6.1 Introduction

Keratoconus is a disorder that results in corneal thinning and is named for the conical shape that the cornea develops. The progressive distortion of corneal shape usually becomes noticeable in early adulthood, causing increasingly severe astigmatism, myopia, and higher-order aberrations that become difficult to correct by spectacles or contact lenses. A number of different therapies have been tested in keratoconus as alternatives to contact lenses. These include, thermokeratoplasty, epikeratophakia, and intracorneal ring segments (Intacs). These treatments do not appear to stop progression of the disease; rather, they are alternatives to manage the refractive abnormalities induced by irregular...
corneal thinning and ectasia, or expansion. When distortions reach the point that refractive correction is no longer possible, corneal transplant is the only option.

Recently, the pioneering work of Wollensak, Seiler, Spoerl, and co-workers demonstrated a potential treatment to arrest progression of keratoconus using photoactivated crosslinking.\textsuperscript{8-21} Deriving from presumed weakened mechanical integrity of the cornea in keratoconus,\textsuperscript{22} this novel crosslinking therapy is directed at increasing the cornea’s strength. The procedure uses ultraviolet light (370 nm) to activate radical generation of riboflavin in the cornea. The treatment consists of removing the corneal epithelium over a 7 mm diameter and then applying 0.1% riboflavin solution 5 minutes before, and then every 5 minutes thereafter, during a 30 minute UV-A irradiation. Other than post-operative pain from corneal epithelial removal, the procedure is well tolerated. In a series of 22 patients with progressive keratoconus, progression was stopped by treatment with UV-A and riboflavin.\textsuperscript{16} Mean follow up was nearly 2 years. In 16 patients there was actual regression of the keratoconus with dioptric corneal flattening of 2 diopters. After the epithelium regenerated, no adverse effects, e.g., on corneal transparency, were observed.

Despite these encouraging results, there are several potential drawbacks to the current method. First, removal of the epithelium is not desirable because the tight junctions of epithelial cells provide the first protective barrier for the cornea and prevent many molecules from penetrating this cell layer. To avoid difficulties of delivering drug through the lipophilic epithelium and into the hydrophilic stroma, the epithelium is typically removed. Although it re-grows, the epithelium is home to nearly 325,000 nerve endings\textsuperscript{23} and its removal risks impaired sensation, leading to complications due to reduced blinking.
reflex (failure to maintain proper corneal hydration and to sense foreign particles on the eye). For this reason, the present study examines the ability of our chosen light-activated drug, Eosin Y, to penetrate into the cornea with and without the epithelium present. Second, UV light is potentially toxic, and when combined with riboflavin activation, has produced toxic effects on keratocytes, the cells responsible for ongoing repair and remodeling of the corneal stroma. We study the ability to induce corneal crosslinking with a visible-light-activated system. Finally, the UV treatment lasts 35 minutes, a relatively long time compared to refractive procedures that last only about two minutes. Our demonstration of crosslinking uses a shorter treatment time totaling 10 minutes (only 5 minutes of irradiation). We compare treatment efficacy of the brief, visible-light protocol to the > 30 minute, UV-activated method using our intact globe expansion test.

6.2 Materials and Methods

6.2.1 Penetration of Molecules—Epithelial Barrier

We have modified a slit lamp with filters and a digital camera to observe the depth profile of the fluorescence of drug molecules in enucleated porcine corneas (Figure 6.1). A sheet of light, sent through a bandpass filter chosen to select light that efficiently excites Eosin Y (Filter 1 passes 500±20 nm), is incident on the cornea and both emitted and scattered light are viewed from an angle. Scattered light, imaged without a filter between the specimen and the camera, is used to record the shape and position of the cornea. The fluorescence of Eosin Y (EY), imaged through Filter 2 (which passes 560±25 nm) as shown in Figure 6.1a,
facilitates visualization of its penetration profile in the stroma (Figure 6.2b). Untreated corneas show no fluorescence (field appears dark with Filter 2 in place). In this manner, we are able to examine the amount of drug that crosses the epithelium and its distribution in the stroma.

Enucleated eyes from 3–4 month old swine were obtained from Sierra for Medical Science. Fresh eyes were shipped in saline, on ice. On arrival, the eyes were immediately (< 42 hours post-mortem) cleaned by removing the tissue still attached to the eye. Then the cornea was photographed in scattering mode, providing initial dimensions of the cornea,
and fluorescence mode, providing baseline measures of background fluorescence. The epithelium was either removed with a scalpel or left intact before the eye was placed in 2 ml of treatment solution (Table 1). Eyes were left in solution for 1 hour and then removed, rinsed with approximately 4 ml of Dulbecco’s PBS and photographed in both scattering and fluorescence mode on the slit lamp. After this treatment, eyes with intact epithelium were found to have very strong absorption and fluorescence associated with their epithelium. To image fluorescence in their stroma, the epithelium was removed by scraping with a scalpel and they were photographed again. Removal of the epithelium at this step allows excitation light to reach the stroma.

<table>
<thead>
<tr>
<th>Treatment Solutions</th>
<th>EosinY (mM)</th>
<th>Triethanolamine (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x EY</td>
<td>0.0289</td>
<td>90</td>
</tr>
<tr>
<td>35x EY</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>350x EY</td>
<td>10</td>
<td>225</td>
</tr>
</tbody>
</table>

Table 6.1. Treatment Solutions in Dulbecco’s Phosphate-Buffered Saline.

Figure 6.2. Slit lamp images of A) scattered light, revealing the full thickness of the treated cornea (without Filter 2), and B) filtered light, isolating the fluorescence emission of Eosin Y (with Filter 2, as shown in Figure 1A)
6.2.2 In Vitro Application and In Vitro Expansion

We use the intact globe method, established in Chapters 2 and 5, to quantitatively compare treated and control eyes with respect to their resistance to corneal expansion.

Tissue Preparation: Eyes from 2–3 week old New Zealand White Rabbits (provided by collaborator Dr. Keith Duncan at the University of California at San Francisco) were stored in saline on ice for use within 48 hours of enucleation. The epithelium was removed by scraping a scalpel blade across the cornea. Removal was confirmed by visual inspection of the corneal surface. The extraorbital tissues were removed to expose the sclera and ensure accurate analysis of the eye shape. The eyes were then put into DPBS until treatment (at least 30 minutes and not more than 120 minutes).

Materials:

We tested three treatment solutions:

“1x EY”: 0.0289 mM EY with 90 mM TEOA in DPBS,

“10x EY”: 0.289 mM EY with 90 mM TEOA and 2% w/w hyaluronic acid (HA) in DPBS, and

“R/UVA”: 2.09 mM (0.1% w/w) riboflavin-5’-phosphate sodium salt hydrate and 20% w/w T-500 Dextran in DPBS.

Respective controls for each group used the treatment vehicle without EY or TEOA (i.e., DPBS, 2% HA in DPBS, and 20% T-500 Dextran in DPBS, respectively).
The addition of 2% HA and dextran are to increase viscosity so that the treatment could be applied topically and to maintain hydration of the cornea while doing so (Table 6.2).

<table>
<thead>
<tr>
<th>Treatment Formulation</th>
<th>Vehicle</th>
<th>Delivery Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x EY Treated</td>
<td>0.0289 mM EY and 90 mM TEOA</td>
<td>DPBS</td>
</tr>
<tr>
<td>1x EY Control</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>10x EY Treated</td>
<td>0.289 mM EY and 90 mM TEOA</td>
<td>2% HA in DPBS</td>
</tr>
<tr>
<td>10x EY Control</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>R/UVA Treated</td>
<td>2.09 mM riboflavin-5′-phosphate</td>
<td>20% Dextran in DPBS</td>
</tr>
<tr>
<td>R/UVA Control</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2. Treatments and Respective Controls Used for Corneal Expansion Studies

Procedure: The intact globes for 1x EY treated and control (fellow eye from the same animal) were placed cornea down in a holder such that 750 μL of their respective solutions just covered the cornea. The eyes were soaked in this manner for 5 minutes in the dark. The eyes were removed from solution and dabbed with a Kimwipe to remove excess solution from the corneal surface. Then the treated and fellow control eyes were placed cornea up in two identical holders, each equipped with a circle of green light-emitting diodes (seven 5-mm LEDs at 525 ± 16 nm, 6–8 mW/cm² in the plane of the cornea). An aluminum foil mask was place over each specimen to cover the sclera, while leaving the cornea exposed. Then the irradiation was applied for 5 minutes.

The 10x EY treated and control corneas had the gel treatment solution spread over the cornea. After allowing 5 minutes of diffusion time, the solution was removed with a spatula and the corneal surface was rinsed with DPBS. Each of the eyes was placed under a green light source consisting of seven 5-mm LEDs at 525 ± 16 nm and 6–8 mW/cm².
Using the same protocol described above, the corneas of the treated and fellow control eyes were irradiated simultaneously for 5 minutes while the remainder of the eye was protected from light exposure using an aluminum foil mask.

The R/UVA treated and control eyes received drops of solution on the cornea for 5 minutes prior to irradiation, and then received fresh drops every 5 minutes during the 30 minute irradiation with 3 mW/cm² of 370±5 nm ultraviolet light for 30 minutes. Both eyes were irradiated at the same time.

After irradiation, all the eyes were placed in DPBS for 30 minutes. Treated and fellow control eyes were mounted into an intact globe expansion apparatus with two sites to ensure that both experienced the same ambient pressure and temperature, and the same imposed intraocular pressure, as described in Chapter 2. To restore the native shape of the globes, they received a “prestress” of 22 mmHg for one hour, based on observations that showed that shape recovery required up to 45 minutes for some specimens. Images were recorded at 15 minute intervals during the prestress period. Then the imposed intraocular pressure was increased to 85 mmHg, a first image was required within a few seconds of the inception of elevated pressure and subsequent images were acquired at 15 minute intervals for 24 hours.
6.3 Results

6.3.1 Penetration of Molecules—Epithelial Barrier

Fluorescence emission from the stroma of eyes treated with the epithelium present is much weaker than that observed in specimens that had the epithelium removed prior to treatment (Figure 6.3): 1x EY penetrates the stroma of eyes with removed epithelium within an hour, but in eyes with the epithelium intact, 1x EY cannot be detected after 6 hours of soaking. This observation accords with prior literature indicating that the epithelium inhibits penetration of compounds closely related to EY (e.g., fluorescein). Nevertheless, significant penetration of EY across the epithelium is evident in corneas treated with high concentration solutions (35x EY and 350x EY). These results indicate that EY penetration through the epithelium is possible. Clinically, it is significant that removal of the epithelium may not be necessary for treatment.

Note that this method has very low sensitivity relative to, for example, laser scanning confocal fluorescence microscopy. Due to low sensitivity, soaking for at least 1 hour was needed to achieve detectable levels of fluorescence for 1x EY even without the epithelium present (Figure 6.3, left, second row). The next section of this chapter discusses treatments with 1x EY that successfully stabilize deepithelialized corneas using a 5 minute soaking time. While slit lamp photography may be used to make qualitative judgments of epithelial penetration, it cannot be used to quantify changes with the sensitivity required for our treatments. Future studies with the more sensitive approach of confocal microscopy could be used to reveal the diffusion rates through the epithelium and within the stroma.
6.3.2 In Vitro Application and In Vitro Expansion

All quantitative comparisons of treated and control eyes were performed using deepithelialized specimens. This decision was based on observations made on intact globes with the epithelium present. An illustration is presented in Figure 6.4 (a and b) where the epithelium was left intact: the cornea behaves similarly for treated and control globes, while the expansion of the sclera is lower for the treated globe than for the fellow control.

In contrast, if the epithelium is removed (Figure 6.4c, d), sufficient drug enters the cornea during the 5 minute soak to prevent corneal expansion (Figure 6.4c). The behavior evident for this pair of eyes is statistically significant: results for a group of 6 treated eyes and their
6 fellow controls (Figure 6.5) show that both cornea and sclera of deepithelialized globes are stabilized by treatment (further information on this set of data is given in Chapter 5).

![Figure 6.4](image)

**Figure 6.4.** Traces of the globe perimeter show the initial shape and final shape after 24 hours of high pressure (85 mmHg) for representative specimens that were fully immersed for 5 minutes in either 1x EY (treated) or DPBS control followed by irradiation of the entire globe for 5 minutes (see Chapter 5 for further details). The epithelium was left in place for the upper pair of eyes (A treated and B control). The epithelium was removed prior to soaking the lower pair of eyes (C treated and D control). Effective stabilization of the cornea is observed in C, but not in A.

Although they are not from the same animal, comparison of the control eyes (Figure 6.4b and d) shows that control eyes with epithelium removed expand similarly to control eyes with epithelium intact. Removal of the epithelium, used in the remainder of this section,
allows drug to penetrate into the corneal stroma with no other discernible effect on the intact globe expansion behavior.

In relation to keratoconus, we examine the changes in dimensions of the cornea (CP, CD, and CL, defined in Figure 6.5). In the control specimens, each of the three dimensions expands significantly under the influence of elevated pressure (approximately 12% for CP, 10% for CD and 18% for CL). The three groups of controls do not differ significantly, indicating that the formulation vehicles do not play a direct role in mechanical stabilization.

**Figure 6.5.** Rabbit eyes with removed epithelium are subjected to a high pressure (85 mmHg) for up to 24 hours, showing the ability of treatment of the entire eye with 1x EY to prevent the expansion of the sclera (top row) and cornea (middle row). The relevant ocular dimensions are illustrated in the inset figure, and n is the number of eyes in each group.
Soaking the cornea in 1x EY for 5 minutes and irradiating with visible light for 5 minutes reduces expansion of the cornea as compared to DPBS controls (Figure 6.6). In anticipation of topical application of a viscous solution or gel on the cornea to administer the drug, a more concentrated solution (10x EY) was made with a “viscoelastic” surgical aid (2% HA) that creates a gel. It was applied to form a coating on the cornea. This treatment showed enhanced ability to prevent expansion (Figure 6.6), consistent with the increased EY concentration. For comparison to the treatment that is currently in FDA clinical trials for the treatment of keratoconus, we include a protocol that uses the same parameter values described for that clinical procedure. The treated eyes in the R/UVA group show similar resistance to expansion as the 10x EY treated group (Table 6.3). The treatment of the whole eye with GA (Figure 2.26) gives an extreme degree of crosslinking that is presented here for comparison (Figure 6.6).

Importantly, the intact globe expansion method provides a way to compare treatments for efficacy. Tests using a few animals (8–10) are able to distinguish treatments and to screen variations on the procedure to improve the treatment. Further, the results of the R/UVA treatment provide a benchmark for identifying clinically relevant treatments.
Successful treatment of keratoconus by crosslinking the constituents of the cornea depends on penetration of the photoactivated formulation into the tissue and the activation of the
drug with safe doses of light to produce a therapeutically significant stabilization of the tissue. The in vitro studies presented in this chapter address each of these requirements. It is shown that EY readily penetrates into the cornea if the epithelium is removed. If the epithelium remains on the cornea, partitioning of Eosin Y into the cornea is greatly reduced. Nevertheless, with sufficiently high driving force (high EY concentration), EY does penetrate into the cornea. Furthermore, it penetrates to an extent visible with a relatively insensitive imaging system—representing a large excess over the concentration required to achieve an effect similar to the riboflavin/UVA treatment. Therefore, it may be possible to deliver treatment to the cornea without removing the epithelium, and future studies on increasing concentrations could achieve the desired mechanical stability. Without the epithelium present, the 10x EY treatment shows strengthening capabilities comparable to the R/UVA treatment. Clinically, the R/UVA treatment involves removing the epithelium, applying topical drops of treatment solution for 5 minutes, and irradiating for 30 minutes with ~3 mW/cm² 370 nm ultraviolet light while applying drops every 5 minutes. The 10x EY treatment we have developed involves removing the epithelium, applying a viscous drug solution for 5 minutes, cleaning off excess drug, and irradiating with ~6 mW/cm² 514 nm visible light for 5 minutes. The 10x EY treatment is shorter and uses visible light instead of UV in order to achieve the same effective prevention of expansion. If the treatment also proves to be non-toxic and the cornea maintains optical clarity in vivo, this would be a potential treatment option for keratoconus.


