Transepithelial Riboflavin Absorption in an Ex Vivo Rabbit Corneal Model

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Submitted: March 18, 2015
Accepted: June 23, 2015

Citation: Gore DM, O'Brart D, French P, Dunsby C, Allan BD. Transepithelial riboflavin absorption in an ex vivo rabbit corneal model. *Invest Ophthalmol Vis Sci*. 2015;56:5006–5011. DOI:10.1167/iovs.15-16903

Purpose. To measure depth-specific riboflavin concentrations in corneal stroma using two-photon fluorescence microscopy and compare commercially available transepithelial corneal collagen cross-linking (CXL) protocols.

Methods. Transepithelial CXL riboflavin preparations—MedioCross TE, Ribocross TE, Paracel plus VibeX Xtra, and iontophoresis with Ricrolin+—were applied to the corneal surface of fresh postmortem rabbit eyes in accordance with manufacturers' recommendations for clinical use. Riboflavin 0.1% (VibeX Rapid) was applied after corneal epithelial debridement as a positive control. After riboflavin application, eyes were snap frozen in liquid nitrogen. Corneal cross sections 35-μm thick were cut on a cryostat, mounted on a slide, and imaged by two-photon fluorescence microscopy. Mean (SD) concentrations were calculated from five globes tested for each protocol.

Results. Peak riboflavin concentration of 0.09% (±0.01) was observed within the most superficial stroma (stromal depth 0–10 μm) in positive controls (epithelium-off). At the same depth, peak stromal riboflavin concentrations for MedioCross TE, Ricrolin+, Paracel/Xtra, and Ribocross TE were 0.054% (±0.01), 0.031% (0.003), 0.021% (±0.001), and 0.015% (±0.004), respectively. At a depth of 300 μm (within the demarcation zone commonly seen after corneal cross-linking), the stromal concentration in epithelium-off positive controls was 0.075% (±0.006), while at the same depth MedioCross TE and Ricrolin+ achieved 0.018% (±0.006) and 0.016% (0.002), respectively. None of the remaining transepithelial protocols achieved concentrations above 0.005% at this same 300-μm depth. Overall, MedioCross TE was the best-performing transepithelial formulation.

Conclusions. Corneal epithelium is a significant barrier to riboflavin absorption into the stroma. Existing commercial transepithelial CXL protocols achieve relatively low riboflavin concentrations in the anterior corneal stroma when compared to gold standard epithelium-off absorption. Reduced stromal riboflavin concentration may compromise the efficacy of riboflavin/ultraviolet corneal CXL.

Keywords: corneal stroma, transepithelial, riboflavin, iontophoresis, two-photon fluorescence microscopy, cross-linking

Corneal collagen cross-linking (CXL), using the original "Dresden" epithelium-off protocol,[1] is effective in arresting disease progression in keratoconus in over 90% of eyes.[2–4] The corneal epithelium is removed in this protocol to facilitate riboflavin absorption and hence treatment efficacy. However, complications including infection, scarring, delayed healing, sterile inflammatory infiltration, tissue melting, and resultant vision loss have all been described in association with corneal epithelial removal in CXL.[5] Due to these complications, CXL is normally reserved for patients with demonstrable disease progression.

Transepithelial CXL, in which riboflavin is applied through an intact epithelium, aims to deliver the benefits of standard epithelium-off treatments without the painful rehabilitation and complications of epithelial removal. However, the only published randomized controlled trial of this newer technique[6] recently concluded that it was not effective compared with epithelium-off CXL. Most commercially available riboflavin formulations for transepithelial CXL are designed to enhance epithelial permeability by the addition of epithelial-toxic agents (Table). Another method involves iontophoresis, in which negatively charged riboflavin is driven through the epithelium down an electrical gradient.[7–9]

Previous studies to quantify riboflavin penetration through an intact epithelium have used high-performance liquid chromatography (HPLC)[10–12] and fluorescence microscopy.[13–17] High-performance liquid chromatography, in which the sample is dissolved in a solvent for analysis, can accurately quantify the concentration of riboflavin in a whole block of tissue, but is unable to provide information about the concentration at different depths within the corneal stroma (unless lamella sections are prepared and separately dissolved).[18] Fluorescence microscopy, both single-photon excitation (i.e., confocal) and multiphoton excitation,[13–17] has the

Table. Commercially Available Riboflavin Preparations

Riboflavin Formulation	Composition
VibeX Rapid	0.1% wt/vol riboflavin 5′-monophosphate, saline, HPMC
Ribocross TE	0.125% wt/vol riboflavin 5′-monophosphate, D-alpha-tocopheryl poly(ethylene glycol) 1000 succinate
MedioCross TE	0.25% wt/vol riboflavin 5′-monophosphate, HPMC, benzalkonium chloride 0.01%
Ricrolin+	0.1% wt/vol riboflavin 5′-monophosphate, sodium edetate, trometamol, sodium dihydrogen phosphate dihydrate, sodium phosphate dibasic dehydrate
Paracel	0.25% wt/vol riboflavin 5′-monophosphate, HPMC, sodium edetate, trometamol, benzalkonium chloride, saline
VibeX Xtra	0.25% wt/vol riboflavin 5′-monophosphate, saline

added advantage of quantifying concentration at depth within the cornea; both excitation techniques, however, suffer from signal attenuation with increasing scan depth, principally attributable to scattering and aberration.[14–16] We have previously reported[14] a novel time-lapse measurement approach to correct for two-photon fluorescence (TPF) signal attenuation; although applicable to epithelium-off absorption, this method has not proven suitable when imaging through an intact epithelium.[19]

Here we describe a new method for quantifying riboflavin concentration within the cornea using an ex vivo animal rabbit eye model and compare the efficacy of current commercially available protocols for transepithelial CXL in promoting riboflavin absorption.

Materials and Methods

Ethical approval for corneal two-photon fluorescent microscopy studies in an ex vivo model was granted by University College London Institute of Ophthalmology (Ref. 10/H0106/57-2012ETR27).

Sample Preparation

Adult pigmented rabbit heads transported on ice in a phosphate-buffered saline (PBS) bath were received within 5 hours post mortem (First Link Ltd., Wolverhampton, UK). Intact globes were enucleated and examined under a low-magnification light microscope to rule out obvious epithelial trauma or scars. The globes were warmed to room temperature in balanced salt solution before different commercially available riboflavin solutions (Table) were applied to the corneas according to the manufacturers' protocols. Corneas soaked for 30 minutes with 0.1% riboflavin in hydroxypropyl methylcellulose (HPMC) and saline (VibeX Rapid; Avedro, Inc., Waltham, MA, USA) after epithelial debridement served as positive controls and unsoaked corneas served as negative controls.

Commercial transepithelial CXL protocols we compared were ($n = 5$ for each protocol) were as follows:

1. MedioCross TE (Peschke Meditrade GmbH, Waldshut-Tiengen, Germany)

A 9-mm vacuum well secured on the cornea was filled with approximately 0.5 mL MedioCross TE solution for 30 minutes. Riboflavin was not rinsed from the cornea.

2. Ribocross TE (IROS Srl, Napoli, Italy)

A 9-mm vacuum well secured on the cornea was filled with approximately 0.5 mL Ribocross TE solution for 30 minutes. Riboflavin was not rinsed from the cornea.

3. Paracel and VibeX Xtra (Avedro, Inc.)

Paracel drops were applied at a rate of one drop every 90 seconds for 4 minutes. The cornea was then rinsed with VibeX Xtra completely coating the cornea. Additional VibeX Xtra was applied at a rate of one drop every 90 seconds for 6 minutes (total riboflavin soak time 10 minutes). Riboflavin was not rinsed from the cornea.

4. Ricrolin+ (Sooft Italia S.p.A., Montegiorgio, Italy)

The iontophoresis system comprised two electrodes: a negatively charged metal grid housed within the corneal applicator and a positively charged 20-gauge needle inserted through the sclera into the vitreous. The corneal applicator was vacuum attached to the cornea. The reservoir was filled with Ricrolin+ above the level of the metal grid before beginning a 5-minute treatment with a continuous direct current of 1 mA (Fig. 1A). After the iontophoretic procedure, the applicator was removed and riboflavin rinsed from the cornea.

Section Preparation

At the end of the riboflavin soak, the globes were immediately immersed in liquid nitrogen for 5 minutes and stored overnight

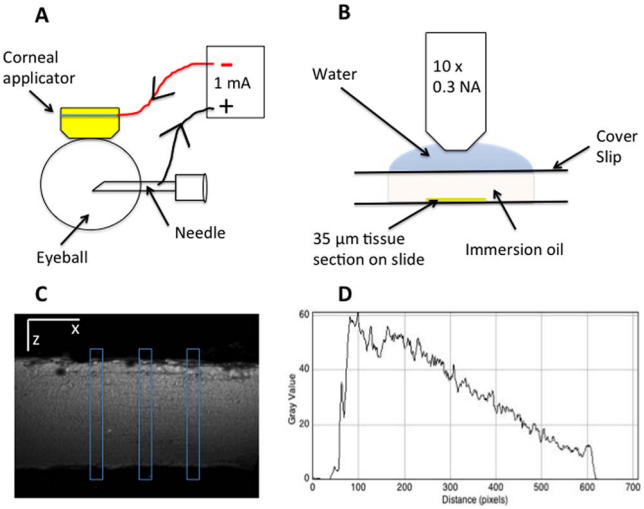

Figure 1. (A) Iontophoresis schematic showing a riboflavin-filled corneal well housing a negative electrode with the return electrode connected to a needle passed into the vitreous cavity. The riboflavin solution acts as an electrical contact between the electrode and the eye. The generator self-checks the continuity and a warning signal sounds in case of current disruption. (B) Slide-mounted 35-µm tissue sections were covered in immersion oil with a coverslip placed on top and coupled to the microscope objective with distilled water. (C) Grayscale two-photon fluorescence image analyzed in ImageJ with three box plots (40 pixels wide) from which to average the signal (D) in each section. The edges of the images were not analyzed to avoid areas of vignetting (grayscale image formatted to increase brightness and clarity).

at −25°C. A broad incision across the posterior globe was made just before immersion to prevent the cornea from splitting open on freezing. For each globe, three 35-μm corneal cross sections were cut on a cryostat 1 mm apart (Supplementary Fig. S1). With the frozen section on the blade platform (maintained at −21°C), a knife was used to cut the cornea free, and the remaining tissue was brushed away. The cornea itself was meticulously brushed to ensure that no ice remained attached that might allow riboflavin to leak out once thawed. The corneal section was then mounted on a slide and covered with fluorescence-free immersion oil (Immersol 518 F; Carl Zeiss Ltd., Cambridge, UK) to prevent any leakage of riboflavin out of the tissue. A coverslip was placed on top prior to imaging under the microscope objective (Fig. 1B). The time taken from the tissue thawing onto the slide to image acquisition on the microscope was approximately 1 minute, and we refer to this earliest imaging time point as $t = 0$ minutes. To investigate riboflavin migration within the thawed slide-mounted tissue, serial images were captured every minute for selected samples. For all other measurements, data were acquired as soon as possible following tissue thawing, that is, after approximately 1 minute.

Two-Photon Microscope Setup and Imaging Protocol

We have previously described the use of TPF microscopy for imaging corneal cross sections.[14] In brief, a Ti:Sapphire laser, operating at a wavelength of 890 nm with a 140-femtosecond pulse duration and 80-MHz pulse repetition rate, was used as the excitation laser source. The excitation laser beam was guided to a Leica DM6000CS upright microscope (Leica Microsystems GmBH, Wetzlar, Germany) where it passes through two galvoscanners, allowing scanning in the *x-y* plane, before being focused into the sample by a Leica 10X/0.3 NA water-immersion objective. Theoretical (full-width half-maximum) axial and lateral resolutions were calculated at 16 and 1.1 μm, respectively. Two-photon fluorescence excitation light of 890-nm wavelength was chosen to correspond with the highest riboflavin absorption peak (445 nm) as determined by spectrophotometry. Emitted riboflavin fluorescence was collected between 525 and 650 nm to avoid overlap with the absorption spectrum of riboflavin. Images (553 × 553 μm [512 × 512 pixels]) were captured at a scan rate of 600 Hz (line average 16) with the pinhole wide open.

Image Analysis

Grayscale images (Fig. 1C) were exported and analyzed using Java-based imaging software (ImageJ, 1.48v, http:// imagej.nih.gov.ij; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). For each image, three separate rectangular regions of interest each 40 pixels wide in the *x*-direction (see Fig. 1C) were manually selected. The intensity profiles along the *z*-direction for each region of interest were then exported to a .txt file (Excel for Mac, 2011; Microsoft Corp., Redmond, WA, USA) (Fig. 1D). The epithelial/stromal junction was then identified in each trace by the abrupt change in signal and confirmed with reference to the corresponding image region of interest. This information was then used to align all three plots along the *z*-axis, and a mean intensity plot was then generated representing the average TPF signal for that image as a function of depth (*z*). Two-photon fluorescence signals were converted to riboflavin concentration by normalizing to the TPF signal achieved in a well-slide reservoir of 0.1% riboflavin solution during the same experimental session. Mean (SD) concentrations were calculated from five globes tested for each protocol.

Results

Riboflavin Migration Within Thawed Tissue

Serial images were captured at time = 0, 1, and 2 minutes ($t = 0$ being the first scan approximately 1 minute after the tissue thawed on the slide). Figure 2 confirms fluorophore migration within the sample, redistributing posteriorly with time including from the epithelium into the underlying stroma. Measured at the beginning of the stroma, this drop in signal is approximately 7% within the first minute. Therefore, all subsequent data were acquired as soon as possible following thawing, that is, after approximately 1 minute.

Depth-Specific Corneal Riboflavin Absorption

No fluorescence was detected by TPF microscopy in the negative (untreated) controls. The maximum riboflavin concentration in positive controls (epithelium-off) was 0.09% (±0.01). Maximum stromal riboflavin concentrations for MedioCross TE, Ricrolin+, Paracel/Xtra, and Ribocross TE were 0.054% (±0.01), 0.031% (0.003), 0.021% (±0.001), and 0.015% (±0.004), respectively. Depth-specific riboflavin concentrations are displayed in Figures 3 through 5. Stromal absorption of riboflavin by iontophoresis was not homogenous, with areas of relative hypo- and hyperfluorescence within each section. In all samples, the epithelial concentration was higher than that in the underlying stroma. A film of higher-signal riboflavin adherent to the epithelium was detected in samples treated with Ribocross TE.

At a depth of 300 μm (within the demarcation zone commonly seen after corneal cross-linking), the stromal concentration in epithelium-off positive controls was 0.075% (±0.006), while at the same depth MedioCross TE and Ricrolin+ achieved 0.018% (±0.006) and 0.016% (0.002), respectively.

None of the remaining transepithelial protocols achieved concentrations above 0.005% at this same 300-μm depth.

Discussion

These data suggest that absorption of riboflavin into the corneal stroma is significantly reduced in current commercial transepithelial CXL protocols in comparison with epithelium-off CXL.

Since most tissue cross-linking is thought to take place in the anterior cornea,[20] determining the availability of key photochemical ingredients (riboflavin, UV light, oxygen) at specific depths may add useful additional information. The method we describe above provides these depth-specific measurements of riboflavin, with the microscope objective and image size determining the resolution available. An additional advantage of our technique is the ability to quantify riboflavin concentration within the epithelium, which, as our results demonstrate, exceeds that in the underlying stroma. In clinical practice, the consequences of loading the corneal epithelium with riboflavin in transepithelial CXL could include epithelial toxicity and UV light shielding. The normal epithelium filters out an average 20% of UVA light radiation passing through the cornea.[20] This UV light attenuation is increased by riboflavin absorption within the epithelium in transepithelial CXL. Enhanced UV absorption with free radical generation within the epithelium could result in "arc eye"-type epithelial toxicity. Epithelial defects are commonly observed on day 1 after transepithelial CXL using currently available protocols,[21] and punctate epitheliopathy is evident in all cases (O'Brart D, oral communication, 2015).

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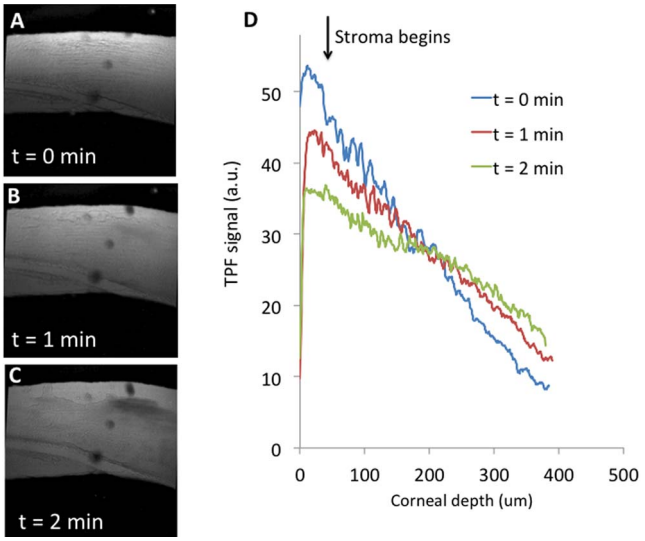

FIGURE 2. Fluorophore migration within the corneal section investigated with serial images of the same tissue location at time = 1, 2, and 3 minutes (**A**–**C**). *Dark circles* are air bubbles within the oil overlying the tissue. All images have been formatted to increase brightness by the same amount to improve view. (**D**) Corresponding TPF signal across samples. Note the loss of the epithelial peak as riboflavin moves into the stroma.

The inability to measure concentration at specific depths in the epithelium and stroma is a significant limitation of HPLC. Furthermore, unless this riboflavin-rich epithelium is specifically removed prior to dissolving the sample, the concentration result will be inappropriately increased. This may have contributed to the observations described by Ostacolo et al.[11] in a study in which Ribocross TE was tested using modified Franz-type diffusion cells with freshly excised porcine corneas (both with and without epithelium). After 40 minutes, riboflavin accumulation through an intact epithelium (0.394 ± 0.02 nmol/mg) matched that achieved epithelium-off (0.396 ± 0.03 nmol/mg). This contrasts with our results showing that Ribocross TE performed poorly compared to other protocols

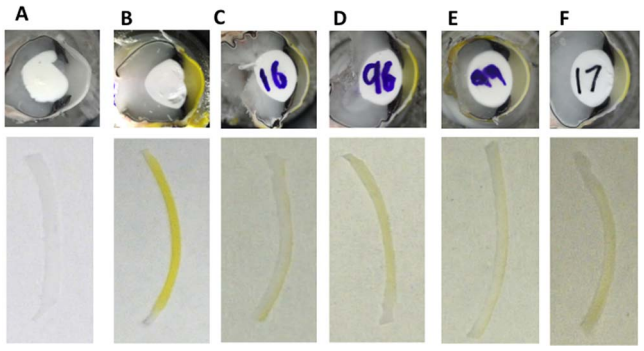

FIGURE 3. Color photographic globe and section examples from each group tested allowing naked eye comparative view of riboflavin absorption through the cornea into the anterior chamber. *Upper row*: sagittal sections of treated globes mounted on a cryostat during section preparation. *Lower row*: 35-μm corneal sections prepared and laid on white paper for photographic contrast (visible shadows indicate section lifting off paper). (**A**) Negative control; (**B**) positive control (epithelium-off) 0.1% riboflavin 30 minutes; (**C**) Ribocross TE 0.125% 30 minutes; (**D**) MedioCross TE 0.25% 30 minutes; (**E**) Paracel 0.25% 4 minutes, VibeX Xtra 0.25% 6 minutes; (**F**) Ricrolin+ 0.1% 1 mA 5-minute iontophoresis.

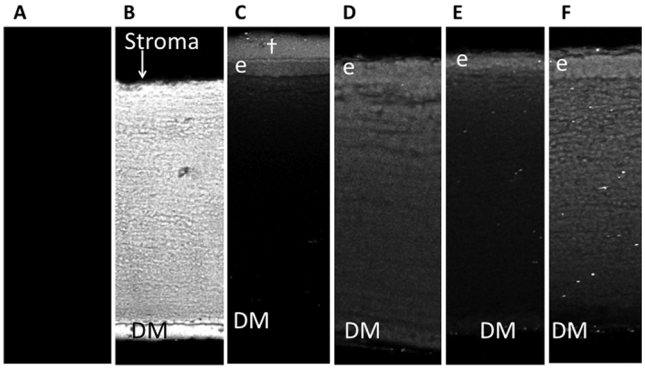

FIGURE 4. Two-photon fluorescence images of tissue sections (grayscale). (**A**) Negative control; (**B**) positive control (epithelium-off) 0.1% riboflavin 30 minutes; (**C**) Ribocross TE 0.125% 30 minutes; (**D**) MedioCross TE 0.25% 30 minutes; (**E**) Paracel 0.25% 4 minutes, VibeX Xtra 0.25% 6 minutes; (**F**) Ricrolin+ 0.1% 1 mA 5-minute iontophoresis. e, epithelium; DM, Descemet's membrane; †Ribocross TE "riboflavin film" on top of epithelium. All images have been formatted to increase brightness by the same amount to improve view. Descemet's membrane scroll artifact visible in some preparations.

tested. As demonstrated in Figures 4 and 5, we observed TFP signals within the epithelium to be higher than in the underlying stroma in all tested protocols. Furthermore, the precorneal film of riboflavin solution exhibited even higher fluorescence signal. Dissolving a full-thickness specimen, including a riboflavin-loaded epithelium and precorneal film, will lead to overestimation of corneal stromal riboflavin concentrations by HPLC. This may explain why Ostacolo et al.[11] recorded only a 4-fold reduction in stromal concentration when their control riboflavin solution (i.e., no vitamin E) was applied to an intact epithelium, compared with epithelium-off application (0.098 ± 0.04 nmol/mg epithelium-on; 0.396 ± 0.03 nmol/mg epithelium-off). Cassagne et al.[12] also used HPLC in an in vivo rabbit eye study of iontophoresis, testing both the dissolved cornea and aqueous sample. Again, the investigators did not remove the epithelium prior to analysis. This may, in part, explain the discrepancy in their results showing 45% less riboflavin in corneas treated using iontophoresis (compared to epithelium-off controls), but 95% less riboflavin in the aqueous of iontophoresis-treated eyes.

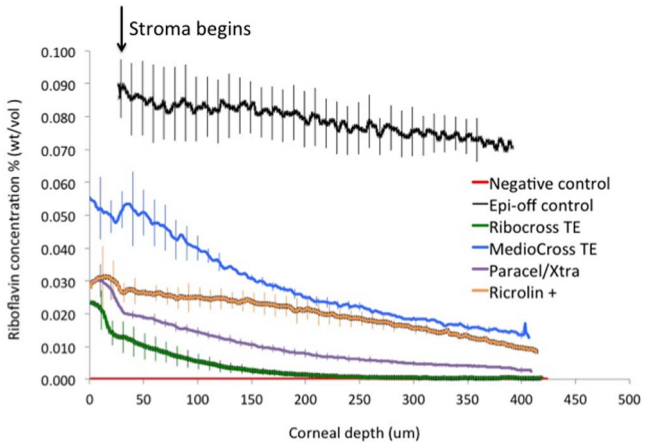

FIGURE 5. Mean concentrations (standard deviation *error bars*) of riboflavin achieved using different transepithelial protocols, compared with epithelium-off absorption ($n = 5$ for each protocol). *Shaded box* denotes epithelium.

There are a number of limitations to this study. Firstly, results in ex vivo rabbit corneas, although a better anatomical match to human corneal epithelial thickness than porcine eyes, may be affected by postmortem changes in epithelial layer integrity. Measures taken to minimize this compromise included a short postmortem interval, immersion in chilled PBS immediately post mortem, and warming to physiological temperature prior to preparation of corneas for experimentation. We observed no obvious stromal swelling (indicative of endothelial pump failure) in negative controls, with similar mean corneal thicknesses to those published for rabbit corneas in vivo (400 μm).[22] Despite this, some epithelial degradation and enhanced permeability are likely, and our results may overestimate stromal riboflavin absorption in clinical transepithelial CXL. Secondly, migration of riboflavin within the snap-frozen tissue would have started as soon as the section thawed on the slide. Even with the cryostat next to the microscope, there was still a delay of up to a minute before image acquisition. This would tend to result in an underestimation of stromal riboflavin concentrations. Finally, imaging through two media of different refractive indices (oil and water either side of the coverslip) may have increased optical aberrations as the laser light passed through. We chose oil to ensure that no (water soluble) riboflavin leaked out of the tissue. When we tried to embed excised corneas in optimal cutting temperature (OCT) compound we observed very prompt dye leakage once thawed on the slide (data not shown). This may have impacted the study of Cassagne et al.[12] For lack of an appropriate oil-immersion objective, we placed a coverslip on top and used a water-immersion objective. Any induced aberrations may have resulted in a small absolute loss of signal, but since this same method was employed for all imaged samples, no relative drop between samples should have been present.

Notwithstanding the above laboratory studies, results from preliminary clinical case series using noniontophoretic transepithelial riboflavin preparations have been equivocal, with some showing similar efficacy to epithelium-off CXL,[23,24] while most have demonstrated less pronounced effects.[6,25–28] Twelve-month results from one recently published randomized controlled trial[6] concluded that transepithelial CXL using Ricrolin TE (Sooft Italia S.p.A.) was not effective at stabilizing corneal shape compared with epithelium-off treatment. For iontophoresis, up to 15-month follow-up data have been published, with reported cessation of disease progression and improvements in keratometric and visual parameters.[7–9] The longer-term relative efficacy of all these transepithelial techniques compared to epithelium-off CXL remains unknown.

In conclusion, the methodology described above provides a quantitative means of measuring riboflavin across the whole cornea in an ex vivo model. We present evidence that the corneal epithelium represents a significant barrier to riboflavin absorption into the stroma. Existing commercial transepithelial CXL protocols load the corneal epithelium with riboflavin and achieve relatively low riboflavin concentrations in the anterior corneal stroma when compared to epithelium-off CXL. Although a key rate-limiting step, the absolute stromal concentration of riboflavin required for effective CXL is not known. Without further evidence, the concentration achieved "epithelium-off" remains the gold standard. The differences in transepithelial absorption demonstrated above may yield different degrees of tissue cross-linking and are, therefore, likely to be clinically significant. The method described above may prove useful in testing candidate protocols prior to future clinical trials.

Acknowledgments

Daniel M. Gore thanks Christopher Thrasivoulou, PhD, imaging team manager at the University College London confocal unit, for microscopy technical support and Grace Lytle, Avedro, Inc., for provision of riboflavin solution.

Supported in part by the Department of Health's National Institute for Health Research (NIHR) Biomedical Research Centre for Ophthalmology at Moorfields Eye Hospital and University College London (UCL) Institute of Ophthalmology; Fight for Sight (1348/9; DMG); the Rosetrees Trust (JS16/M282; DMG); the Ian Collins Rayner Fellowship (Rayner Intraocular Lenses Ltd., Hove, UK, and United Kingdom and Ireland Society of Cataract and Refractive Surgeons [UKISCRS]); DMG); and the Special Trustees of Moorfields Eye Hospital (ST1415A; DMG). The views expressed in the publication are those of the authors and not necessarily those of the Department of Health.

Disclosure: **D.M. Gore**, Avedro, Inc. (F), **D. O'Brart**, None; **P. French**, None; **C. Dunsby**, None; **B.D. Allan**, None

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