UV Cross-linking of Donor Corneas Confers Resistance to Keratolysis

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Purpose: The aim of this study was to develop a modified ex vivo corneal cross-linking method that increases stromal resistance to enzymatic degradation for use as a carrier for the Boston keratoprosthesis.

Methods: Ex vivo cross-linking of human corneas was performed using Barron artificial anterior chambers. The corneas were deepithelialized, pretreated with riboflavin solution (0.1% riboflavin/20% dextran), and irradiated with ultraviolet A (UV-A) light (λ = 370 nm, irradiance = 3 mW/cm^2) for various durations. The combined effect of UV-A and gamma (γ) irradiation was also assessed using the commercially available γ-irradiated corneal donors. The corneas were then trephined and incubated at 37°C with 0.3% collagenase A solution. The time to dissolution of each cornea was compared across treatments.

Results: Deepithelialized corneas (no UV light, no riboflavin) dissolved in 5.8 $\pm$ 0.6 hours. Cross-linked corneas demonstrated increased resistance to dissolution, with a time to dissolution of 17.8 $\pm$ 2.6 hours ($P < 0.0001$). The corneal tissues' resistance to collagenase increased with longer UV-A exposure, reaching a plateau at 30 minutes. Cross-linking both the anterior and posterior corneas did not provide added resistance when compared with cross-linking the anterior corneas only ($P > 0.05$). γ-irradiated corneas dissolved as readily as deepithelialized controls regardless of whether they were further cross-linked (5.6 $\pm$ 1.2 hours) or not (6.1 $\pm$ 0.6 hours) ($P = 0.43$).

Conclusions: Collagen cross-linking of the deepithelialized anterior corneal surface for 30 minutes conferred optimal resistance to in vitro keratolysis by collagenase A.

Key Words: corneal collagen cross-linking, gamma (γ) irradiation, collagenase, keratolysis

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Sterile corneal melting or keratolysis is a serious and potentially vision-threatening condition that involves the destruction of the stromal extracellular matrix. Melting may develop after a variety of insults, ranging from autoimmune diseases (eg, 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 and many other iatrogenic sources (eg, mitomycin C, topical nonsteroidal antiinflammatory eye drops).[1–8] The prevention of corneal melting is of particular interest to our group because 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 after 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 (polymethyl methacrylate; 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 and ocular mucous membrane pemphigoid have a significantly higher risk of keratolysis after B-KPro.[11–13] The prevention of corneal melting after B-KPro implantation has always been a priority because 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, and 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. Furthermore, γ-irradiation results in the devitalization of corneal cells and, consequently, in reduced allogenicity of the transplanted tissue.[18] In addition to these interesting characteristics, γ-irradiated corneal tissue has been suggested to be equivalent to fresh tissue when used as a carrier for the B-KPro.[16,19]

Corneal collagen cross-linking with riboflavin and ultraviolet A (UV-A) light has been shown to increase biomechanical strength,[20,21] decrease tissue permeability,[22] and increase resistance of the cross-linked 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 cross-linking has also been used to treat recalcitrant ulcerative corneal infections.[6,25] The corneal collagen cross-linking technique combines the use of riboflavin (vitamin B2) and UV-A 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/UV-A–induced cross-linking between proteoglycan core proteins (i.e., keratocan, lumican, mimecan, and decorin) as well as linkages between collagen and proteoglycans.[27]

In this study, we explore various cross-linking protocols including γ-irradiation alone or followed by cross-linking 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 cross-linked ex vivo before B-KPro surgery, the cross-linking treatment options are not limited to those available for in vivo cross-linking of corneas ectasias. For instance, because healthy endothelial cells are not needed to maintain a clear visual axis after B-KPro,[15] it becomes permissible to cross-link both the anterior and posterior surfaces of carrier grafts. Moreover, the impact of concurrent γ-irradiation and cross-linking was examined.

MATERIALS 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, MO) and 50 mL of 20% dextran (wt/wt, 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 freshly prepared before every experiment using phosphate-buffered saline. Both solutions were covered using aluminum foil to protect from the light and were 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 Cross-linking

All corneas were deepithelialized and pretreated with 0.1% riboflavin solution every 2 minutes for 15 minutes as previously described.[30] The corneas were irradiated with UV-A 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 cross-linked for either 7½, 15, 30, 60, or 90 minutes. In addition, other treatment groups included the cross-linking of both the anterior and posterior corneal surfaces for either 15 or 30 minutes (Table 1). Treatment groups are described using the nomenclature A_xP_y, where x is the duration of cross-linking of the anterior (A) surface of the cornea and y is the duration of cross-linking of the posterior (P) surface of the cornea in minutes. When γ-irradiated corneas were used, cross-linking was applied to the anterior surface of the cornea for 30 minutes. The following groups served as negative controls: (1) untreated corneas, (2) deepithelialized corneas with application of 0.1% riboflavin solution but no UV-A exposure (riboflavin only), and (3) deepithelialized corneas with UV-A exposure without prior instillation of riboflavin (UV-A 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 with untreated corneas.

Data Analysis

Statistical analysis was performed using GraphPad Instat 3.10. The results were reported as mean ± SD. Normality was tested using the Kolmogorov–Smirnov test, and nonparametric tests were used when indicated. One-way analysis of variance

TABLE 1. Study Groups

Study Group	Duration of Cross-linking of Anterior Corneal Surface, min	Duration of Cross-linking of Posterior Corneal Surface, min
$A_{7.5}$	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
γ + A_{30}	30	0

A, anterior corneal surface; P, posterior corneal surface; γ, gamma irradiation.

and Kruskal–Wallis test were used to compare times to complete dissolution between groups. Mann–Whitney *U*-statistics was used for comparing nonparametric nonmatched groups. The tests were performed using a 2-tailed *P* value of 0.05.

RESULTS

Treatment

Cross-linked corneas were more resistant to enzymatic degradation when compared with untreated corneas (Fig. 1). The resistance of corneas that underwent cross-linking of the anterior surface only reached a plateau after 30 minutes of treatment ($P < 0.05$). Corneas that underwent 15 minutes of cross-linking 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 UV-A only control groups. Untreated corneas dissolved after 5.8 ± 0.6 hours, whereas corneas cross-linked for 30 minutes (A_{30}) dissolved after 17.8 ± 2.6 hours ($P < 0.0001$).

γ-irradiated corneas (without cross-linking) showed similar resistances to degradation as untreated corneas (non–γ-irradiated, non cross-linked) with times to dissolution of 6.1 ± 0.6 hours and 5.8 ± 0.6 hours, respectively ($P = 0.91$) (Fig. 2). Interestingly, the time to dissolution of γ-irradiated corneas that were cross-linked for 30 minutes (5.6 ± 1.2 hours) was comparable with both untreated corneas and γ-irradiated corneas that were not cross-linked ($P = 0.75$). In addition, cross-linked γ-irradiated corneas ($\gamma + A_{30}$) were less resistant to degradation than non–γ-irradiated corneas that were cross-linked 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 cross-linking protocols were investigated in an effort to maximize the corneal resistance to enzymatic degradation. Clinical relevance of ex vivo collagen cross-linking lies in the potential use of cross-linked 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 cross-linking 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 cross-linking and the time to complete dissolution by collagenase. However, this dose–response curve was achieved only for the first 30 minutes of UV-A exposure. Longer UV-A exposures did not yield additional improvement in the tissues' resistance to collagenase.

The effects of cross-linking the anterior and posterior corneal surfaces have not been previously reported. Although it is not feasible to treat both corneal surfaces during in vivo cross-linking 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, cross-linking of the posterior corneal surface did not result in any additional resistance compared with cross-linking of the anterior cornea only.

To the best of our knowledge, this is the first study to compare the collagenase resistance of corneal tissue after γ-irradiation alone, collagen cross-linking alone, and the combination of both γ-irradiation and cross-linking. Interestingly, γ-irradiated corneas were no more resistant to enzymatic degradation than untreated corneas. Furthermore, γ-irradiation seemed to offset the effect of cross-linking. Compared with non–γ-irradiated cross-linked corneas, γ-irradiated cross-linked 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 and cross-linking between peptides.[31,32] Protein chain degradation and depolymerization seem to be the predominant effects, leading to faster enzymatic degradation and 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,

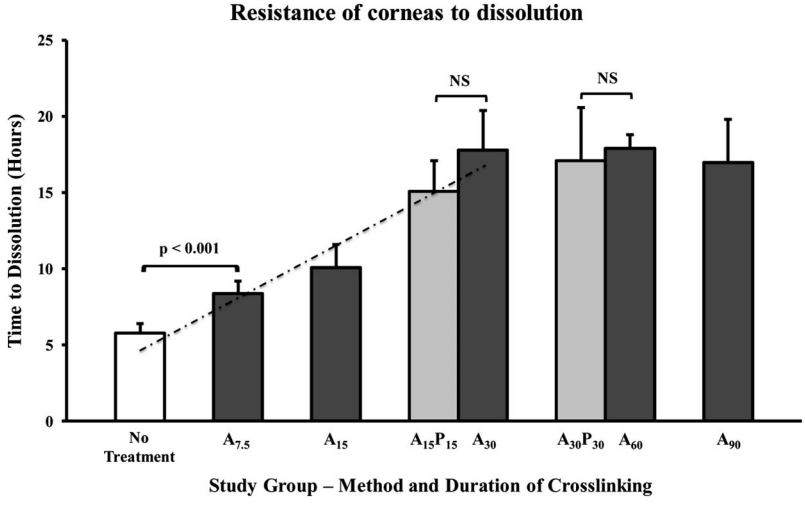

FIGURE 1. UV cross-linked corneas are relatively resistant to degradation in 0.3% collagenase A. Through 30 minutes of treatment, a linear correlation was demonstrated between the time to dissolution and the duration of cross-linking (dashed line, $R^2 = 0.98$, $P < 0.05$). No increase in resistance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

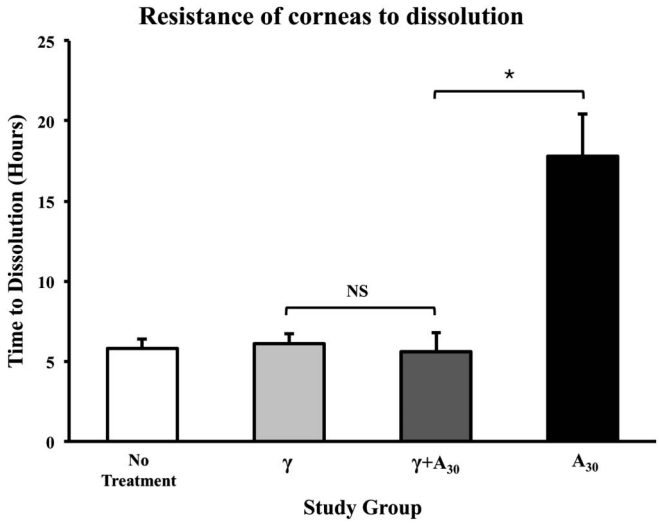

FIGURE 2. γ-irradiated corneas with and without subsequent cross-linking were no more resistant to degradation in 0.3% collagenase A than untreated corneas. Cross-linked γ-irradiated corneas where less resistant than non γ-irradiated corneas that were cross-linked for the same duration (30 minutes). Non–γ-irradiated, cross-linked corneas were more resistant to degradation than untreated and γ-irradiated corneas. $*P < 0.05$; NS, not significant.

pepsin, trypsin, and proteinase K after γ-irradiation.[35] However, to the best of 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 after γ-irradiation. Therefore, it is hypothesized that the γ-irradiation-induced weakening of corneal collagen was sufficient to annul any further cross-linking effect using riboflavin and UV-A.

The increased resistance of cross-linked corneas to enzymatic degradation found in this study supports previous research 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. Also, a higher concentration of collagenase A (0.3%) was used 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 because of 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 cross-linking experiments were performed using a UV-A emitter designed to emit a single irradiation energy (3 mW/cm^2). According to the Bunsen–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] Although higher irradiation energies can theoretically accelerate the cross-linking 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 cross-linking (for example, 7 mw/cm^2 for 15 minutes),[28,44] future ex vivo cross-linking studies should include the assessment of higher UV-A fluence and the effect of debriding versus leaving the epithelium intact. Indeed, these parameters have the potential benefits of decreasing the duration of UV-A exposure and cross-linking treatment, thus enhancing the feasibility of preoperative ex vivo cross-linking. 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 cross-linked corneal buttons were submerged in collagenase A, allowing the tissue to be degraded from the anterior and posterior surfaces. However, in vivo corneal melting seems to occur only from the anterior cornea.[31] Nevertheless, cross-linking of donor carrier corneas for B-KPro seems promising, and clinical studies are indicated in patients with high risk of keratolysis, who are also in need of a KPro.

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