3 minutes of euthanatization and were transferred to seawater on ice. Corneas then were removed with a 4-mm scleral rim, rinsed 2× in running sea water, quick-frozen in liquid nitrogen, and stored at −20°C. In preparation for RFUVA experiments, frozen corneas were thawed by immersion in three successive baths of sea water (10 minutes total thawing time). Frozen rabbit corneas from the Emory University preparation were thawed by total immersion in a succession of two immersions for 5 minutes each in Hanks BSS. The treated corneas were then cut into two strips of 2-mm width across the central cornea and subjected to the destructive tension testing to determine tissue tensile strength.

At Kansas State University (Manhattan, Kansas), isolated corneas of spiny dogfish (obtained at MDI by the procedures described) were thawed in 0.53 M NaCl isotonic sea water and then de-epithelialized. Corneas were removed from frozen whole young rabbit eyes (Pel-Freez Biologicals, Rogers, AR) while the whole globe was still frozen; the isolated corneas were then thawed in 1× PBS and subsequently de-epithelialized. Radial cuts were made with a scalpel in preparation for pretreatment with reagents and RFUVA.

RFUVA Treatments and Tension Measurements

Rabbit and shark corneas were treated identically during the RFUVA treatments (Fig. 1A). Surfaces of de-epithelialized corneas were treated topically for 30 minutes at room temperature by the addition of 20 μL of 0.1% RF (wt/vol; 2.657 mM) in 20% dextran (wt/vol) in isotonic saline (Medio-Cross; Steffens'-Kronoen Apotheke, Kiel, Germany) every 2 minutes for 30 minutes. For the next 30 minutes, topical application of the RF solution continued, and simultaneously the anterior stromal surface was irradiated with long-wavelength ultraviolet light (365 nm, UVA, 3 mW/cm^2 intensity) at a distance of 50 mm using a radiation system (UV-X Radiation System for Corneal Cross-linking CXL; IROC Medical, Zurich, Switzerland). After treatment, a central 4-mm band of each cornea was immediately cut in half across its diameter to yield two 2-mm wide strips, sclera-to-sclera, from each half cornea (Fig. 1A). Then each strip was mounted in a device (G&R Manufacturing, Manhattan, KS) with the tissue mount spanning the corneal-scleral junction of the tissue on both edges. Constant tension was applied by pulling at a constant rate of 6 mm/min on the mounted strip until each tissue strip ruptured in the central region of the cornea. The amount of force being exerted on the tissue sample was monitored every 0.1 seconds with a digital force gauge (model GS; Dillon Quantrol, Fairmont, MN) mounted along the axis of tension, which generated a graph of tension force exerted × time (seconds) while tension was being applied (Fig. 1B) in acquisition software (Collect Data; Labtronics, Guelph, ON, Canada). The maximum tension value before rupture was taken as the maximum destructive tension for the sample. Such data were then collected from the control and experimental groups to generate mean values.

Statistical Analysis

Data from each experimental group were compared with the data from the relevant control using a Student's *t*-test assuming unequal variance to determine statistical significance. Confidence intervals of 95% were also calculated for each group and were reported in the appropriate figure. All mean values are reported as mean ± 95% confidence interval.

Preparation of Riboflavin Solutions

RF-only control groups were treated topically with standard clinical RF solution (Medio-Cross; Steffens'-Kronoen Apotheke), containing 0.1%

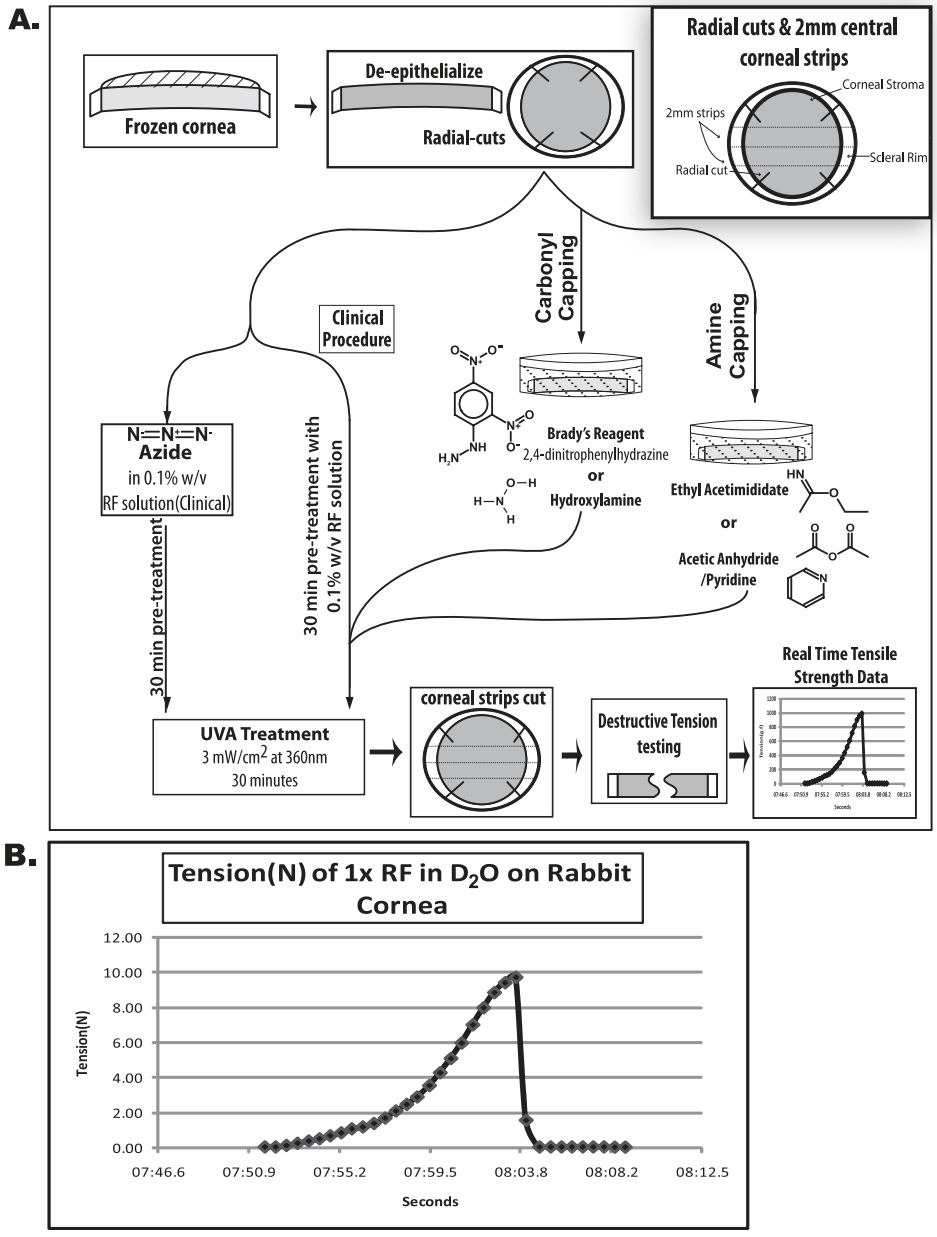

FIGURE 1. (**A**) Tissue manipulations for experimentation and tension measurements. Chemical treatments used for capping carbonyl groups in tissue or for capping free amine groups, both before the standard RFUVA treatment, are shown. (**B**) Example of the tension versus time profile recorded while destructive tension was applied to a strip of corneal tissue. All strips displayed plots of this shape. Destructive tension was the maximum tension exerted just before the strip broke. Strips broke within the central region of the cornea. Two such 2-mm strips were assayed from each cornea, with four corneas assayed per treatment group; thus, eight strips were assayed per treatment group (total, $n = 8$).

wt/vol (2.657 mM) RF dissolved in 20% wt/vol dextran 500 (Fisher Scientific, Pittsburgh, PA).

Other solutions were also used for topical application to the stromal surface in place of the RF solution. UVA-only control corneal stromas were treated topically with a 20% wt/vol dextran 500 (Fisher Scientific) Hanks balanced saline solution. RF solution + azide (248 mM or 495 mM) was prepared from a 0.1% wt/vol solution of RF in Hanks balanced saline with 20% wt/vol dextran 500 (Fisher Scientific). RF was dissolved in 100% D_2O (RF, 0.265 mM [0.1×] or 2.65 mM [1×]; Sigma-Aldrich, St. Louis, MO) with 20% wt/vol dextran 500 (Fisher Scientific).

^{1}H Nuclear Magnetic Resonance Detection of 2,3-Butanedione

For ^{1}H nuclear magnetic resonance (NMR) detection of 2,3-butanedione, 600 μL (corresponding to total treatment solution volume over 1-hour treatment) of a 0.025 M solution of RF was made in D_2O (Sigma Aldrich) and charged to a 3-mL quartz cuvette with 0.65 mL deuterated chloroform ($CDCl_3$; Cambridge Isotope Laboratories, Andover, MA) spiked with equimolar lipophilic internal standard (relative RF) of 1,1,2,2-tetrachloroethane. After addition, the cuvette was grease-sealed and irradiated with the UV-X light system for the 30-minute treatment interval. The mixture was shaken every 5 minutes to sequester any 2,3-butanedione produced and to minimize H-D exchange to allow for more accurate integration of product peaks. The ^{1}H NMR spectra were then taken on a 400-MHz NMR spectrometer (Varian; INOVA, Palo Alto, CA).

Carbonyl Identification and Capping

Free carbonyl groups in corneal tissue, assumed to be represented mostly as aldehyde groups but also potentially as ketones, were identified using the water-based ligation protocol of Dirkson and Dawson[39] with minor modifications to account for the dramatically increased scale of the reactions. Commercially available Alexa Fluor 488 hydrazide (sodium salt; Invitrogen, Carlsbad, CA) was dissolved in 1.117 mL of 3× glass-distilled H_2O to make approximately 1 mM solution (here

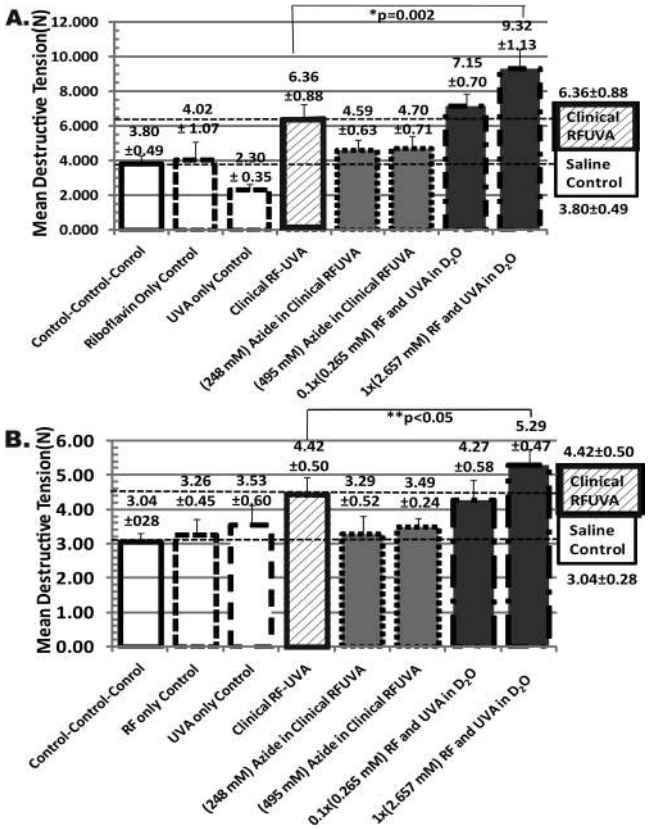

FIGURE 2. Mean destructive tension for control and oxygen-manipulated corneas. Effects of azide and deuterium oxide on destructive tension values for corneal strips. (**A**) Rabbit. (**B**) Shark. Mean destructive tension for nontreated saline control corneas and for RFUVA-treated corneas are shown in this figure and in Figures 4 to 7 as *horizontal dashed lines* to provide a convenient visual gauge of the degree to which each chemical treatment affects the degree of cross-linking with respect to those standard values. A 2-fold increase in azide concentration still caused the same degree of inhibition. A 10-fold decrease in RF concentration from clinical values still caused significantly greater stimulation of cross-linking when it was dissolved in D_2O. Each group, $n = 8$. $^*P < 0.01$; $^{**}P < 0.05$.

referred to as A). Another stock solution of 200 mM aniline (Sigma-Aldrich) in 1× PBS was prepared immediately before use (here referred to as B). The corneas were immersed in a minimal volume (roughly 12 mL/6 corneas) of B in light-excluded containers. Into the immersion mixture, 240 μL A was added to give a final hydrazide molarity of approximately 24 μM. The combined solution of A+B with corneas was agitated with the exclusion of light for 6 hours. After treatment, corneas were washed twice for 1 hour at room temperature with equal volumes of 1× PBS to completely remove excess fluorescent reagent and aniline.

Fluorescent images were captured with a dissecting microscope (MZ16F FLUO COMBI III; Leica, Wetzlar, Germany) and filter set for GFP2 (Leica).

Carbonyls in the corneal tissue were blocked/capped (Fig. 1A) with either 2,4-dinitrophenylhydrazide (DNPH; Brady's reagent)[40] to form a hydrazone or hydroxylamine to form oximes,[41] as follows: Suspend 2 g of 2,4-dinitrophenylhydrazine in 100 mL methanol. Cautiously and slowly add 4 mL concentrated sulfuric acid. Filter if necessary. Final solution molarity is 0.1 M capping reagent. The hydroxylamine solution was prepared in an identical fashion from hydroxylamine HCl in 25:1 methanol/sulfuric acid. Corneas were incubated for 3 hours at 25°C with constant agitation.

On completion of the methanol treatment, corneas were removed from the methanol solution and reacclimated to physiological pH and osmolarity. DNPH- and hydroxylamine-treated corneas were reacclimated in a slightly different way to account for the different solubilities of the capping reagents in water (to ensure total removal of residual capping reagent). DNPH corneas were washed with 50 mL methanol to remove residual DNPH (poorly water soluble) for 1.5 hours. Next, the corneas were washed in a series of two 50-mL PBS washes of appropriate osmolarity for the given species. Hydroxylamine-corneas bypassed the methanol wash because of high water solubility and ease of removal of excess capping reagent in buffered solution. At the end of the reacclimation, the corneas were in species-specific buffered solutions of physiologically correct osmolarity.

Capping efficiency was tested fluorescently via the aforementioned labeling protocol. Capped corneas were treated with the hydroxylamine capping protocol, reacclimated to physiological saline, and subjected to fluorescence labeling.

Amine Capping

Free amino groups in corneal tissue were blocked/capped with acetic anhydride in pyridine[42,43] or with ethyl acetimidate in pyrophosphate buffer, pH 9.2[44] (Fig. 1A). The pH of a 1% solution of sodium pyrophosphate (PPi) in isotonic saline solution containing 0.8% NaCl and 0.04% KCl was adjusted to pH 8.8. To the previously prepared PPi stock saline solution (100 mL), 0.274 g ethyl acetimidate HCl salt (Sigma-Aldrich) was added 45 seconds before the addition of the corneas, and the pH was readjusted to a final reaction pH of 9.36, with a final reactant molarity of 0.200 M ethyl acetimidate. Corneas were incubated for 6 hours at 25°C with constant agitation. Free amino groups were detected by reaction with ninhydrin.[45,46]

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

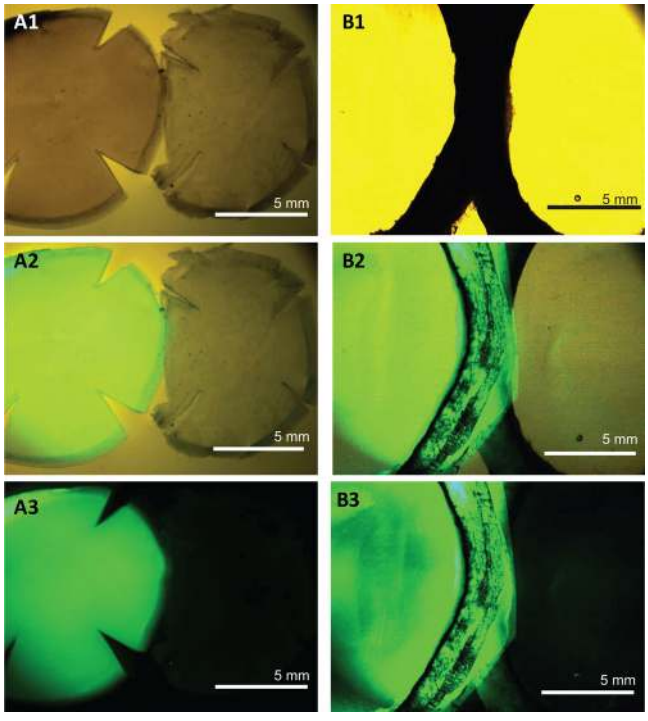

FIGURE 3. Available carbonyl groups in corneas were labeled with fluorescent marker Alexa Fluor 488 hydrazide versus saline-treated controls at pH 7.5 in water. (**A1–A3**) Rabbit corneas. (**B1–B3**) Shark corneas. (**A1**, **B1**) One cornea of each pair was ligated with carbonyl marker Alexa Fluor 488 hydrazide; the other cornea was a saline-treated control. Bright-field illumination only. (**A2**, **B2**) Fluorescence and bright-field illumination. (**A3**, **B3**) Fluorescence illumination only. Scale bar, 5 mm.

RESULTS

Effect of RFUVA on Strength of Rabbit and Shark Corneas: Effects of Azide and D_2O Indicate a Requirement for Singlet Oxygen

After exposure to RFUVA treatment, corneas increased in breaking strength compared with saline controls (rabbit, 167% of saline-treated controls [Fig. 2A]; 145%, shark [Fig. 2B]). For both rabbit and shark corneas, RFUVA treatment causes significant tissue strengthening (RFUVA cross-linking), indicating the generality of the cross-linking reaction mechanism of two other species, in addition to humans. The standard levels of breaking strength determined for saline controls and for RFUVA-treated corneas of each respective type, rabbit and shark, are represented in Figures 2 to 7 as horizontal dashed lines to provide a convenient visual gauge of the degree to which each chemical treatment affects the degree of cross-linking with respect to those standard values.

Control treatments with RF alone and UVA alone generated the same degree of tissue strength as saline controls (Figs. 2A, 2B). Thus, simultaneous presence of both RF and UVA is required to produce significant cross-linking.

For both rabbit and shark corneas, the RFUVA cross-linking reaction(s) can be blocked (decreased destructive tension relative to the clinical RFUVA strength) by the presence of sodium azide, indicating a requirement for singlet oxygen ($^1O_2$) as a reactive intermediate (Figs. 2A, 2B). Azide almost totally prevented RFUVA cross-linking of both rabbit and shark corneas at two different azide concentrations (rabbit, 495 mM azide, 123% of saline controls, 248 mM azide, 121%; sharks, 495 mM azide, 115% of saline controls, 248 mM azide, 108%). Therefore, we concluded that singlet oxygen is a necessary reactant and intermediate for the cross-linking reaction.

Conversely, for both rabbit and shark corneas, RFUVA cross-linking is greatly enhanced in the presence of deuterium oxide (D_2O; Figs. 2A, 2B). Moreover, in the presence of D_2O, RF at even one-tenth of the currently used clinical concentration, a value that is below the threshold for RF concentration-independent tensile strength increases,[47] still induces increased cross-linking to the extent of the RFUVA control. D_2O enhances RFUVA cross-linking of both rabbit and shark corneas. When using the current 1× clinical concentration of RF (2.65 mM, 0.1% wt/vol), the breaking strength of rabbit corneas increases to 245% of saline controls and to 147% of RFUVA treated without D_2O and to sharks 174% of saline controls and 120% of RFUVA treated without D_2O. Even when the RF concentration was reduced to one-tenth of the normal clinical concentration, the presence of D_2O still allowed cross-linking to be enhanced over levels in the absence of D_2O or be equivalent to it (rabbits, 188% of saline controls and 113% of RFUVA treated without D_2O; sharks, 141% of saline controls and 97% of RFUVA treated without D_2O).

Data in Figures 2A and B indicate that RFUVA cross-linking is a $^1O_2$-mediated process for both rabbit and shark corneas.

Endogenous Carbonyls Are a Detectable Constituent of the Native Extracellular Matrix

Carbonyls were ligated with a fluorescent hydrazide tag under physiological conditions. Strong fluorescence indicates that ligation to endogenous carbonyls occurred in the native extracellular matrix of both rabbit and shark corneas (Fig. 3).

Tissue Carbonyl Groups Play a Major Role in RFUVA Cross-linking Reactions

Preexisting carbonyl groups in corneal tissue were blocked/capped by treatment with either DNPH (Brady's reagent) or with hydroxylamine. Results indicated significant inhibition of cross-linking in rabbit corneas (Fig. 4A) and in shark corneas (Fig. 4B), whereas control samples exposed to similar acidic conditions required for capping exhibited essentially normal levels of RFUVA cross-linking.

To qualitatively assess the degree of capping, fluorescent ligation of carbonyls was performed on control treated compared with capped corneas (Fig. 4C). The underlying chemistry (hydrazone formation centered on a C = N double bond) is identical in the case of Alexa Fluor 488 hydrazide and in the case of Brady's reagent, despite the milder fluorescence labeling protocol. Capping caused a dramatic reduction in the amount of labeling.

The slight degree of fluorescence observed in the capped corneas is to be expected because of the ligation protocol. Aniline, the operative nucleophilic catalyst that imparts the speed and effectiveness of the ligation protocol, speeds both the ligation reaction of the hydrazide to free carbonyls and aids in the equilibration of capped species with the fluorescent hydrazide. Ordinarily, in water, the K_{eq} of bound oximes (hydroxylamine-capped carbonyls) is more than 10^8 and approximately 10^5 for hydrazones (DNPH and fluorophore-capped carbonyls).[39] When the oxime-capped carbonyls from the treated corneas are placed in the fluorescent ligation solution, there is a new equilibrium established between the fluorescent hydrazide and the capped oxime. Although the oxime cap is still favored by 3 orders of magnitude, there will be some incorporation of the fluorescent label into the capped cornea because of the relative abundance of dye molecules in the system.

Thus, carbonyl-capped corneas showed dramatically reduced levels of RFUVA cross-linking compared with control corneas. This conclusion was true for both rabbit and shark corneas, whose carbonyl groups had been capped with either DNPH or hydroxylamine.

Tissue Primary Amine Groups Do Not Play a Major Role in the RFUVA Cross-linking Reactions

Treatment of rabbit corneas with RFUVA in the solution containing PPi buffer at pH 9.2 and ethyl acetimidate to cap free amine groups caused an increase in tissue strength to 123% that of nonirradiated controls, almost the same amount as when corneas are immersed in simple saline (167% of corresponding controls; Fig. 5A [see the dotted lines for those reference standards]). Treatment of corneas with ninhydrin showed no reaction after treatment with ethyl acetimidate, whereas the controls treated only with PPi buffer reacted strongly (data not shown), indicating that corneas treated with ethyl acetimidate contained no detectable free amine groups. Thus, in rabbit corneas, amine groups participate in RFUVA cross-linking, but their role is minor compared with that of carbonyl-dependent mechanisms.

These same conclusions can be made from the behavior of shark corneas treated with pyridine and acetic anhydride or with PPi buffer and ethyl acetimidate (Fig. 5B; both similarly became ninhydrin negative after treatment with acetic anhydride or ethyl acetimidate). Treatment of shark corneas with RFUVA in the solution containing PPi buffer at pH 9.2 and ethyl acetimidate to cap-free amine groups caused an increase in tissue strength to 122% of nonirradiated controls, similar to that for corneas immersed in saline (145% of corresponding controls; Fig. 5B [see the dotted lines for those reference standards]). Treatment of shark corneas with RFUVA in pyridine and acetic anhydride to cap free amine groups caused an increase in tissue strength to 149% that of nonirradiated controls, identical with that when corneas are immersed in saline (145% of corresponding controls). Thus, almost normal de-

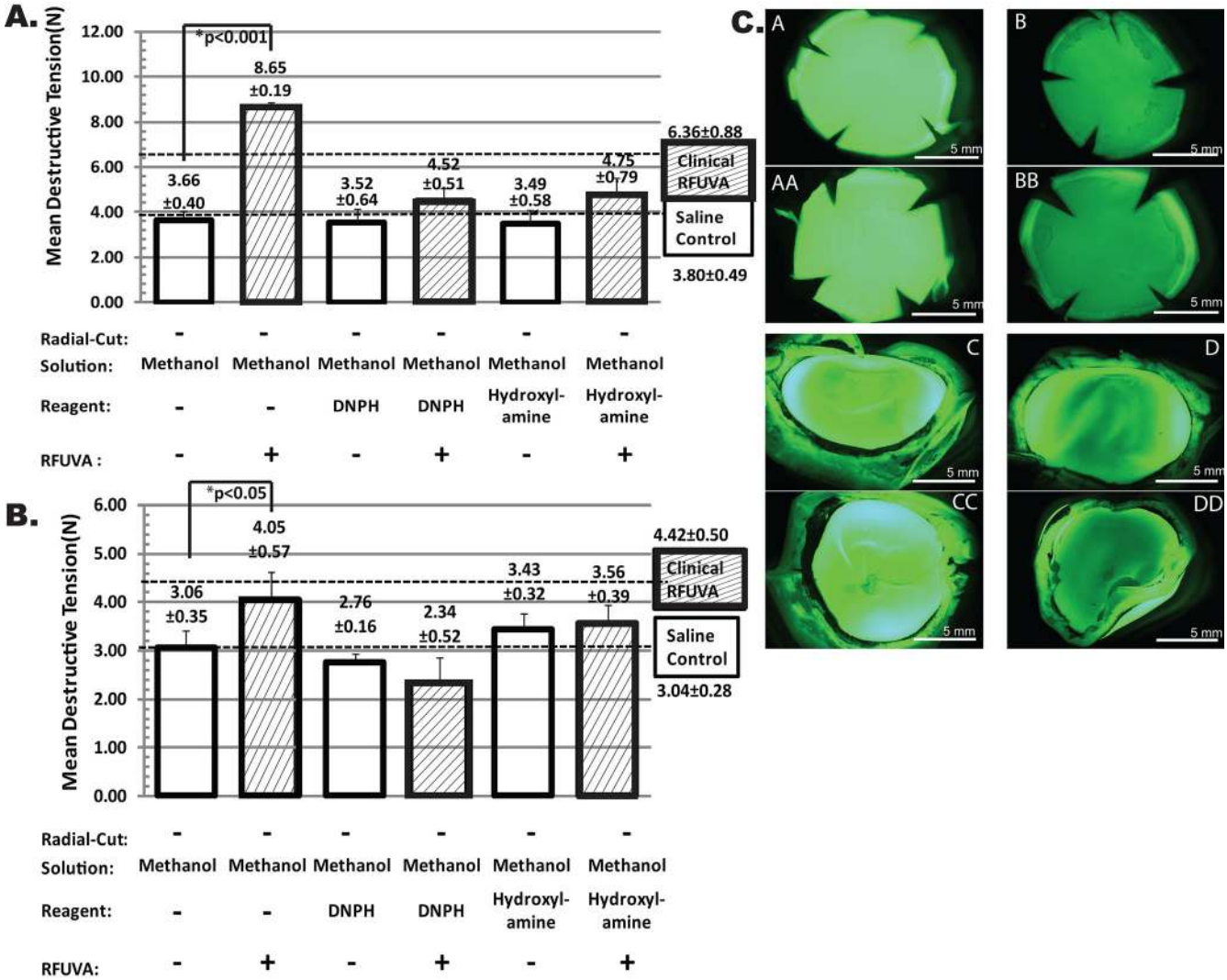

Figure 4. Mean destructive tension for carbonyl-capped corneas. Effects of acidic methanol control treatments and subsequent carbonyl group capping with either DNPH or hydroxylamine, both dissolved in acidic methanol, before RFUVA treatment. (**A**) Rabbit, $^{*}P < 0.001$. (**B**) Shark, $^{**}P < 0.05$. Each group, $n = 8$. (**C**) Available carbonyl groups in corneas were labeled with fluorescent marker Alexa Fluor 488 hydrazide before and after carbonyl capping reaction. (**A**, **AA**) Rabbit acidic methanol control corneas labeled with Alexa Fluor 488 hydrazide. (**B**, **BB**) Rabbit corneas carbonyl capped with hydroxylamine before labeling with Alexa Fluor 488 hydrazide. Degree of labeling is diminished by capping carbonyl groups. (**C**, **CC**) Shark acidic methanol control corneas labeled with Alexa Fluor 488 hydrazide. (**D**, **DD**) Shark corneas carbonyl capped with hydroxylamine before labeling with Alexa Fluor 488 hydrazide. Degree of labeling is diminished by capping carbonyl groups. Scale bar, 5 mm.

grees of RFUVA cross-linking occurred in shark corneas even in the absence of free amino groups generated by either of two standard blocking techniques.

Solvent effects were also noted for both rabbit and shark corneas, as their tensile strength increased in response to the PPi buffer containing ethyl acetimidate (Figs. 5A, 5B). Nonetheless, such corneas still undergo increased cross-linking in response to RFUVA compared with corneas in saline.

Conclusions: RFUVA cross-linking does not appear to involve or require cross-linking of amine groups.

Blocking Both Singlet Oxygen and Tissue Carbonyl Groups Blocks All RFUVA Reactions That Increase Corneal Strength

The previous data reveal the effects of individually inhibiting putative mechanisms involving singlet oxygen, tissue carbonyl (aldehyde and ketone) groups, or tissue primary amine groups. However, to evaluate whether other undetected cross-linking reactions were occurring, a combination of two blocking treatments was applied to determine whether together they would inhibit all stromal cross-linking. Thus, corneas treated with hydroxylamine to block participation of tissue carbonyl groups were, in addition, treated with azide to block the participation of singlet oxygen in cross-linking reactions. The results indicate that such corneas display no remaining mechanisms by which stromal cross-linking occurs in response to RFUVA, both in rabbits (Fig. 6A) and in sharks (Fig. 6B). Thus, in RFUVA-mediated corneal cross-linking, only reactions requiring singlet oxygen-mediated groups, tissue carbonyl groups, or both are involved.

Solvent Effects on Corneal Strength

It is customary to cap free amine groups by treating samples with acetic anhydride dissolved in pyridine.[42,43] However, when rabbit corneas were treated with pyridine or with pyridine containing acetic anhydride, the mean destructive tension

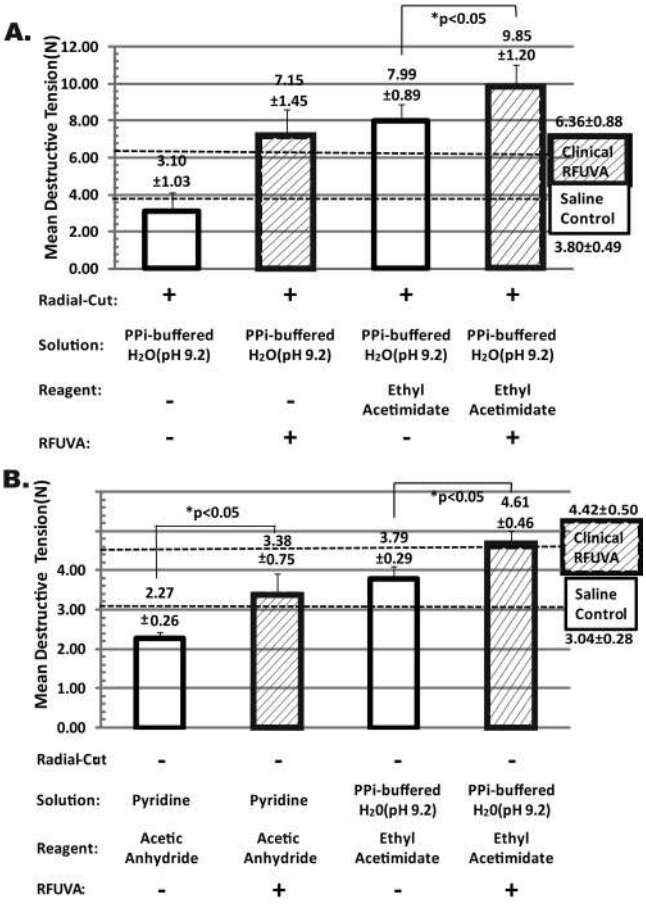

FIGURE 5. Mean destructive tension for amine-capped corneas. Effects of blocking tissue amine groups. (**A**) Rabbit, with ethyl acetimidate in pH 9.2 aqueous pyrophosphate buffer; $^{*}P < 0.05$. (**B**) Shark, with acetic anhydride in pyridine or with ethyl acetimidate in pH 9.2 aqueous pyrophosphate buffer; $^{*}P < 0.05$. Each group, $n = 8$.

increased significantly (Fig. 7). Dimethylformamide (DMF), a similar solvent but a solvent unsuitable for acetic anhydride, caused rabbit corneas to increase significantly in strength. Despite the increase in strength that occurred with rabbit corneas in either pyridine or in DMF, RFUVA caused increases in tissue strength compared with controls in saline (Fig. 7). When acetic anhydride was dissolved in pyridine to block/cap free amine groups, with or even without treatment with RFUVA, rabbit corneas strength increased maximally and displayed no further increases in cross-linking in response to RFUVA.

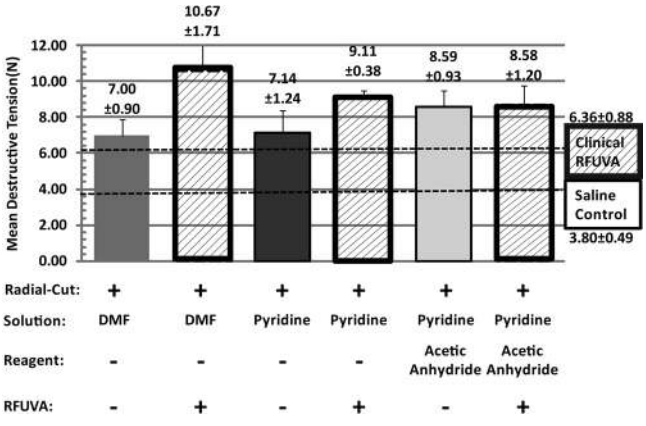

FIGURE 7. Mean destructive tension for solvent-treated rabbit corneas. Effects of common organic solvents DMF, pyridine, and acetic anhydride in pyridine on mean destructive tension of rabbit corneas. Each group, $n = 8$.

In contrast to rabbit corneas, when shark corneas were treated with pyridine, they underwent significant weakening yet still showed normal amounts of RFUVA cross-linking, even after amine groups were blocked by treatment with acetic anhydride (Fig. 5B).

Thus, for both rabbit and shark corneas, amine groups can be blocked/capped with ethyl acetimidate, and shark corneas can also even be blocked/capped with acetic anhydride in pyridine. In both cases this does not significantly inhibit RFUVA cross-linking. Thus, RFUVA cross-linking does not require the presence of free amine groups in the tissue.

2,3-Butanedione Detection

Huang et al.[17] showed with the use of gas chromatography-mass spectrometry that 2,3-butanedione was a primary product of RF degradation with $^{1}O_2$. To detect the production of 2,3-butanedione during the clinical irradiation procedure, ^{1}H NMR was used to detect and quantify butanedione (data not shown). Through the use of chloroform extraction of the aqueous RF solution through the UVA treatment regime, a peak corresponding to the hydrate of 2,3-butanedione was detected, showing 3 mol% yield relative to an equimolar (relative to RF concentration) nonvolatile internal standard (1,1,2,2-tetrachloroethane) in the deuterated chloroform ($CDCl_3$) solution. Thus, 2,3-butanedione is an available potential reactant produced during the standard RFUVA clinical protocol.

DISCUSSION

The RFUVA clinical protocol represents a significant advance in the treatment of patients with keratoconus[2,3,7–11] and is the

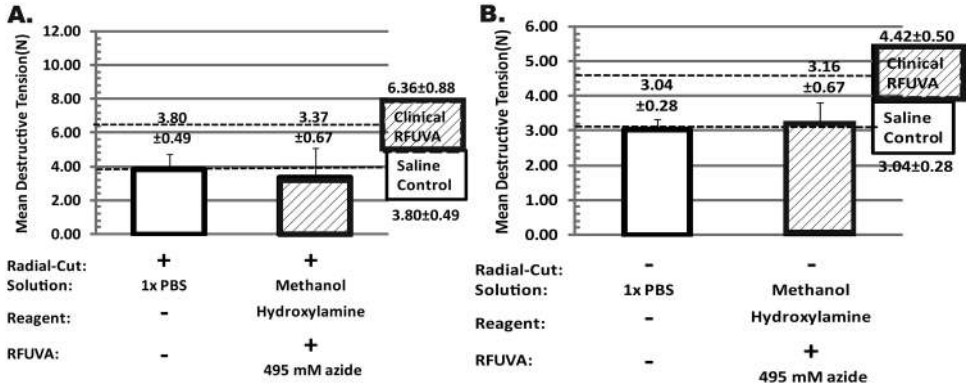

FIGURE 6. Mean destructive tension for $^{1}O_2$-quenched and carbonyl-capped corneas. Effects of both blocking tissue carbonyl groups with hydroxylamine in methanol and quenching $^{1}O_2$ by the presence of azide during RFUVA. All cross-linking is blocked. (**A**) Rabbit. (**B**) Shark. Each group, $n = 8$.

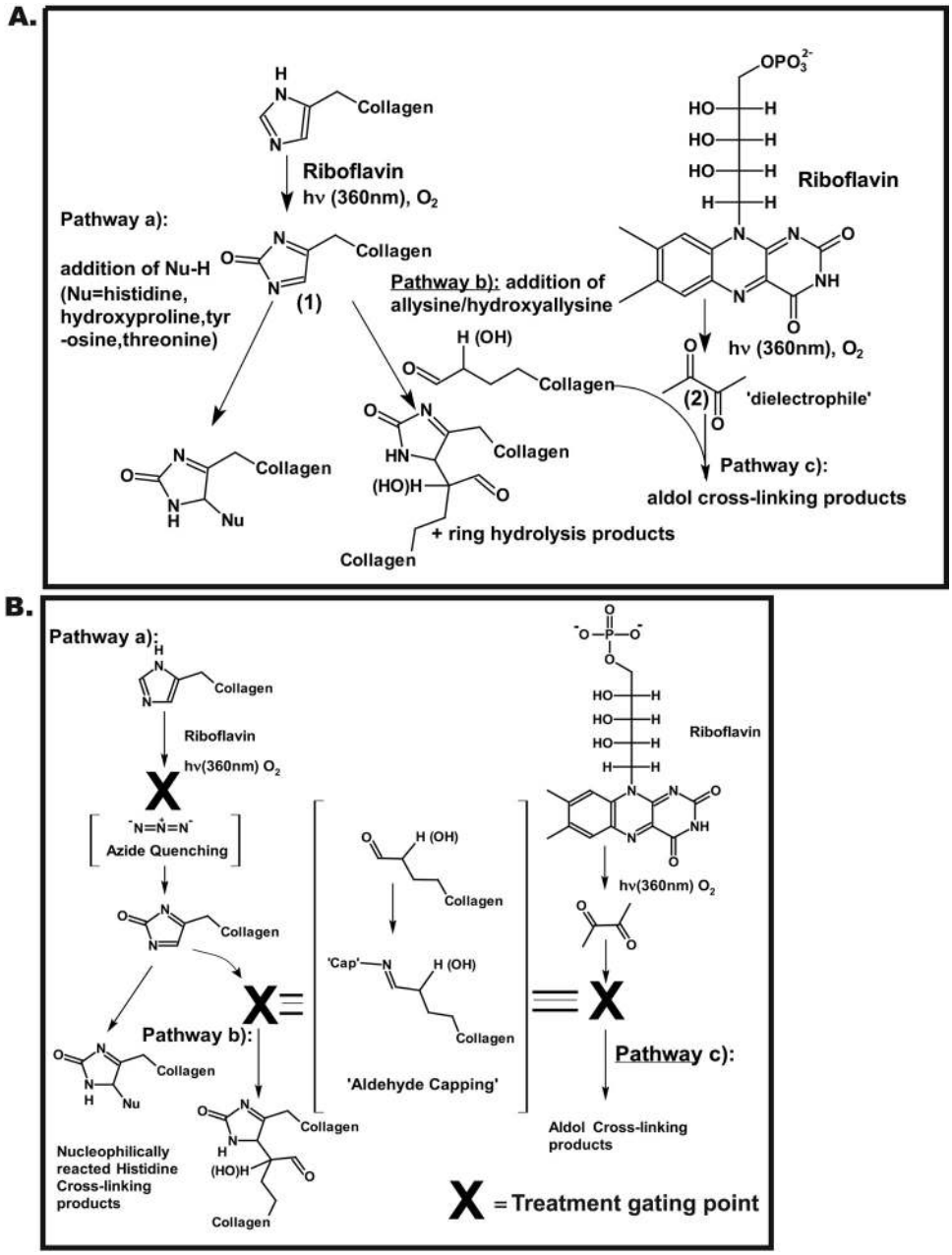

FIGURE 8. (**A**) Proposed mechanisms by which RF can induce cross-linking of collagen molecules in the presence of UVA. Pathway (**a**) is singlet oxygen-dependent, which produces imidazolone (compound 1). This short-lived intermediate can then react with an uncapped nucleophile (Nu). Pathway (**b**) invokes endogenous carbonyls (allysine) as a nucleophile in a subsidiary $^1O_2$-dependent pathway. Pathway (**c**) suggests that a self-activation product of RF, 2,3-butanedione (compound 2) could be formed on excitation of the RF, yielding an additional $^1O_2$ -dependent pathway that would react strongly with endogenous carbonyls. (**B**) Experimental gating of the putative reaction mechanisms. X represents the point along each of the three pathways at which the reactions would be blocked in those corneas treated with azide or with any of the carbonyl-capping protocols shown in Figure 1A.

topic of US clinical trials (www.clinicaltrials.gov NIH clinical trials registry number, Clinical Study NCT00647699). It is, therefore, important to understand precisely the mechanisms involved in the tissue cross-linking that appear to be the basis for increased corneal stromal tensile strength.

Studies reported here sought to determine whether reactive oxygen species (singlet oxygen [$^1O_2$]) were involved and the extent to which carbonyl or free amine groups, or both, were involved in forming the functional cross-links. Functional stromal cross-linking was determined by measuring the amount of force needed to tear the corneal tissue entirely in two after various chemical treatments.

Singlet oxygen ($^1O_2$) was involved with the RFUVA-catalyzed reactions because cross-linking was significantly inhibited by the presence of azide but, conversely, was greatly stimulated by the presence of heavy-water, deuterium oxide, D_2O. Azide is known to inhibit reactions that require $^1O_2$,[17–19] whereas D_2O is known to prolong the half-life of $^1O_2$.[29]

Free aldehyde and ketone groups (i.e., carbonyl groups) within tissues are associated with many types of reactions, including reactions with drugs,[48] general protein cross-linking,[49] and collagen cross-linking.[36] Endogenous populations of carbonyl groups are likely maintained through steady state action of lysyl oxidase,[50] which is actively produced in both keratocytes[51] and the corneal endothelium[52] and through potential products of glycation-oxidation.[53] In the present study, free carbonyl groups in tissues were fluorescently detected in native tissue and manipulated by blocking/capping with Brady's reagent (DNPH) or with hydroxylamine before rinsing and then application of the RFUVA protocol. In both rabbit and shark corneas, endogenous carbonyls were detected, and application of capping reagents dramatically blocked the ability of the RFUVA protocol to cause increased tissue strength, suggesting that, for the corneas of both animal species, free carbonyl groups in tissues are normally present and essential for RFUVA cross-linking.

Free amine groups within tissues are associated with a variety of reactions, including lipid oxidation, leading to protein cross-links,[54] nonenzymatic reactions between sugars and free amino groups on proteins leading to cross-links,[55] and cross-linking of collagens.[56] In the present study, abundant free amino groups were detected by reaction with ninhydrin[45,46] and blocked/capped by reaction with acetic anhydride[42,43] or with ethyl acetimidate.[44] However, after such treatment, corneas were still able to undergo significant cross-linking in response to RFUVA, indicating the minimal role of free amino groups for RFUVA cross-linking in the corneal stroma.

Solvents can have marked effects on tissue architecture irrespective of RFUVA-catalyzed reaction mechanisms, and some were observed here. For example, both DMF and pyridine, without treatment with RF or UVA, caused increased tensile strength in rabbit corneas (Fig. 7). However, after treatment with either DMF or pyridine and the resultant increase in strength, the tissue still responded to RFUVA treatment with a significant increase in strength (Fig. 7), indicating that the relevant reactive groups were still available.

The same cross-linking groups remained available even after free amine groups were blocked/capped with ethyl acetimidate (Fig. 5A), indicating the noninvolvement of free amino groups in the RFUVA-catalyzed reactions. Inexplicably, rabbit corneas treated with pyridine containing acetic anhydride underwent no further cross-linking in response to RFUVA (Fig. 7).

The results presented in this study suggest it is likely that increased stromal tensile strength in RFUVA-treated corneas involves reactions such as those shown in Figure 8A. Thus, it is likely that collagen molecules may become cross-linked in the simultaneous presence of RF and UVA because of the involvement of histidine, hydroxyproline, hydroxylysine, tyrosine, and threonine in cross-links. There is growing evidence indicating that the histidine side chain is a primary target for singlet oxygen generated in biological systems[28] and that a large reduction in the amount of histidine occurs.[26] On reaction, the imidazole moiety is turned into an electrophilic imidazolone that is highly reactive and susceptible to attack, forming covalent cross-links.[28] It is reasonable to assume, given that sterically hindered and biologically less common oxygen-centered nucleophiles (hydroxyproline, threonine, tyrosine) are immune to the capping procedures performed, that these secondary and phenolic alcohols represent several potential nucleophiles in bond-forming covalent pathways that may serve as an explanation for the increase in strength observed in RFUVA-treated tissue. In association with the experimentally verified involvement of carbonyl (likely aldehyde) groups, the indications of 2,3-butanedione production during the RFUVA protocol also necessitated incorporation of this potential in situ-generated reactant into any scheme that relies on RF photoexcitation in the presence of molecular oxygen. The steps in this scheme that appear to have been inhibited by azide or by capping of aldehyde groups are shown in Figure 8B. The data presented notably deviate from the trend of singlet oxygen independence observed by Kato et al.[26] in collagen aggregation and indicate that the RFUVA process chemically differs from simple aggregation. The data, unlike in collagen aggregation, implicate the spatial proximity of the cross-links because of the high degree of preorganization within the tissue. Although the involvement of collagen molecules is emphasized in Figures 8A and B, it also is likely that RFUVA could cause cross-linking of other classes of macromolecules of the corneal stroma, such as proteoglycans, to one another or to collagen molecules. Finally, the results in Figure 2A strongly suggest that the current concentration of RF could be reduced as much as 10-fold in the presence of D_2O and that the use of an RF in D_2O solution could greatly increase corneal strength and degree of cross-linking.

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References

1. Wollensak G, Spoerl E, Seiler T. Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus. *Am J Ophthalmol.* 2003;135:620–627.
2. Wollensak G. Crosslinking treatment of progressive keratoconus: new hope. *Curr Opin Ophthalmol.* 2006;17:356–360.
3. Caporossi A, Baiocchi S, Mazzotta C, Traversi C, Caporossi T. Parasurgical therapy for keratoconus by riboflavin-ultraviolet type A rays induced cross-linking of corneal collagen: preliminary refractive results in an Italian study. *J Cataract Refract Surg.* 2006; 32:837–845.
4. 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;29:1780–1785.
5. Seiler T, Hafezi F. Corneal cross-linking-induced stromal demarcation line. *Cornea.* 2006;25:1057–1059.
6. Kohlhaas M, Spoerl E, Schilde T, Unger G, Wittig C, Pillunat LE. Biomechanical evidence of the distribution of cross-links in corneas treated with riboflavin and ultraviolet A light. *J Cataract Refract Surg.* 2006;32:279–283.
7. Wittig-Silva C, Whiting M, Lamoureux E, Lindsay RG, Sullivan LJ, Snibson GR. A randomized controlled trial of corneal collagen cross-linking in progressive keratoconus: preliminary results. *J Refract Surg.* 2008;34:S720–S725.
8. Vinciguerra P, Albè E, Trazza S, et al. Refractive, topographic, tomographic, and aberrometric analysis of keratoconic eyes undergoing corneal cross-linking. *Ophthalmology.* 2009;116:369–378.
9. Kymionis GD, Diakonis VF, Kalyvianaki M, et al. One-year follow-up of corneal confocal microscopy after corneal cross-linking in patients with post laser in situ keratomileusis ectasia and keratoconus. *Am J Ophthalmol.* 2009;147:774–778.
10. Agrawal V. Corneal collagen cross-linking with riboflavin and ultraviolet-A light for keratoconus: results in Indian eyes. *Ind J Ophthalmol.* 2009;57:111–114.
11. Raiskup-Wolf F, Hoyer A, Spoerl E, Pillunat LE. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. *J Cataract Refract Surg.* 2008;34:796–801.
12. Spoerl E, Wollensak G, Seiler T. Increased resistance of crosslinked cornea against enzymatic digestion. *Curr Eye Res.* 2004;29:35–40.
13. Spörl E, Raiskup-Wolf F, Pillunat LE. Biophysical principles of collagen cross-linking *Klin Monatsbl Augenheilkd.* 2008;225:131–137.
14. Spoerl E, Huhle M, Seiler T. Induction of cross-links in corneal tissue. *Exp Eye Res.* 1998;66:97–103.
15. Wollensak G, Redl B. Gel electrophoretic analysis of corneal collagen after photodynamic cross-linking treatment. *Cornea.* 2008; 27:353–356.
16. Min DB, Boff JM. Chemistry and reaction of singlet oxygen in foods. *Compr Rev Food Sci Food Saf.* 2002;1:58–72.
17. Huang R, Choe E, Min DB. Kinetics for singlet oxygen formation by riboflavin photosensitization and the reaction between riboflavin and singlet oxygen. *J Food Sci.* 2004;69:C726–C732.
18. Choe E, Huang RM, Min DB. Chemical reactions and stability of riboflavin in foods. *J Food Sci.* 2005;70:R28–R36.
19. Choe E, Min DB. Chemistry and reactions of reactive oxygen species in foods. *Crit Rev Food Sci Nutr.* 2006;46:1–22.
20. Spikes JD, Shen HR, Kopecková P, Kopecek J. Photodynamic crosslinking of proteins, III: kinetics of the FMN- and rose Bengal-sensitized photooxidation and intermolecular crosslinking of model tyrosine-containing N-(2-hydroxypropyl)methacrylamide copolymers. *Photochem Photobiol.* 1999;70:130–137.
21. de La Rochette A, Birlouez-Aragon I, Silva E, Morlière P. Advanced glycation endproducts as UVA photosensitizers of tryptophan and ascorbic acid: consequences for the lens. *Biochim Biophys Acta.* 2003;1621:235–241.
22. Dalsgaard TK, Otzen D, Nielsen JH, Larsen LB. Changes in structures of milk proteins upon photo-oxidation. *J Agric Food Chem.* 2007;55:10968–10976.

23. Slifkin MA. Interaction of amino-acids with riboflavin. *Nature.* 1963;197:275–276.
24. LaBella F, Waykole P, Queen G. Formation of insoluble gels and dityrosine by the action of peroxidase on soluble collagens. *Biochem Biophys Res Commun.* 1968;30:333–338.
25. Waykole P, Heidemann E. Dityrosine in collagen. *Connect Tissue Res.* 1976;4:219–222.
26. Kato Y, Uchida K, Kawakishi S. Aggregation of collagen exposed to UVA in the presence of riboflavin: a plausible role of tyrosine modification. *Photochem Photobiol.* 1994;59:343–349.
27. Ishitsuka Y, Maniwa F, Koide C, et al. Detection of modified tyrosines as an inflammation marker in a photo-aged skin model. *Photochem Photobiol.* 2007;83:698–705.
28. Au V, Madison A. Effects of singlet oxygen on the extracellular matrix protein collagen: oxidation of the collagen crosslink histidinohydroxylysinonorleucine and histidine. *Arch Biochem Biophys.* 2000;384:133–142.
29. Wright A, Bubb WA, Hawkins CL, Davies MJ. Singlet oxygen-mediated protein oxidation: evidence for the formation of reactive side chain peroxides on tyrosine residues. *Photochem Photobiol.* 2002;76:35–46.
30. Baier J. *Lumineszenz-Untersuchungen zur Generierung und Relaxation von Singulett-Sauersto in zellulärer Umgebung* (dissertation). Regensburg, Germany: Universitat Regensburg; November. 2005.
31. Wollensak G, Wilsch M, Spoerl E, Seiler T. Collagen fiber diameter in the rabbit cornea after collagen crosslinking by riboflavin/UVA. *Cornea.* 2004;23:503–507.
32. Iozzo RV. The biology of the small leucine-rich proteoglycans: functional network of interactive proteins. *J Biol Chem.* 1999;274:18843–18846.
33. Meek KM, Quantock AJ, Boote C, Liu CY, Kao WW. An X-ray scattering investigation of corneal structure in keratocan-deficient mice. *Matrix Biol.* 2003;22:467–475.
34. Hayashida Y, Akama TO, Beecher N et al. Matrix morphogenesis in cornea is mediated by the modification of keratan sulfate by GlcNAc 6-O-sulfotransferase. *Proc Natl Acad Sci U S A.* 2006;103:13333–13338.
35. Bowes JH, Raistrick AS, Moss JA. Reactivity of the epsilon-amino group of collagen. *Nature.* 1965;208:1094–1095.
36. Tanzer ML. Cross-linking of collagen. *Science.* 1973;180:561–566.
37. Conrad GW, Paulsen AQ, Luer CA. Embryonic development of the cornea in the eye of the clearnose skate, *Raja eglanteria*, I: stromal development in the absence of an endothelium. *J Exp Zool.* 1994;269:263–276.
38. Goldman JN, Benedek GB. The relationship between morphology and transparency in the nonswelling corneal stroma of the shark. *Invest Ophthalmol.* 1967;6:547–600.
39. Dirksen A, Dawson PE. Rapid oxime and hydrazone ligations with aromatic aldehydes for biomolecular labeling. *Bioconjug Chem.* 2008;19:2543–2548.
40. Brady OL, Elsmie GV. The use of 2:4-dinitrophenylhydrazine as a reagent for aldehydes and ketones. *Analyst.* 1926;51:77–78.
41. Ahluwalia VK, Dhingra S. *Comprehensive Practical Organic Chemistry: Qualitative Analysis.* Hyderabad, India: Universities Press; 2004.
42. McManus JFA, Cason J. Carbohydrate histochemistry studied by acetylation techniques. *J Exp Med.* 1950;91:650–655.
43. Lillie RD. Histochemical acylation of aldehydes produced by periodic acid oxidation. *J Histochem Cytochem.* 1966;14:529–537.
44. Makoff AJ, Malcolm AD. Properties of methyl acetimidate and its use as a protein-modifying reagent. *Biochem J.* 1981;193:245–249.
45. Troll W, Cannan RK. A modified photometric ninhydrin method for the analysis of amino and imino acids. *J Biol Chem.* 1953;200:803–811.
46. Friedman M. Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *J Ag Food Chem.* 2004;52:385–406.
47. Schreiber J. *Verfestigung der Hornhaut durch UVA 365 nm und Riboflavin oder durch Glutaraldehyd* (dissertation). Dresden, Germany: Dresden University of Technology; 2003.
48. Oster KA, Oster JG. Reaction of tissue aldehydes with certain drugs. *J Pharmacol Exp Ther.* 1945;85:332–335.
49. O'Brien PJ, Siraki AG, Shangari N. Aldehyde sources, metabolism, molecular toxicity mechanisms, and possible effects on human health. *Crit Rev Toxicol.* 2005;35:609–662.
50. Reiser K, McCormick RJ, Rucker RB. Enzymatic and nonenzymatic cross-linking of collagen and elastin. *FASEB J.* 1992;6:2439–2449.
51. Kipling D, Jones DL, Smith SK, et al. A transcriptomic analysis of the EK1.Br strain of human fibroblastoid keratocytes: the effects of growth, quiescence and senescence. *Exp Eye Res.* 2009;88:277–285.
52. Fujimaki T, Hotta Y, Sakuma H, Fujiki K, Kanai A. Large-scale sequencing of the rabbit corneal endothelial cDNA library. *Cornea.* 1999;18:109–111.
53. Sady C, Khosrof S, Nagaraj R. Advanced Maillard reaction and cross-linking of corneal collagen in diabetes. *Biochem Biophys Res Commun.* 1995;214:793–797.
54. Kikugawa K, Kato T, Beppu M, Hayasaka A. Fluorescent and cross-linked proteins formed by free radical and aldehyde species generated during lipid oxidation. *Adv Exp Med Biol.* 1989;266:345–356.
55. Forbes JM, Cooper ME, Oldfield MD, Thomas MC. Role of advanced glycation end products in diabetic nephropathy. *J Am Soc Nephrol.* 2003;14:S254–S258.
56. Barnes CP, Pemble CW, Brand DD, Simpson DG, Bowlin GL. Cross-linking electrospun type II collagen tissue engineering scaffolds with carbodiimide in ethanol. *Tissue Eng.* 2007;13:1593–1605.