

# Cross-Linking with Ultraviolet-A and Riboflavin Reduces Corneal Permeability

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**PURPOSE.** To investigate the effect of cross-linking treatment on corneal permeability in a live animal model.

**METHODS.** Rabbit eyes were selected at random to be left unoperated or to undergo epithelial debridement with or without treatment consisting of cross-linking (CXL) with riboflavin and ultraviolet-A. Nine eyes received a total dose of 3.6 J/cm<sup>2</sup> and after epithelial healing the corneas were placed in a two-chamber system for quantification of the diffusion of fluorescein compared with controls. Thirty eyes received a total dose of 5.4 J/cm<sup>2</sup> and, after epithelial healing, in vivo corneal permeability was quantified as the pupillary response over a 30-minute period to a dose of topical pilocarpine compared with controls.

**RESULTS.** In the ex vivo assay, the mean permeability coefficient in the CXL group ( $2.42 \times 10^{-7}$ ) was reduced when compared with the unoperated controls ( $3.73 \times 10^{-7}$ ;  $P = 0.007$ ) and to the eyes that received epithelial debridement alone ( $3.74 \times 10^{-7}$ ;  $P = 0.01$ ). In the in vivo permeability assay, the change in pupillary diameter at 30 minutes after pilocarpine administration was smaller in the CXL group (−1.9 mm), compared with the epithelial debridement group (−2.6 mm;  $P < 0.001$ ) and with the unoperated controls (−2.7 mm;  $P = 0.003$ ).

**CONCLUSIONS.** Corneal cross-linking with ultraviolet-A and riboflavin results in a statistically significant reduction in corneal permeability. These findings suggest that dosing of topical medications may need to be increased in eyes with a history of CXL to achieve expected therapeutic effects, and they may have implications for the long-term health of the cornea. (*Invest Ophthalmol Vis Sci.* 2011;52:9275-9278) DOI:10.1167/iov.11-8155

In recent years corneal cross-linking treatments have gained widespread acceptance in the treatment of corneal ectatic disorders.<sup>1-6</sup> The application of ultraviolet-A irradiation to a riboflavin-soaked cornea has been shown to result in an increase in biomechanical strength of the cornea<sup>7,8</sup> with lasting clinical stabilization of conditions such as keratoconus or ectasia after refractive surgery. It is believed that stiffening of the cornea occurs through an increase in cross-link bonds in the stroma, resulting from the release of free radicals by the inter-

action of riboflavin and ultraviolet energy.<sup>9,10</sup> The procedure, known as cross-linking (CXL) in its current form, has been found to be generally well tolerated by the cornea, and therefore it has been evaluated for a growing number of indications.<sup>11-13</sup>

Beyond its strengthening effect on the cornea, the increased cross-link density in the stroma might be expected to have other effects on the tissue's behavior and its physiology. With age, the cornea accumulates collagen cross-links, both enzymatic (lysyl-oxidase dependent) and non-enzymatic (through glycation via Maillard-type reactions).<sup>14,15</sup> Additionally, diseases such as diabetes can result in increased corneal cross-linking through the sustained elevation of glucose levels.<sup>16</sup> In an in vitro model of these glycation-related changes in the corneal stroma, we have shown that cross-links impair corneal permeability.<sup>17</sup> Other cross-linking agents such as glutaraldehyde have been found to have similar effects in the sclera.<sup>18</sup> Because CXL works to increase the stromal cross-link density, it is reasonable to presume that the treatment would also impede diffusion through the cornea. The cornea relies on diffusion of water and solutes for the nutrition of its cellular components and maintenance of clarity.<sup>19-22</sup> Additionally, transcorneal penetration is a principal mechanism through which topically applied medications enter the eye.<sup>23</sup> Thus, it is important to understand the effect on corneal permeability of an increasingly popular treatment modality such as CXL.

We hypothesized that CXL results in a significant reduction in corneal permeability. To investigate this possibility, we performed CXL in rabbits and studied the effect on both corneal permeability to solute diffusion (measured ex vivo) and topical drug penetration to the anterior chamber (measured functionally in vivo).

## METHODS

### Animal Surgery

Animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the University of California San Francisco Committee on Animal Research. Forty-seven New Zealand White female rabbits (approximately 4.0 kg) from the Western Oregon Rabbit Company (Philomath, OR) were used in the study. Rabbits underwent treatment of only one eye at a time. Eyes were assigned randomly to three groups. The first group remained unoperated as controls, the second group underwent corneal epithelial debridement, and the third group received CXL treatment, consisting of epithelial debridement with topical riboflavin treatment and UV light irradiation. For eyes receiving epithelial debridement with or without CXL, treatment was performed on the second eye after the first eye had fully recovered, with no remaining epithelial defect or apparent inflammation.

### Epithelial Debridement

For eyes assigned to either epithelial debridement alone or epithelial debridement plus CXL, rabbits were anesthetized by intramuscular

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injection of 0.03 mg/kg buprenorphine followed by ketamine (35 mg/kg)/xylazine (5 mg/kg). Eyes were bathed in povidone-iodine solution and rinsed with sterile balanced salt solution. For additional local anesthesia, proparacaine eye drops were administered onto the eyes. Corneal thickness was measured using an ultrasonic pachymeter (Pachette3; DGH Technology, Inc., Exton, PA). The recorded reading was the average of 25 measurements. A lid speculum was placed into the fornix of the treated eye and the central 8 mm of the corneal epithelium was mechanically removed using a number 69 Beaver blade. The thicknesses of postdebridement corneas were then measured. At the end of the procedure, 150  $\mu$ L of cefazolin was injected subconjunctivally as prophylaxis against infection. In addition, 0.5% erythromycin ointment was applied topically, and buprenorphine and carprofen were administered systemically. Postoperative care is described separately below.

### Riboflavin Treatment

In eyes undergoing CXL, after epithelial debridement the corneal thickness was confirmed to be at least 400  $\mu$ m, and then 0.1% riboflavin solution in PBS containing 20% dextran (riboflavin 5'-phosphate sodium salt hydrate, phosphate-buffered solution, and dextran, all from Sigma-Aldrich, St. Louis, MO; 1.37 mg per mL of riboflavin 5'-phosphate in 20% dextran solution to achieve a final riboflavin concentration of 1 mg/mL or 0.1%; pH 6.7) was applied topically at 3-minute intervals for 30 minutes before the irradiation and then during the 30-minute irradiation. One drop was given at each time point.

### Cross-Linking with UV at a Dose of 3.6 J/cm<sup>2</sup>

In the first phase of the experiments, irradiation for eyes assigned to CXL was performed using a custom-built UV light-emitting diode apparatus (NS370L-5RLO, Roithner Lasertechnik, Vienna, Austria), as previously described,<sup>2,8</sup> at an irradiance of 2.0 mW/cm<sup>2</sup> (dosage of 3.6 J/cm<sup>2</sup>) for 30 minutes. The surface irradiance at the cornea was confirmed using a UV meter (Lasermate-D, Coherent, Auburn, CA). Antibiotics were then given as described for epithelial debridement eyes, and postoperative care is described below.

### Cross-Linking with UV at a Dose of 5.4 J/cm<sup>2</sup>

In the second phase of the experiment, the UV-X ultraviolet light source (Iroc, Zurich, Switzerland) was used for the CXL treatment. Eyes treated with this apparatus received an irradiance of 3.0 mW/cm<sup>2</sup> (dosage of 5.4 J/cm<sup>2</sup>). The treatment and follow-up procedures were otherwise identical with that described above.

### Postoperative Care and Euthanization

After either epithelial debridement or CXL, rabbits were examined once a day for 5 days for any signs of inflammation including ptosis, blepharospasm, redness of the eye, discharge, or photophobia. Treatment with analgesics was repeated as needed. Corneas were stained with 1% sodium fluorescein solution (Sigma, St. Louis, MO) to confirm that the epithelial wound was healed, at which point the second eye was treated according to its assigned group (observation, epithelial debridement, or CXL). Rabbits were kept in animal housing for more than 2 weeks after treatment of the second eye, to allow relative stabilization of corneal stromal changes.<sup>24</sup> Rabbits were then euthanized with an overdose of 150 mg/kg intracardiac sodium pentobarbital. Both eyes were enucleated and used immediately in subsequent studies.

### In Vivo Evaluation of Drug Penetration

Drug penetration was measured in vivo in eyes assigned to either observation, epithelial debridement, or CXL at a dose of 5.4 J/cm<sup>2</sup>. Before euthanization, animals were anesthetized, and penetration of topical pilocarpine into the eye, as an indirect measure of corneal permeability, was detected by quantifying pupil constriction as previously described.<sup>25,26</sup> While the rabbit was anesthetized, pupillary di-

ameter was measured using a pupillometer (Colvard; Oasis Medical, Glendora, CA). Then a single eye drop containing preservative-free pilocarpine 1% solution (Sigma) was applied to the cornea, and the eyelids were closed. Pupillary diameter was then measured again every 3 minutes for the next 30 minutes. In some instances, approximately 20 minutes into the 30-minute measurement period, a single drop of balanced salt solution was placed on the cornea if the investigators judged that the eye appeared dry. Net average change in pupillary diameter at 30 minutes was compared between groups using Student's *t*-test, and all measurements were plotted versus time for each group. These eyes were subsequently enucleated and used in ex vivo experiments not presented in this report.

### Ex Vivo Diffusion Assay

Diffusion of fluorescein across the cornea was measured after enucleation in eyes assigned to either observation, epithelial debridement, or CXL at a dose of 3.6 J/cm<sup>2</sup>. The method has been described previously.<sup>17</sup> The cornea was excised from the globe, blotted dry, and placed between two stainless steel annular platens having 6.35 mm central apertures. The tissue was mounted without stretching so as to minimize asymmetric stresses. This assembly was placed between the two reservoir chambers of a housing constructed from PVC unions, and this was threaded together to provide the compression necessary to prevent leakage around the specimen. The open ends of the union were sealed (Parafilm; Pechiney Plastic Packaging, Chicago, IL) after both chambers were filled with PBS and visually inspected for leakage. The apparatus was incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 1 hour to restore normal hydration and temperature.

The medium in the reservoir facing the intraocular side of the cornea (receiver chamber) was replaced by 9 mL of fresh PBS at 37°C, whereas the reservoir facing the extraocular side (donor chamber) was filled with an equal volume of PBS containing 1 mg/mL sodium fluorescein (Sigma). The apparatus was returned to the tissue incubator and placed under constant agitation using a rotary hybridization oven. The chamber was removed from the incubator and shaken with a mixer before samples measuring 200  $\mu$ L were removed from each reservoir at 30-minute intervals for 4 hours and stored in the dark at -80°C.

Fluorescence was measured at room temperature with a fluorescence spectrophotometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA). Excitation and emission wavelengths were 492 and 520 nm, respectively. Standard curves of fluorescence versus concentration were obtained by serial dilution of fluorescein in PBS. Concentrations of samples were determined by linear regression analysis within the linear portion of the standard curve. The values of steady state flux were estimated from the linear portion of the cumulative amount of drug permeated ( $\mu$ g/cm<sup>2</sup>) versus time (minutes) profile in each case. All the permeation studies were carried out in duplicate, and the results were expressed as mean  $\pm$  SD. Diffusion from the donor chamber to the receiver chamber was characterized by the apparent permeability coefficient, *P* (cm/second), which was calculated using the following equation:

$$P = \frac{dQ}{dt} \frac{1}{AC}$$

where  $dQ/dt$  is the permeability rate of fluorescein ( $\mu$ g/second), *A* is the cross-sectional area of specimen exposed to the solution (cm<sup>2</sup>), and *C* is the initial donor solute concentration ( $\mu$ g/mL).

## RESULTS

### Recovery After Corneal Treatment

In the group receiving epithelial debridement without UV irradiation, the epithelium healed by a mean of 3.2 days (SD  $\pm$  0.9). These eyes were enucleated at a mean of 7 days after

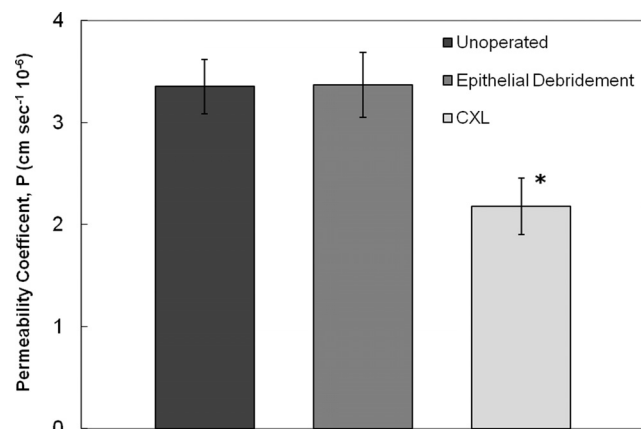
treatment in the ex vivo experiment and 14 days in the in vivo experiment. In the group receiving epithelial debridement with CXL at a dose of 3.6 J/cm<sup>2</sup>, the epithelium healed by a mean of 4.6 days (SD ± 0.9). These eyes were enucleated at a mean of 18 days after treatment. In the group receiving epithelial debridement with CXL at a dose of 5.4 J/cm<sup>2</sup>, the epithelium healed by a mean of 7.0 days (SD ± 3). These eyes were enucleated at a mean of 43 days after treatment. The groups had different mean times to enucleation because efforts were made initially to ensure at least 2 weeks of recovery after CXL, and then later in the course of the experiment the authors decided that an even longer recovery time would be preferable to approximate more steady state conditions in the stroma after CXL. Corneal clarity was achieved in all groups when the epithelium healed. No corneal infections or other complications developed in any group.

### Ex Vivo Diffusion of Fluorescein through the Cornea

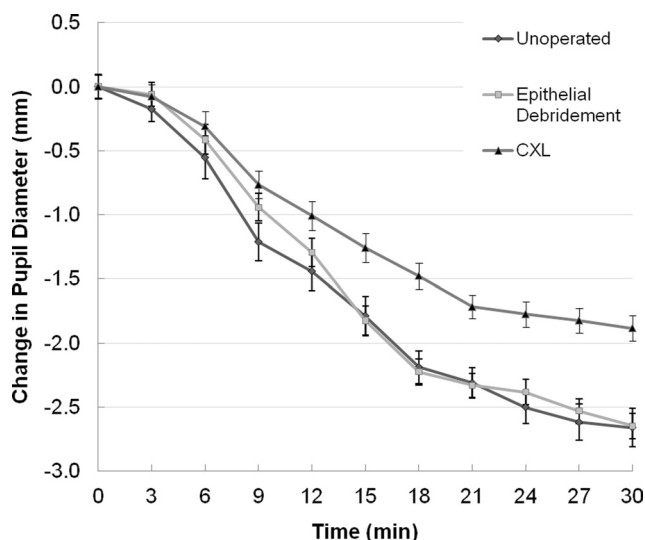
The number of live rabbit eyes in each group in this phase of the experiment was as follows: 10 unoperated controls, 10 epithelial debridement without UV irradiation, and 9 epithelial debridement with CXL at a dose of 3.6 J/cm<sup>2</sup>. CXL in live rabbit eyes resulted in a statistically significant reduction of fluorescein permeation through the cornea in the ex vivo assay (Fig. 1). The permeability coefficient in the treated group ( $2.18 \pm 0.28 \times 10^{-6}$ ;  $n = 9$ ) decreased a significant amount when compared with the unoperated controls ( $3.35 \pm 0.27 \times 10^{-6}$ ;  $n = 10$ ;  $P = 0.007$ ) and eyes that received epithelial debridement alone ( $3.37 \pm 0.32 \times 10^{-6}$ ;  $n = 10$ ;  $P = 0.01$ ). Corneas fully healed from epithelial debridement were no different from unoperated controls ( $P = 0.97$ ).

### In Vivo Evaluation of Drug Penetration

The number of live rabbit eyes in each group in this phase of the experiment was as follows: 17 unoperated controls, 17 epithelial debridement without UV irradiation, and 30 epithelial debridement with CXL at a dose of 5.4 J/cm<sup>2</sup>. Penetration of topical pilocarpine into the eye was impeded by CXL treatment to a significant degree, compared with unoperated controls and eyes receiving only epithelial debridement, as reflected by a smaller degree of pupillary constriction measured at 30 minutes.<sup>25,26</sup> There was no significant difference be-



**FIGURE 1.** Permeability coefficients of ex vivo rabbit cornea for each treatment group (unoperated,  $n = 10$ ; epithelial debridement,  $n = 10$ ; CXL,  $n = 9$ ). Epithelial debridement and CXL groups were tested after the epithelium had healed, mean 7 and 18 days after surgery, respectively. Error bars, SE. \* $P < 0.05$ ; statistically significant difference from untreated and epithelial debridement groups.



**FIGURE 2.** Plot of pupillary constriction over time in live rabbit eyes after topical administration of one drop of 1% pilocarpine solution (unoperated,  $n = 17$ ; epithelial debridement,  $n = 17$ ; CXL,  $n = 30$ ). Epithelial debridement and CXL groups were tested after the epithelium had healed, mean 14 and 43 days after surgery, respectively. Pupilary diameter was measured every 3 minutes for 30 minutes. Error bars, SE.

tween unoperated controls and the epithelial debridement group. The net average change in pupillary diameter at 30 minutes after pilocarpine administration was  $-1.9$  mm in the CXL group,  $-2.6$  mm in the epithelial debridement group, and  $-2.7$  mm in the unoperated controls ( $P = 0.003$  unoperated control vs. CXL;  $P < 0.001$  epithelial debridement vs. CXL;  $P = 0.96$  unoperated control vs. epithelial debridement). The plot of pupillary diameter versus time for all three groups is presented in Figure 2.

### DISCUSSION

This study demonstrated that that CXL results in a statistically significant reduction in corneal permeability compared with untreated corneas. Chronic compromise of nutrient diffusion through the cornea might be expected to impact the health of its cellular constituents, particularly if these are already weakened due to other causes such as surgery, disease, or pharmacologic toxicity. Additionally, as demonstrated herein, patients undergoing CXL will likely achieve less intraocular penetration of topically applied medications over a given time period when compared with other patients. The cross-links created by CXL appear to be affecting permeability in the same fashion as those resulting from nonenzymatic glycation.<sup>17</sup> It seems likely that regardless of the specifics of the chemical reaction that create the bonds between collagen fibrils, the effect on the matrix properties will be the same, namely a reduction in permeability along with the expected increase in stiffness. Indeed, for the same solute (fluorescein), the reduction in permeability associated with CXL in our ex vivo assay is similar in magnitude to that resulting from glycation.<sup>17</sup>

In this study, corneal permeability was quantified approximately 3 to 6 weeks after CXL (mean 18 days for the ex vivo assay and 43 days for the in vivo assay). It is known that the stroma continues to undergo structural and cellular changes for months after treatment.<sup>24</sup> Therefore, it seems possible that the permeability of the tissue varies somewhat in conjunction with these evolving changes and that the findings of our study reflect the matrix properties at a given time point during this

posttreatment healing process. Nevertheless, because the mechanical effects seen early after treatment persist over a prolonged follow-up period,<sup>8</sup> it is likely the changes in the collagen network of the stroma are lasting. Because the latter effects are thought to be responsible for the reduction in permeability after CXL, it seems probable that the permeability effect also persists. This could be confirmed by further studies with longer-term follow-up.

An additional limitation of the experimental methods in this study is that two different systems were used to deliver the ultraviolet irradiance for the cross-linking reaction in the cornea. The *in vivo* permeability assay was performed in rabbits treated at a standard dose with the UV-X light source system, which is commonly used in patients at the present time, whereas the *ex vivo* permeability testing was performed on corneas that had been treated at a dose that was 33% lower and using a diode array assembled in the laboratory, before we were able to obtain the UV-X system. The fact that statistically significant reductions in corneal permeability were detected in both instances strengthens the conclusion of the study by confirming that the effect is independent of the diode arrangement that delivered the ultraviolet energy. Future studies using the rabbit model could determine whether a dose-response effect for CXL's effect on corneal permeability exists, as has been seen in corneas cross-linked with glycation.<sup>17</sup> Finally, a riboflavin-only control group, in which corneas are not exposed to any ultraviolet irradiation at all, could be used in future studies to provide additional evidence that the corneal cross-linking, rather than an independent effect of riboflavin, is responsible for the permeability reduction seen with CXL.

The implications of our findings may extend to the sclera, for which therapeutic cross-linking has been proposed as a treatment for degenerative myopia. Because the sclera also consists of a network of collagen fibrils (albeit organized differently than in the cornea), it seems likely that these treatments would also reduce scleral permeability. Although the effect of CXL on fluid flow across the cornea was not characterized in this study, we have previously demonstrated that chemical cross-linking of both the cornea and sclera reduces specific hydraulic conductivity.<sup>17,27</sup> Whether this would result in any clinical adverse effects such as uveal effusion or increased intraocular pressure, from an inability of intraocular fluid to egress across the sclera, remains to be seen.

In summary, we have shown that CXL in rabbits results in a statistically significant reduction in corneal permeability. Future studies in this animal model as well as longitudinal studies in patients will be necessary to characterize the nature and clinical effects of the permeability reduction resulting from corneal cross-linking.

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