UV Crosslinking of Donor Corneas Confers Resistance to Keratolysis

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Abstract

Purpose—To develop a modified *ex vivo* corneal crosslinking method that increases stromal resistance to enzymatic degradation for use as a carrier for the Boston keratoprosthesis.

Methods—*Ex vivo* crosslinking of human corneas was performed using Barron® artificial anterior chambers. The corneas were de-epithelialized, pre-treated with riboflavin solution (0.1% riboflavin/20% dextran) and irradiated with ultraviolet A (UVA) light (λ=370nm, irradiance=3mW/cm^2) for various durations. The combined effect of UVA and gamma (γ) irradiation was also assessed using the commercially available γ-irradiated corneal donors. The corneas were then trephined and incubated at 37 degrees Celsius with 0.3% collagenase A solution. The time to dissolution of each cornea was compared across treatments.

Results—De-epithelialized corneas (no UV light, no riboflavin) dissolved in 5.8 ± 0.6 hours. Crosslinked corneas demonstrated increased resistance to dissolution, with a time to dissolution of $17.8 +/- 2.6$ hours ($p < 0.0001$). The corneal tissues' resistance to collagenase increased with longer UVA exposure, reaching a plateau at 30 minutes. Crosslinking both the anterior and posterior corneas did not provide added resistance when compared to crosslinking the anterior corneas only ($p>0.05$). γ-irradiated corneas dissolved as readily as de-epithelialized controls regardless of whether they were further crosslinked (5.6 ± 1.2 hours) or not (6.1 ± 0.6 hours) ($p=0.43$)

Conclusions—Collagen crosslinking of the de-epithelialized anterior cornea surface for 30 minutes conferred optimal resistance to *in vitro* keratolysis by collagenase A.

Keywords

Corneal collagen crosslinking; gamma (γ) irradiation; collagenase; keratolysis

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INTRODUCTION

Sterile corneal melting or keratolysis, is a serious and potentially vision-threatening condition that involves the destruction of the stromal extracellular matrix (ECM). Melting may develop following a variety of insults, ranging from autoimmune diseases (rheumatoid arthritis, Stevens-Johnson syndrome, peripheral ulcerative keratitis), chemical burns, atopic disease, infectious keratitis, neurotrophic states, chronic exposure, disruption of local nutrition by intrastromal corneal ring segments, corneal inlays or keratoprosthesis as well as many other iatrogenic sources (mitomcyin C, topical non-steroidal anti-inflammatory eye drops).[1–8] The prevention of corneal melting is of particular interest to our group as it was, historically, a common complication that limited the use of early keratoprostheses[3] and remains one of the most common causes of keratoprosthesis losses.[9]

Keratoprostheses are used in patients at high risk of failure following conventional corneal transplantation. The Boston Keratoprosthesis (B-KPro) is the most commonly used keratoprosthesis in North America and elsewhere in the world. It is composed of synthetic materials ([PMMA] and titanium) and requires a donor cornea to function as a carrier for the device.[10] Modifications to the B-KPro design and postoperative management have improved the retention rate, but keratolysis remains the most common cause of B-KPro failure. In the most recent report from the Boston Keratoprosthesis Type 1 Study Group, 43% of the B-KPro loss could be attributed to sterile keratolysis.[9] Patients with underlying autoimmune conditions such as Stevens-Johnson syndrome (SJS) and ocular mucous membrane pemphigoid have a significantly higher risk of keratolysis following B-KPro.[11–13] The prevention of corneal melting after B-KPro implantation has always been a priority as corneal melting may lead to permanent loss of vision or loss of the globe, thus precluding future visual rehabilitation.

Because of the limited availability of corneal donor grafts in many parts of the world, other tissue options have been explored as carriers for the B-KPro. These include ipsilateral corneal autografts, frozen corneal grafts as well as gamma (γ)-irradiated corneal grafts.[14–16] γ-irradiation sterilizes the graft and allows for tissue storage at room temperature for prolonged periods.[17] These characteristics have the potential of improving the availability of corneal donors. Further, γ-irradiation results in the devitalization of corneal cells and consequently, in reduced allogenicity of the transplanted tissue.[18] In addition to of these interesting characteristics, γ-irradiated corneal tissue has been suggested to be equivalent to fresh tissue when used as a carrier to the B-KPro.[16, 19]

Corneal collagen crosslinking with riboflavin and ultraviolet A (UVA) light has been shown to increase biomechanical strength,[20, 21] decrease tissue permeability[22] and increase resistance of the crosslinked cornea to enzymatic degradation.[23] The procedure is primarily used to slow or arrest the progression of corneal ectasias and is currently undergoing clinical trials in the United States.[24] Corneal crosslinking has also been used to treat recalcitrant, ulcerative corneal infections.[6, 25] The corneal collagen crosslinking technique combines the use of riboflavin (B2) and UVA to induce free oxygen radical formation and chemical covalent bonding between the amino groups of collagen fibrils within the stroma.[26] Controlled *in vitro* and *ex vivo* studies have demonstrated riboflavin/UVA-induced

crosslinking between proteoglycan (PG) core proteins (keratocan, lumican, mimecan and decorin) as well as linkages between collagen and PGs.[27]

In this report, we explore various crosslinking protocols including γ-irradiation alone or followed by crosslinking and compare their effectiveness as pertains to the tissue's resistance to enzymatic degradation. Ultimately, the use of collagenase-resistant carrier grafts may improve the retention of the B-KPro device.[28] Given that carrier corneas can be crosslinked *ex vivo* prior to B-KPro surgery, the crosslinking treatment options are not limited to those available for *in vivo* crosslinking of corneas ectasias. For instance, as healthy endothelial cells are not needed to maintain a clear visual axis following B-KPro,[15] it becomes permissible to crosslink both the anterior and posterior surfaces of carrier grafts. Moreover, the impact of concurrent gamma-irradiation and crosslinking was examined.

MATERIAL AND METHODS

Reagent preparation

Riboflavin (0.1% or 1 mg/mL) solution was prepared by thoroughly mixing 50 mg of riboflavin 5′-phosphate sodium salt hydrate (Sigma Aldrich, St. Louis, Missouri) and 50 mL of 20% dextran (w/w, Sigma Aldrich, St. Louis, MO). Collagenase A (matrix metalloproteinase 1a or EC 3.4.24.3, 0.3% (3 mg/mL), Sigma Aldrich, St. Louis, MO) was prepared fresh before every experiment using phosphate-buffered saline (PBS). Both solutions were covered in aluminum foil to protect from the light and stored at 4°C until use.

Tissue preparation

Human research corneas were obtained from Tissue Banks International (Baltimore, MD) and North Carolina Eye Bank (Winston-Salem, NC). The corneal tissues were provided in OptiSol® solution and stored at 4°C until use. All experiments were performed within 2 weeks of the donor's death. Each cornea was fit into a Barron® artificial anterior chamber (Katena Eye Instruments, Denville, NJ) and maintained with balanced salt solution. In addition, corneas were γ-irradiated in accordance with ISO standards for the terminal sterilization of allografts and obtained from Tissue Banks International (TBI VisionGraft, Baltimore, MD).[29]

Corneal crosslinking

All corneas were de-epithelialized and pre-treated with 0.1% riboflavin solution every 2 minutes for 15 minutes as previously described.[30] The corneas were irradiated with UVA light using the VEGA LED-based UV emitter (Costruzione Strumenti Oftalmici, Firenze, Italy) at a wavelength of 370 nm, irradiance of 3 mW/cm^2 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. Consistent with the clinical technique used to treat corneal ectasia *in vivo*, the anterior surface of the corneas were crosslinked for either 7 ½, 15, 30, 60 or 90 minutes. In addition, other treatment groups included the crosslinking of both the anterior and posterior cornea surfaces for either 15 or 30 minutes (see table 1). Treatment groups are described using the nomenclature A_xP_y, where *x* is the duration of crosslinking of the anterior (A) surface of the cornea and *y* is the

duration of crosslinking of the posterior (P) surface of the cornea in minutes. When γ-irradiated corneas were used, crosslinking was applied to the anterior surface of the cornea for 30 minutes. The following groups served as negative controls: 1) untreated corneas, 2) de-epithelialized corneas with application of 0.1% riboflavin solution but no UVA exposure (riboflavin only) and 3) de-epithelialized corneas with UVA exposure without prior instillation of riboflavin (UVA only).

Enzymatic degradation

Corneas in each treatment group (N = 5) were trephined into 8.5-mm buttons and incubated with 0.3% collagenase A solution at 37°C on a plate shaker set at 150 rotations-per-minute. The corneas were observed hourly for the first 12 hours, and then every 30 minutes until complete dissolution was achieved. The time to dissolution of the corneal button was recorded and all groups were compared to untreated corneas.

Data analysis

Statistical analysis was performed using Graphpad Instat 3.10. The results were reported as mean ± standard deviation. Normality was tested using the Kolmogorov-Smirnov test, and non-parametric tests were used when indicated. One-way analysis of variance (ANOVA) and Kruskal-Wallis test were used to compare times to complete dissolution between groups. Mann-Whitney U-statistics was used for comparing non-parametric non-matched groups. The tests were performed using a two-tailed p-value of 0.05.

RESULTS

Treatment

Crosslinked corneas were more resistant to enzymatic degradation when compared to untreated corneas (Figure 1). The resistance of corneas that underwent crosslinking of the anterior surface only reached a plateau after 30 minutes of treatment (p<0.05). Corneas that underwent 15 minutes of crosslinking of the anterior and posterior surfaces ($A_{15}P_{15}$) were no different than the corneas that underwent 30 minutes of the anterior surface (A_{30}). There was also no significant difference between treatment of both anterior and posterior corneal surfaces for 15 minutes ($A_{15}P_{15}$, 15 ± 1.5 hours) or 30 minutes ($A_{30}P_{30}$, 17.9 ± 4.9 hours, p=0.24). There was no difference between untreated, riboflavin only, and UVA only control groups. Untreated corneas dissolved after 5.8 ± 0.6 while corneas crosslinked for 30 minutes (A_{30}) dissolved after 17.8 ± 2.6 hours (p < 0.0001).

γ-irradiated corneas (without crosslinking) showed similar resistances to degradation as untreated corneas (non-γ-irradiated, not crosslinked) with times to dissolution of 6.1 ± 0.6 hours and 5.8 ± 0.6 hours, respectively (p=0.91) (Figure 2). Interestingly, the time to dissolution of γ-irradiated corneas that were crosslinked for 30 minutes (5.6 ± 1.2 hours) was comparable to both untreated corneas and γ-irradiated corneas that were not crosslinked (p=0.75). In addition, crosslinked γ-irradiated corneas (γ+A_{30}) were less resistant to degradation than non-γ-irradiated corneas that were crosslinked for the same duration (5.6 ± 1.2 hours vs. 17.8 ± 2.6 hours; p<0.0001).

DISCUSSION

In this study, several *ex vivo* collagen crosslinking protocols were investigated in an effort to maximize the corneal resistance to enzymatic degradation. The clinical relevance of *ex vivo* collagen crosslinking lies in the potential use of crosslinked corneal donors as carriers for B-KPro or as tectonic grafts in ocular surface diseases with predisposition to corneal melting. Our results demonstrated that collagen crosslinking of the anterior corneal surface exhibited a dose-response increase in the tissue's resistance to enzymatic degradation. Indeed, a strong linear relationship ($R^2 = 0.98$) existed between the duration of crosslinking and the time to complete dissolution by collagenase. However, this dose-response curve was achieved only for the first 30 minutes of UVA exposure. Longer UVA exposures did not yield additional improvement in the tissues' resistance to collagenase.

The effects of crosslinking the anterior and posterior corneal surfaces have not been previously reported. While it is not feasible to treat both corneal surfaces during *in vivo* crosslinking of ectatic corneas, such a treatment is possible in donor tissue destined to act as a carrier for B-KPro or as a corneal patch graft where a viable endothelium is not required. However, crosslinking of the posterior corneal surface did not result in any additional resistance compared to crosslinking of the anterior cornea only.

To our knowledge, this is the first study to compare the collagenase resistance of corneal tissue following γ-irradiation alone, collagen crosslinking alone and the combination of both γ-irradiation and crosslinking. Interestingly, γ-irradiated corneas were no more resistant to enzymatic degradation than untreated corneas. Furthermore, γ-irradiation seemed to offset the effect of crosslinking. Compared to non-γ-irradiated crosslinked corneas, γ-irradiated crosslinked corneas demonstrated significantly less resistance to enzymatic degradation. This is consistent with the existing literature, where γ-irradiation of tendon and bone collagen has been shown to cause a dose-dependent fragmentation of collagen fibers as well as crosslinking between peptides.[31, 32] Protein chain degradation and depolymerization appear to be the predominant effects, leading to faster enzymatic degradation as well as decreased mechanical properties.[33, 34] For example, the acellular tissue matrix AlloDerm® (LifeCell Corporation, Bridgewater, NJ) was digested at a faster rate by type I collagenase, pepsin, trypsin and proteinase K following γ-irradiation.[35] However, to our knowledge, there are no such studies evaluating the effect of γ-irradiation on corneal collagen. Similar to other collagen-based tissues, corneas are also likely to undergo collagen fiber breakdown following γ-irradiation. Therefore, it is hypothesized that the γ-irradiation-induced weakening of corneal collagen was sufficient to annul any further crosslinking effect using riboflavin and UVA.

The increased resistance of crosslinked corneas to enzymatic degradation found in this study supports previous research done by Spoerl *et al.*[23] Our study involved several modifications. First, the submerged tissue was incubated at a higher temperature (37°C), to optimize the activity of the collagenase enzymes. As well, a higher concentration of collagenase A (0.3%) was employed here to hasten the time to complete degradation. As such, time to dissolution was measured over the course of hours, rather than days.

Corneal melting occurs due to dysregulated and excessive matrix metalloproteinase (MMP) production by epithelial cells and/or inflammatory cells.[36] MMPs are zinc-dependent endopeptidases that are capable of degrading virtually all components of the extracellular matrix and basement membrane.[37, 38] Increased MMP activity has been shown in the tears of patients with ocular infections, corneal ectasias, and ocular surface diseases at risk of developing sterile corneal ulcers.[39–42] Collagenase A, a member of the MMPs family, is recognized for its potency in degrading all forms of collagen and extracellular matrix and was thus selected for use in this study.

A limitation in our study was that all crosslinking experiments were performed using a UVA emitter designed to emit a single irradiation energy (3 mW/cm^2). According to the Bunson-Roscoe law of reciprocity, a biological effect is directly proportional to the total energy dose and this dose is a product of the intensity and the duration of exposure.[43] While higher irradiation energies can theoretically accelerate the crosslinking and produce enzymatically resistant corneas at a fraction of the time,[44] this was not possible with our current instrumentation. Because good clinical outcomes have been described using higher fluence, accelerated CXL (for example, 7 mw/cm^2 for 15 minutes),[28, 44] future *ex vivo* CXL studies should include the assessment of higher UVA fluence and the effect of debriding versus leaving the epithelium intact. Indeed, these parameters have the potential benefits of decreasing the duration of UVA exposure and CXL treatment, thus enhancing the feasibility of preoperative *ex vivo* CXL. Finally, controlled *ex vivo* experiments likely do not reproduce all of the various factors, enzymes and mechanisms involved in keratolysis *in vivo*. In this study, the crosslinked corneal buttons were submerged in collagenase A, allowing the tissue to be degraded from the anterior and posterior surfaces. However, *in vivo* corneal melting appears to occur only from the anterior cornea.[31] Nevertheless, crosslinking of donor carrier corneas for B-KPro appears promising, and clinical studies are indicated in patients with high risk of keratolysis, who are also in need of a KPro.

Acknowledgments

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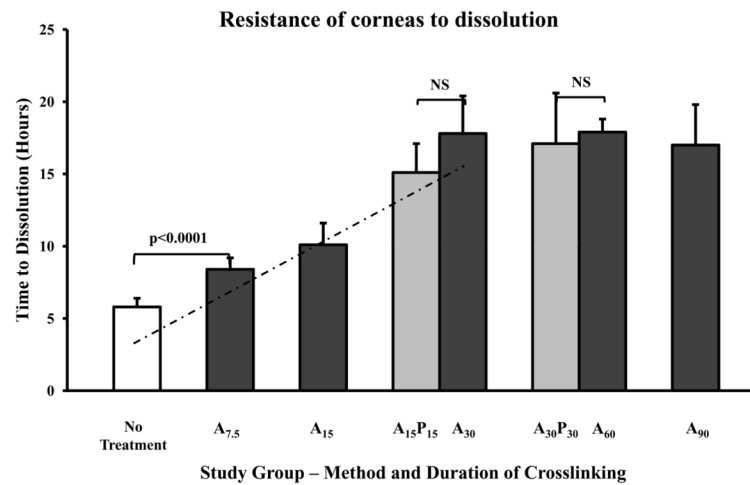
Figure 1.
UV crosslinked corneas are relatively resistant to degradation in 0.3% collagenase A. Through 30 minutes of treatment, a linear correlation was demonstrated between the time to dissolution and the duration of crosslinking (dashed line, $R^2 = 0.98$, p<0.05). No increase in resistance was observed when both the anterior and posterior corneas were crosslinked. NS = not significant.
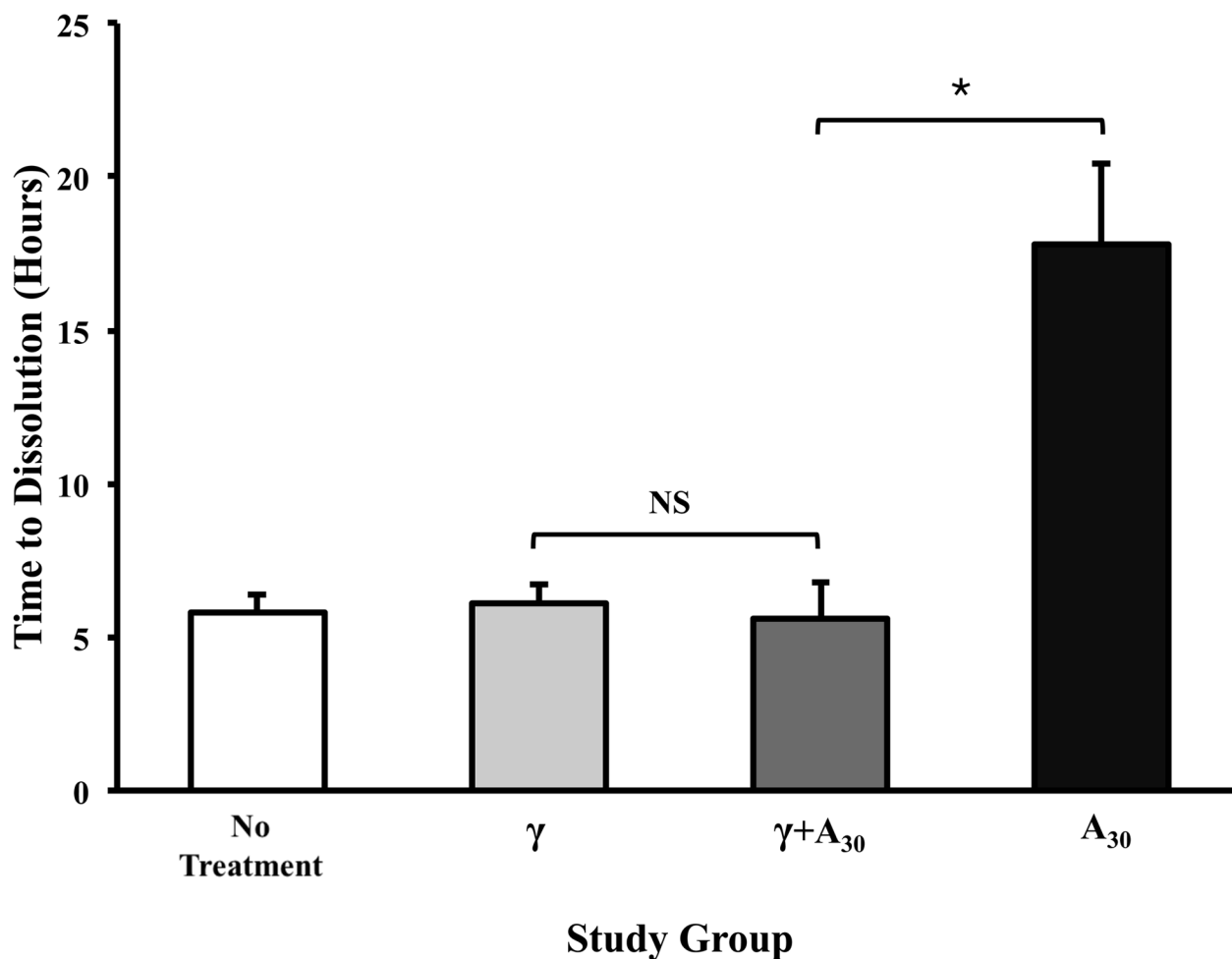
Resistance of corneas to dissolution

Figure 2.
γ-irradiated corneas with and without subsequent crosslinking were no more resistant to degradation in 0.3% collagenase A than untreated corneas. Crosslinked γ-irradiated corneas where less resistant than non γ-irradiated corneas that were crosslinked for the same duration (30 minutes). Non γ-irradiated, crosslinked corneas were more resistant to degradation than untreated and γ-irradiated corneas. $*p<0.05$, NS = not significant.

Table 1

Study Groups

Study Group	Duration of crosslinking of anterior corneal surface (minutes)	Duration of crosslinking of posterior corneal surface (minutes)
$A_{7.5}$	7.5	-
A_{15}	15	-
A_{30}	30	-
A_{60}	60	-
A_{90}	90	-
$A_{15}P_{15}$	15	15
$A_{30}P_{30}$	30	30
γ	0	0
$\gamma + A_{30}$	30	0

γ = gamma irradiation, A = Anterior corneal surface, P = Posterior corneal surface