

Corneal Resistance to Keratolysis After Collagen Crosslinking With Rose Bengal and Green Light

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PURPOSE. The purpose of this study was to evaluate the resistance to degradation by collagenase A of corneas that have been crosslinked with Rose Bengal and green light (RGX).

METHODS. The ex vivo crosslinking procedure was performed on enucleated rabbit corneas. Corneas were deepithelialized after applying 30% alcohol. Corneas were stained with Rose Bengal (RB, 0.1%) for 2 minutes and then exposed to green light (532 nm) at 0.25 W/cm² for times to deliver doses of 50, 100, 150, or 200 J/cm² ($n = 5$ per group). Five corneas were pretreated with riboflavin solution (0.1% riboflavin) for 15 minutes and irradiated with ultraviolet A (UVA) light (370 nm, 3 mW/cm²) for 30 minutes. Five corneas underwent only de-epithelialization and were otherwise untreated. Five corneas were stained with RB without light exposure. The central corneas of each group was removed with a 8.5-mm trephine and incubated at 37°C in 0.3% collagenase A solution. Time to dissolution of each cornea was compared across treatments.

RESULTS. Corneas treated with RGX were treated with light fluences of 50, 100, 150, and 200 J/cm²; these corneas dissolved completely at 8.3 ± 1.2 , 11.1 ± 1.4 , 12.4 ± 1.7 , and 15.7 ± 1.8 hours, respectively. Corneas treated by riboflavin and UVA light dissolved at 15.7 ± 1.7 hours, and nontreated corneas dissolved at 6.1 ± 1.3 hours. Corneas treated with only RB (no green light) dissolved at 9.3 ± 1.7 hours. Compared with the untreated corneas, all of the RB groups and the riboflavin-UVA-treated group of corneas degraded statistically significantly slower than untreated corneas ($P < 0.05$).

CONCLUSIONS. Crosslinking with RGX increased corneal resistance to digestion by collagenase comparable to that produced by riboflavin and UVA treatment.

Keywords: crosslinking, rose bengal, green light

In most parts of the world, the management of corneal ectasia involves visual rehabilitation and slowing disease progression. Collagen crosslinking (CXL) using riboflavin and ultraviolet A (RF-UVA) light has been used to strengthen the corneal stromal collagen and is effective in decreasing progression in keratoconus.¹

Treating corneas with Rose Bengal (RB) topically and exposing the tissue to green light (RGX) has been proposed as an alternative collagen crosslinking technology to RF-UVA crosslinking.² Corneal ectatic disorders are characterized by biomechanical instability of the cornea with increased proteolytic enzymes expression. Treatment with RGX has been shown to be safe and significantly increase corneal stiffness using a treatment time of 12 minutes.^{2,3} The biochemical effect engendered by RGX has not been addressed by previous studies. More recently, investigators have used resistance to collagenase degradation to test the effectiveness of RF-UVA.^{1,4-7} Here, we compare CXL by different RGX dosing regimens to that of RF-UVA using a collagenase degradation assay.

Using a collagenase degradation assay previously described,⁵ a comparative study of the resistances of corneas

crosslinked by RGX or RF-UVA to collagenase A degradation was performed.

METHODS

Experimental Design

A comparative ex vivo study of the resistances of nonirradiated rabbit corneas crosslinked by RGX or by RF-UVA to collagenase A degradation was performed.

Reagent Preparation

Rose Bengal (1 mg/mL or 0.1%) solution was prepared from Rose Bengal sodium salt (Sigma-Aldrich Corp., St. Louis, MO, USA) thoroughly mixed in PBS. Riboflavin (1 mg/mL or 0.1%) solution was prepared from riboflavin-5'-phosphate sodium salt hydrate (Sigma-Aldrich Corp.) in 20% dextran (w/w) (Sigma-Aldrich Corp.). Collagenase A (matrix metalloproteinase 1a or EC 3.4.24.3, 0.3% [3 mg/mL]; Sigma-Aldrich Corp.) was freshly prepared before every experiment in PBS. All solutions were



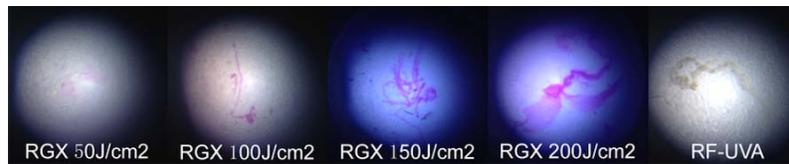


FIGURE 1. Crosslinked corneal button 24 hours after collagenase A digestion. Five groups are shown in this picture: RB50, RB100, RB150, RB200, and RF-UVA.

covered with aluminum foil to protect them from light and stored at 4°C until use.

Tissue Preparation

Frozen mature New Zealand White rabbit eyes were obtained frozen (Pei-Freez Biologicals, Rogers, AR, USA) and thawed in 1× PBS at room temperature for 1 hour before use. Each cornea was fitted into a Barron artificial anterior chamber (Katena Eye Instruments, Denville, NJ, USA) filled with balanced salt solution. The corneal epithelium was removed by debridement in the RF-UVA group and using 30% ethanol for 15 seconds in the RGX group and control group.

Corneal Crosslinking Procedure

Riboflavin/Ultraviolet Light. All corneas were pretreated with 0.1% riboflavin solution every 2 minutes for 15 minutes according to previously published CXL methods that were analyzed by a collagenase degradation assay.⁵ The corneas were irradiated with UVA light using the VEGA light emitting diode (LED)-based UV emitter (Costruzione Strumenti Oftalmici, Firenze, Italy) at a wavelength of 370 nm, irradiance of 3 mW/cm², and distance of 54 mm from the cornea. The UV emitter was calibrated before every experiment. Drops of riboflavin were applied at 5-minute intervals during the irradiation treatment. The corneas were irradiated for 30 minutes.

Rose Bengal/Green Light. All corneas were pretreated with 0.1% RB solution for 2 minutes. Corneas were irradiated with green light using a cw KTP frequency (continuous wave potassium titanyl phosphate crystal) doubled solid-state laser (Oculight OR; IRIDEX Corporation, Mountain View, CA, USA) at a wavelength of 532 nm that is strongly absorbed by the RB. The laser light from an optical fiber was expanded to a 1.2-cm-diameter beam to deliver an irradiance of 0.25 W/cm² on the central cornea. Corneas were treated for 3.25, 6.5, 10, or 13.25 minutes (to deliver fluences of 50, 100, 150, or 200 J/cm², respectively) according to the protocol of Cherfan et al.² This protocol was shown not to cause toxicity to stromal keratocytes *ex vivo* and can be used to stiffen corneas thinner than 400 μm.² Thirty-five enucleated rabbit eyes were divided into the following subgroups: (1) nontreated control group (five eyes), (2) RB-stained control group (RB, five eyes), (3) 50-J/cm² RGX group (RB50, five eyes), (4) 100-J/cm² RGX group (RB100, five eyes), (4) 150-J/cm² RGX group (RB150, five eyes), (6) 200-J/cm² RGX group (RB200, five eyes), and (7) 3-mW/cm² RF-UVA group (five eyes).

Enzymatic Degradation

Corneas were trephined into 8.5-mm buttons and incubated with 0.3% collagenase A solution at 37°C on a plate shaker at 150 rotations per minute. The corneas were observed hourly for the first 12 hours and then every 2 hours until complete dissolution was achieved. A 12-well cell culture plate was used to contain the corneas and run collagenase experiments.

Measurement Methods

The measurements were made in a masked fashion. One investigator treated the corneas, photographed the tissue, and coded the images; corneal measurements were taken from these photographs by a second masked observer who did not have access to the sample code. Two different measurement methods were used to assess enzymatic degradation. The observation method was as follows: direct visual observation of tissue degradation—complete tissue disappearance or a “string tissue pattern” was considered as the time of tissue dissolution (see Fig. 1). The “string pattern” is a corneal tissue end product resisting collagenase A for more than 7 days. The photography method was as follows (Supplementary Materials): corneas were photographed (Nikon D3200 Camera, 24.2 MP; Nikon, Sendai, Japan) and ImageJ software (ImageJ 1.49v; <http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) was used to calculate the remaining cornea area in millimeters squared. The camera was fixed at 20 cm from the bottom of the well containing the cornea and collagenase solution, and uniform lighting conditions were used for all photography. The 12-well cell culture plate was placed under the camera on a millimeter-calibrated graph paper to calculate corneal area, and images were taken for every well against a white and then a black background. The color of the RB was very slight when the tissue was nearly entirely digested. We took photographs with white and black backgrounds and calculated the corneal size (in mm²) based on the photographs using ImageJ software (ImageJ 1.49v; National Institutes of Health).

Statistical Analysis

Statistical analysis was done using SPSS 13.0 software. The Kruskal-Wallis 1-way ANOVA test was used to compare nonparametric nonmatched groups. We used Kruskal-Wallis because of the small sample size in each group ($n = 5$) and because we cannot assume a normal distribution of the variables. P values were ascertained using unpaired, 2-tailed Student's t -tests. Data were considered as significantly different at $P < 0.05$.

RESULTS

Corneas were treated with RB and green light fluences of 50, 100, 150, and 200 J/cm²; with riboflavin and UVA light; and with only RB (no green light). Time to dissolution and percent of corneal surface were compared with the untreated corneas to study the resistance to enzymatic degradation. The time to dissolution of the corneal button was recorded, and all groups were compared. Crosslinking increased cornea resistance to enzymatic degradation (Figs. 2–4).

Observation Method (Fig. 2)

RGX Group. Enzymatic degradation time increased with higher green light dose. The corneal buttons were dissolved at

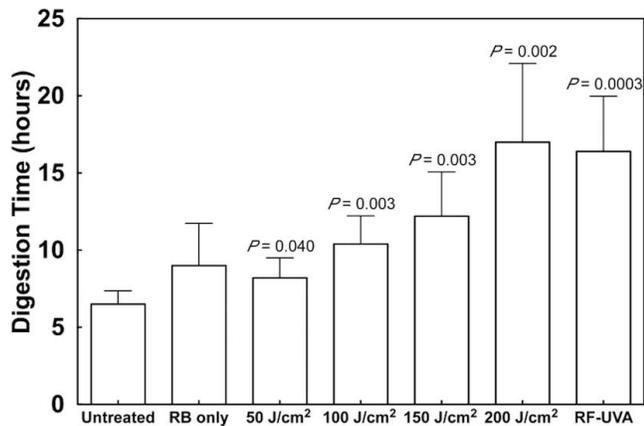


FIGURE 2. Digestion time using observation method. *P* value represents comparison to untreated group as determined by Kruskal-Wallis 1-way ANOVA test. Groups included in comparison: untreated corneas, corneas pretreated with RB only, corneas stained with RB and then exposed to green light for times to deliver doses of 50, 100, 150, or 200 J/cm², and corneas pretreated with RF-UVA. *n* = 5 per group.

8.3 ± 1.2 hours for RB50, 11.1 ± 1.4 hours for RB100, 12.4 ± 1.7 hours for RB150, and 15.7 ± 1.8 hours for RB200. Statistically significant difference exists between different RGX groups: RB200 shows higher degradation time compared with other groups (*P* < 0.05 for RB50, RB100, and RB150 using Kruskal-Wallis 1-way ANOVA test).

Rose Bengal corneas were dissolved at 9.3 ± 1.7 hours compared with 6.1 ± 1.3 hours for the untreated corneas; RB-alone treatment increased the collagenase resistance compared with the untreated group and a similar degradation time compared with RB50 (*P* = 0.03 and *P* = 0.402, respectively, using Kruskal-Wallis 1-way ANOVA test). Compared with RB alone, RB100, RB150, and RB200 had significantly greater resistance (*P* = 0.007, *P* = 0.004, and *P* = 0.003, respectively, using Kruskal-Wallis 1-way ANOVA test).

Riboflavin-UVA Group. Corneal buttons crosslinked with riboflavin and UVA light (RF-UVA) were dissolved at 15.7 ± 1.7

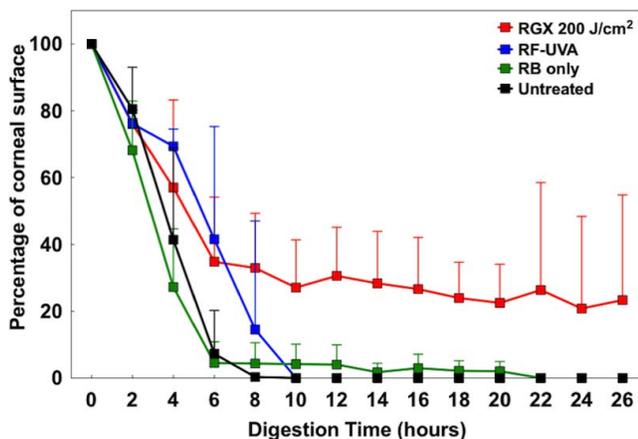


FIGURE 3. Percent change in corneal surface area as measured by photographs taken over 26 hours following the emersion of corneal tissue groups in collagenase. The following treatment groups are represented: untreated corneas, corneas pretreated with RB only, corneas stained with RB and exposure to green light for a treatment dose of 200 J/cm², and corneas pretreated with RF-UVA. *n* = 5 per group.

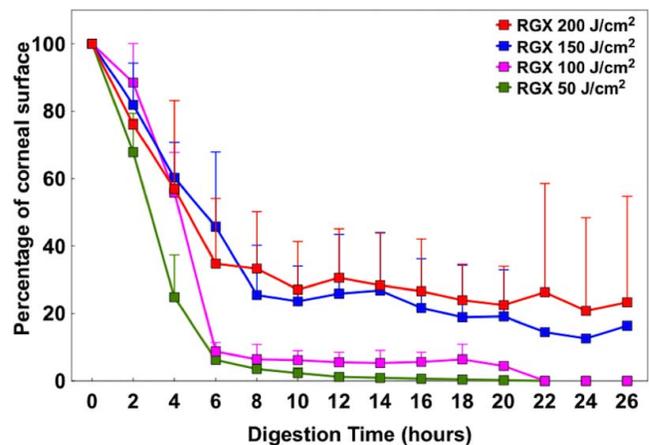


FIGURE 4. Percent change in corneal surface area as measured by photographs taken over 26 hours following the emersion of corneal tissue groups in collagenase. The following treatment groups are represented: corneas stained with RB and then exposed to green light for the following doses: 50, 100, 150, or 200 J/cm². *n* = 5 per group.

hours (G5); RF-UVA exhibited higher degradation time compared with untreated corneas (*P* = 0.0003) and similar degradation time compared with RB200 (*P* = 0.501).

Photography Method (Figs. 3, 4)

Figures 3 and 4 show percent change in corneal surface area as measured by photographs taken over 26 hours following the immersion of corneal tissue groups in collagenase. There is an initial decrease in area between the first 10 hours and then a nearly constant remaining area at longer times corresponding to undigested stroma described as “string pattern” in the observation method. At 6 hours, controls (control and RB) and low-dose green light RGX groups (RB50 and RB100) have nearly disappeared, whereas the high-dose green light RGX groups (RB150 and RB200) and RF-UVA have remaining material (*P* < 0.01). This difference is maintained for the rest of the period tested. Corneas appeared to be totally digested by 10 hours. However, small amounts of cornea in the RF-UVA group at longer incubation times were not readily detected by this method because they were not stained in contrast to the pink color of the RB-stained cornea.

DISCUSSION

In this study, we found an increase in the collagenase A digestion time in corneas crosslinked with RB and green light. This finding reflects the biochemical effect of the crosslinking treatment in addition to the already known biomechanical effect.²

Resistance to collagenase digestion is an essential aspect in the efficacy of the crosslinking treatment in keratoconus because increased collagenolytic activity is one of the most important mechanisms leading to corneal ectasia.^{8,9} Tissue collagenases have also been especially implicated in infectious and noninfectious corneal ulcers such as chemical burns and interstitial corneal inflammation.¹⁰⁻¹³ In the present study, we used bacterial collagenase (collagenase A) as part of a standard testing of the enzymatic resistance of biomaterials.¹⁴ The bacterial collagenase is more potent and efficient than mammalian collagenase because it attacks multiple sites along the collagen helix and has a broader specificity spectrum digesting all types of collagen.¹⁵⁻¹⁷

The stabilizing biochemical effect of RB by crosslinking can be explained by the changes of the tertiary structure of the collagen fibrils induced by crosslinking preventing access of the proteolytic enzymes to their specific cleavage sites.¹⁸ Rose Bengal may produce sufficient bridges between collagen molecules to alter the molecular organization of the stroma to increase its stiffness. The green light will generate singlet oxygen or other reactive species, which react with certain amino acids to initiate covalent bonds between protein molecules.^{19–21} In our study, RGX increased corneal resistance to keratolysis with collagenase A, and this result was comparable to the effect of RF-UVA. This was shown in the observation method (Fig. 2), although not in the photography method (Figs. 3, 4). This difference is likely due the inability to the photographic method to detect the uncolored RF-UVA-treated tissue; RGX results in a pink stained tissue that is more readily visualized by photography.

It has been shown that RB with no light increases corneal stiffness compared with the control group.² In our study, RB with no light seems to increase significantly corneal resistance to keratolysis. When green light is added with a dose of 50 J/cm², resistance to keratolysis seems to be comparable to RB with no light. Higher light doses yield higher keratolysis time, and the difference was significant between the RB150 and RB50. At 200 J/cm², time to keratolysis was comparable to RF-UVA (Fig. 3). This biochemical effect of RF-UVA has already been shown.^{5–7,22} The mechanisms for protein–protein crosslinking by both RF-UVA and RGX are oxygen dependent.^{23–25}

Application of RGX in corneal ectasia could be a new method in the treatment of corneal ectatic disorders. Because RB does not penetrate as deep through the stroma, photosensitized damage to the endothelial layer is less of a concern, and it may be used to stiffen corneas thinner than 400 μm.² RGX delivers an irradiance of 3.47 mW/cm² on the retina when a light delivery device frequency-doubled is used with a diverging beam with a 12-mm image on the retina.² This irradiance is more than a factor of 20 below the thresholds for 532-nm light set by the American National Standard Institute of 75.5 and 159 mW/cm² for thermal and photochemical damage.^{26,27} RGX has been shown not to cause more than 1-month loss of keratocytes in the superficial stroma and to be safe on retinal vasculature and RPE under treatment conditions that stiffened the cornea.³

There are several limitations to our study. The thickness of the button could not be used as a reliable parameter because of edema in the untreated corneas compared with the crosslinked buttons. The observation method is a subjective tool to evaluate tissue degradation, and the image processing software cannot be used to measure for tissue thickness variation. During keratolysis, corneal tissue tends to bend on itself, making area measurements less reliable. In addition, we did not test for stromal depth variation of crosslinking. Another limitation is the lack of an observer masking technique during degradation time. Rose Bengal and green light results in a pink-stained tissue, rendering masking impossible. Another limitation is the soaking time for 15 minutes, which is shorter than some of the well-established methods. Different pretreatment times have been described in literature, and it varies from 5 minutes in accelerated protocols to 15–30 minutes in epithelial OFF classical technique.²⁸ The final limitation is the use of rabbit corneas. Porcine corneas and human corneas were used in previous studies, which makes the comparison with our RF-UVA group more difficult.^{5,22}

In summary, photochemical crosslinking of the cornea using Rose Bengal and green light results in a markedly increased resistance versus a collagenase-digesting enzyme. Rose Bengal and green light and RF-UVA have comparable

keratolysis time, and both techniques yield a higher corneal resistance compared to control group. The findings support the potential use of RGX in the setting of corneal ectatic disorders. Future studies may help to elucidate whether collagenase resistance is due to collagen crosslink or other factors such as RB binding to the collagen molecule.

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