A Comparison of Different Corneal Iontophoresis Protocols for Promoting Transepithelial Riboflavin Penetration

Daniel M. Gore,[1] David P. O'Brart,[2] Paul French,[3] Chris Dunsby,[3,4] and Bruce D. Allan[1]

[1]Moorfields Eye Hospital, London, United Kingdom
[2]Keratoconus Research Institute, Department of Ophthalmology, St. Thomas' Hospital, London, United Kingdom
[3]Department of Physics, Imperial College London, South Kensington, London, United Kingdom
[4]Centre for Histopathology, Imperial College London, London, United Kingdom

Correspondence: Dan Gore, Moorfields Eye Hospital, 162 City Road, London EC1V 2PD, UK; dan.gore@moorfields.nhs.uk.

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Purpose. To measure corneal riboflavin penetration using different transepithelial iontophoresis protocols.

Methods. Freshly enucleated rabbit eyes were divided into nine treatment groups of 4 eyes. One group, in which 0.1% wt/vol riboflavin was applied for 30 minutes without iontophoresis after corneal epithelial debridement, acted as a control. The remaining groups were treated with an intact epithelium using different riboflavin formulations and varying iontophoresis current, soak, and rinse times. After riboflavin application, eyes were snap frozen in liquid nitrogen. Corneal cross sections 35 μm thick were then imaged immediately by two-photon fluorescence microscopy, using image processing software to quantify stromal riboflavin concentration at different corneal depths.

Results. In the epithelium-on iontophoresis treatment groups, greater stromal riboflavin penetration was achieved with higher-concentration riboflavin solutions, greater iontophoresis dosage, and longer solution contact times. A protocol utilizing 0.25% wt/vol riboflavin with benzalkonium chloride (BAC) 0.01% and two cycles of applied current and subsequent soaking (1 mA 5 minutes, soak 5 minutes; 0.5 mA 5 minutes, soak 5 minutes) achieved similar stromal riboflavin penetration to epithelium-off controls. The best-performing non-BAC-containing protocol produced stromal riboflavin penetration approximately 60% that of epithelium-off controls. Riboflavin solutions containing saline resulted in minimal stromal penetration. Riboflavin loading within the epithelium was equivalent to or higher than that in the subjacent stroma, despite rinsing the ocular surface with balanced salt solution.

Conclusions. Modified iontophoresis protocols can significantly improve transepithelial riboflavin penetration in experimental corneal collagen cross-linking.

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

Riboflavin/ultraviolet A corneal collagen cross-linking (CXL) is the first intervention shown to halt disease progression in keratoconus.[1–4] Riboflavin acts as a photosensitizer for the production of free radicals that drive the cross-linking process.[5] While riboflavin is hydrophilic with a molecular weight of 340 Daltons (Da), the corneal epithelium is lipophilic and has a reduced permeability to molecules larger than 180 Da.[6,7] Thus standard CXL requires debridement of the central epithelium to facilitate stromal riboflavin penetration and achieve adequate efficacy.[7] Epithelial debridement, however, is associated with postoperative pain and delayed visual recovery and increases the risks of infection, corneal melt, and scarring.[8] As a result, CXL is normally reserved for patients with documented disease progression.

Transepithelial (epithelium-on) CXL aims to avoid the pain, delayed recovery, and complications of epithelial removal inherent in epithelium-off CXL. The original dextran-containing solutions have been shown in both laboratory and clinical studies to be ineffective for transepithelial cross-linking.[9–12] A number of formulations of riboflavin designed to facilitate penetration across an intact corneal epithelium are currently marketed.[13] Most contain toxic agents to increase epithelial permeability, including benzalkonium chloride (BAC), high-concentration sodium chloride, sodium ethylenediaminetetraacetic acid (EDTA), or trometamol. Despite these chemical enhancers, results in clinical studies with transepithelial riboflavin preparations have been equivocal: Some studies report similar efficacy to epithelium-off CXL,[14,15] but most report inferior results.[10–12,16–18]

Iontophoresis, in which an electrical gradient is used to drive negatively charged riboflavin molecules across the intact epithelium, may further enhance riboflavin penetration in transepithelial CXL. Laboratory studies of iontophoresis have been encouraging, demonstrating enhanced transepithelial riboflavin penetration and improvement of corneal biomechanics.[19–23] Initial clinical studies report improvements in keratometric and visual parameters.[24–26]

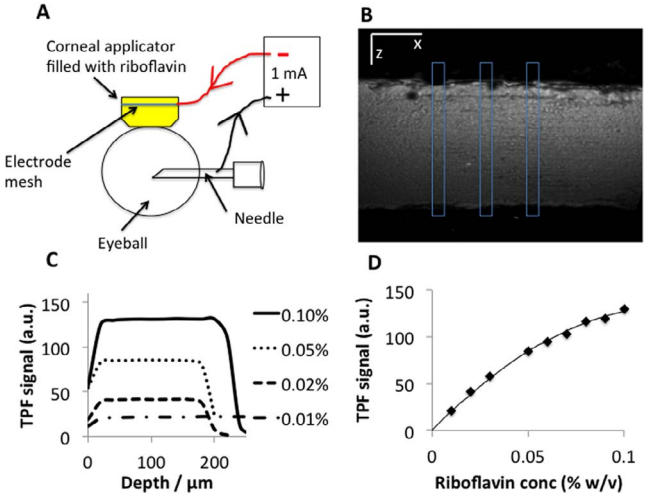

Figure 1. (**A**) Iontophoresis riboflavin delivery system modified for use in ex vivo eyes. The system comprised two electrodes: a negatively charged metal grid housed within the corneal applicator and a positively charged 20-gauge needle inserted through the sclera into the vitreous. (**B**) Grayscale two-photon fluorescence image analyzed in ImageJ with three box plots (40 pixels wide) from which to average the signal in each section. The edges of the images were not analyzed to avoid areas of vignetting. (**C**) TPF signals achieved with depth through diluted riboflavin solutions in a well slide. (**D**) Calibration curve used to calculate corneal riboflavin concentration from TPF signal.

We have previously investigated transepithelial stromal riboflavin penetration of commercially available protocols in an ex vivo rabbit eye model using two-photon fluorescence (TPF) microscopy, and have found that none are able to match the stromal riboflavin concentrations achieved epithelium-off.[13] Here, using the same method, we investigated whether modified iontophoresis protocols designed by one of the authors (DPO) can enhance riboflavin penetration across an intact epithelium.

Methods

Ethical approval for corneal TPF 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) to minimize epithelial cell layer degeneration following death. 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 PBS before the iontophoresis system was set up.

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 (Fig. 1A). The corneal applicator was vacuum-attached to the cornea. Once set up, intraocular pressure was assessed manually by touch to ensure that globe tension remained grossly normal. The reservoir was filled with differing riboflavin formulations (Table 1) to above the level of the metal grid before beginning the iontophoresis treatment. Protocols varied by either iontophoresis dosage, soak time, or riboflavin formulation, and full parameters are given in Table 2. Four globes were studied for each protocol except for protocol F, where $n = 5$. After the iontophoretic procedure, the applicator was removed and the corneas were rinsed with PBS both to remove any riboflavin on the corneal surface and to try to reduce riboflavin concentration within the epithelium. Corneas soaked for 30 minutes with 0.1% wt/vol riboflavin in hydroxypropyl methylcellulose (HPMC) and saline (Vibex Rapid; Avedro, Inc., Waltham, MA, USA) after epithelial debridement served as controls.

Section Preparation and Imaging Protocol

We have previously described the use of TPF microscopy for imaging transepithelial riboflavin penetration.[13,27] Briefly, following iontophoresis, the globes were immediately immersed in liquid nitrogen. Corneal cross sections (35 μm) were cut on a cryostat 1 mm apart (including the central meridian) and 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. For lack of an appropriate oil-immersion objective, we placed a coverslip on top prior to imaging under a Leica (Wetzlar, Germany) 10×/0.3 NA (numerical aperture) water immersion objective. The time taken from the tissue thawing on the slide to image acquisition on the microscope was approximately 1 minute. This interval was kept as short as possible to minimize migration of fluorescein within the thawed tissue. A mode-locked Ti:Sapphire laser (Coherent, Ely, UK), operating with a 140-fs pulse duration and 80-MHz pulse repetition rate, was used as the excitation laser source. 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.[28] Emitted riboflavin fluorescence was collected in the backward (epi) configuration onto a descanned Leica HyD detector with the pinhole opened to its maximum setting. To avoid overlap with the absorption spectrum of riboflavin, fluorescence was detected in the range between 525 and 650 nm. (We previously investigated[13] the presence of endogenous fluorescence within this spectral band by imaging negative controls without riboflavin and detected negligible TPF signal).

Table 1. Riboflavin Formulation

Brand Name (Manufacturer)	Composition
Vibex Rapid (Avedro, Inc., Waltham, MA, USA)	0.1 % wt/vol riboflavin 5′-monophosphate, saline, HPMC
Medio-Cross TE (Peschke Meditrade GmbH, Waldshut-Tiengen, Germany)	0.25 % wt/vol riboflavin 5′-monophosphate, HPMC, 0.01% benzalkonium chloride, disodium hydrogenphosphate, sodium dihydrogenphosphate, water
*Ricrolin + 0.25% (Sooft Italia S.p.A., Montegiorgio, Italy)	0.25 % wt/vol riboflavin 5′-monophosphate, sodium edetate, trometamol, sodium dihydrogenphosphate dihydrate, sodium phosphate dibasic dehydrate
Ricrolin + 0.1% (Sooft Italia S.p.A.)	0.1 % wt/vol riboflavin 5′-monophosphate, sodium edetate, trometamol, sodium dihydrogenphosphate dihydrate, sodium phosphate dibasic dehydrate

* Noncommercial formulation.

Table 2. Treatment Protocols

Protocol	Solution	Current Dose I	Soak I	Current Dose II	Soak II	Rinse
Control	Vibex Rapid	–	30 min	–	–	–
A	MedioCross TE	1 mA 5 min	5 min	0.5 mA 5 min	5 min	5 min*
B	MedioCross TE	1 mA 5 min	15 min	–	–	1 min
C	Ricrolin + 0.25%	1 mA 5 min	5 min	0.5 mA 5 min	5 min	5 min*
D	Ricrolin + 0.25%	1 mA 10 min	–	–	–	5 min
E	MedioCross TE	1 mA 5 min	–	–	–	1 min
F	Ricrolin + 0.1%	1 mA 5 min	–	–	–	1 min†
G	Vibex Rapid	1 mA 5 min	–	–	–	1 min
H	Vibex Rapid	1 mA 5 min	5 min	0.5 mA 5 min	5 min	5 min

$n = 4$ for all protocols except F ($n = 5$).
* St. Thomas'/Cardiff Iontophoresis protocol.
† Previously published.[13]

Image Analysis and Concentration Calibration

Grayscale images 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 40-pixel-wide rectangular regions of interest were manually selected (Fig. 1B). The intensity profiles with depth for each region of interest were then exported to a .txt file. The epithelial/stromal junction was then identified in each trace by the abrupt change in signal and confirmed with reference to the corresponding image of the region of interest. This information was used to align all three plots, and a mean intensity plot was generated representing the average TPF signal for that image as a function of depth.

In order to convert measured TFP signal into riboflavin concentration, we performed a calibration measurement. Each point on the calibration curve was produced by placing a known concentration of riboflavin solution on a well slide. The riboflavin solution was then covered with a layer of immersion oil and a coverslip in order to exactly replicate the measurement conditions used for imaging tissue. Image z-stacks were then acquired throughout the full depth of the well slide using the same Leica 10×/0.3 NA water immersion objective as used for the tissue imaging. The resulting TPF signals are plotted as a function of depth in Figure 1C for selected riboflavin concentrations. As expected, the TPF signal is constant with depth. The average raw TPF signal measured at 60 μm below the riboflavin surface was then plotted against riboflavin concentration (Fig. 1D). A quadratic equation was then fitted to the experimental data, and the resulting curve was used to convert the TPF signal measured in corneal sections into riboflavin concentration. The TPF signal from a well slide reservoir of 0.1% wt/vol riboflavin solution was acquired for every measurement session, and the resulting data were used to correct for drifts caused by variations in laser output power.

Mean (SD) concentrations were calculated from four globes tested for each protocol. Unpaired Student's *t*-tests were used to compare riboflavin concentrations achieved at a depth of 300 μm. A P value less than 0.05 was considered significant. Analyses were performed in Excel for Mac 2011 (Microsoft Corp., Redmond, WA, USA).

Results

Peak riboflavin concentration of 0.094% (±0.002) wt/vol was recorded in epithelium-off controls. At a depth of 300 μm corresponding to the demarcation line commonly observed after corneal cross-linking, the riboflavin concentration was 0.082% (±0.005) wt/vol. Stromal riboflavin concentrations achieved through an intact epithelium with different iontophoresis protocols, along with corresponding color photograph and fluorescence microscopy images, are shown in Figures 2 through 4 and summarized in Table 3. Higher concentrations were achieved with longer iontophoretic dosage and longer solution contact (diffusion) time with the cornea. The best protocol (A), utilizing 0.25% wt/vol riboflavin with BAC and two separate iontophoretic treatments followed

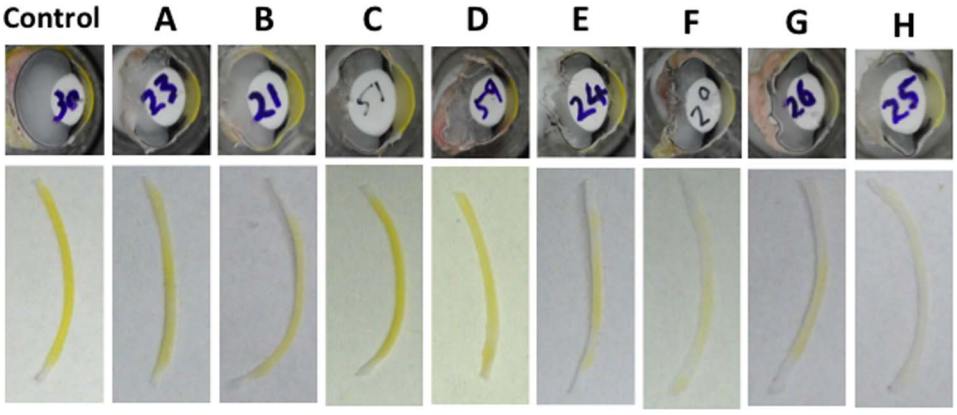

Figure 2. Color photographic globe and section examples from each group tested allowing macroscopic comparative view of riboflavin penetration through the cornea into the anterior chamber. *Upper row*: Sagittal sections of treated globes mounted on a cryostat during section preparation. *Lower row*: 35-μm corneal sections prepared and laid on white paper for photographic contrast (visible shadows indicate section lifting off paper). Protocols A through H as in Table 2.

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Table 3. Corneal Riboflavin Concentrations

Protocol	Riboflavin Concentration ± SD % Wt/Vol			*P* Value*
	Epithelium	Peak Stroma	300 μm	
Control	–	0.094 ± 0.002	0.082 ± 0.005	–
A	0.054 ± 0.004	0.078 ± 0.008	0.075 ± 0.007	0.63
B	0.042 ± 0.006	0.054 ± 0.005	0.053 ± 0.005	0.016
C	0.050 ± 0.008	0.057 ± 0.008	0.049 ± 0.008	0.001
D	0.035 ± 0.004	0.039 ± 0.007	0.026 ± 0.003	<0.001
E	0.029 ± 0.003	0.029 ± 0.002	0.016 ± 0.003	<0.001
F	0.021 ± 0.002	0.018 ± 0.003	0.010 ± 0.001	<0.001
G	0.005	0.008	0.007	<0.001
H	0.005	0.006	0.005	<0.001

* Unpaired Student's *t*-test comparing riboflavin concentration versus control at a depth of 300 μm (depth chosen to correlate with demarcation zone commonly seen after corneal cross-linking).

by soak periods of 5 minutes to allow diffusion of riboflavin from the subepithelial tissues deeper into the stroma (the "St. Thomas'/Cardiff Iontophoresis protocol," total time 20 minutes), was not significantly different with respect to stromal riboflavin concentrations compared with epithelium-off controls (0.082% [±0.005] wt/vol versus 0.075% [±0.007] wt/vol at a depth of 300 μm, $P = 0.63$, Table 3). The best-performing protocol (C), not containing BAC but with 0.25% wt/vol riboflavin and the same iontophoresis treatments and soak times as protocol A, achieved a mean stromal concentration of 0.049% (±0.008) wt/vol at 300 μm (Table 3).

Near-uniform stromal riboflavin penetration with depth was observed in longer-duration protocols (A–C), as compared with shorter treatments in which the concentration fell by up to 40% within the anterior 300 μm (protocols D–F). Riboflavin solutions containing saline (protocols G, H) resulted in minimal stromal penetration (maximum 0.01%). Of note, corneal thickness was observed to increase by approximately 50 μm following these saline-based iontophoretic applications.

The concentration of riboflavin within the epithelium was equivalent to or higher than that in the immediate underlying stroma. Extended rinsing of the surface effectively washed away any precorneal riboflavin film but did not selectively reduce the fluorophore load within the epithelium itself. A 5-minute corneal rinse after protocols A and B (MedioCross TE; Peschke Meditrade GmbH, Waldshut-Tiengen, Germany) resulted in a washout effect of both the epithelium and anterior stroma up to an approximate depth of 200 μm.

Discussion

In this study we have demonstrated that corneal stromal riboflavin penetration in iontophoretic transepithelial CXL can be improved by increasing soak time, solution concentration, and iontophoretic dosage.

The corneal epithelium has been previously shown to be a significant barrier to riboflavin penetration into the stroma.[9,29,30] This has been confirmed by a series of experiments using spectrophotometry as an indirect measure of stromal riboflavin concentration. Using our TPF methodology, which can directly quantify riboflavin concentration through the entire depth of the cornea,[13,28,31] we previously confirmed these findings, demonstrating very limited riboflavin diffusion through an intact epithelium with any of the several current commercially available transepithelial riboflavin formulations and protocols.

Riboflavin is water soluble and negatively charged at physiological pH, and laboratory studies suggest that iontophoresis can be applied effectively to enhance riboflavin penetration in transepithelial CXL. Several groups have reported similar increases in corneal biomechanics[19,22,23] in animal models as compared with epithelium-off CXL. Preliminary clinical studies have also been encouraging, with reported cessation of disease progression with up to 15 months follow-up and improvements in keratometric and visual parameters.[24–26] However, the relative efficacy of this technique compared to epithelium-off CXL remains to be determined especially over longer-term follow-up, and current randomized prospective studies comparing the two techniques (clinicaltrials.gov.uk NCT02117999, NCT01868620, ISRCT 04451470) have yet to be reported.

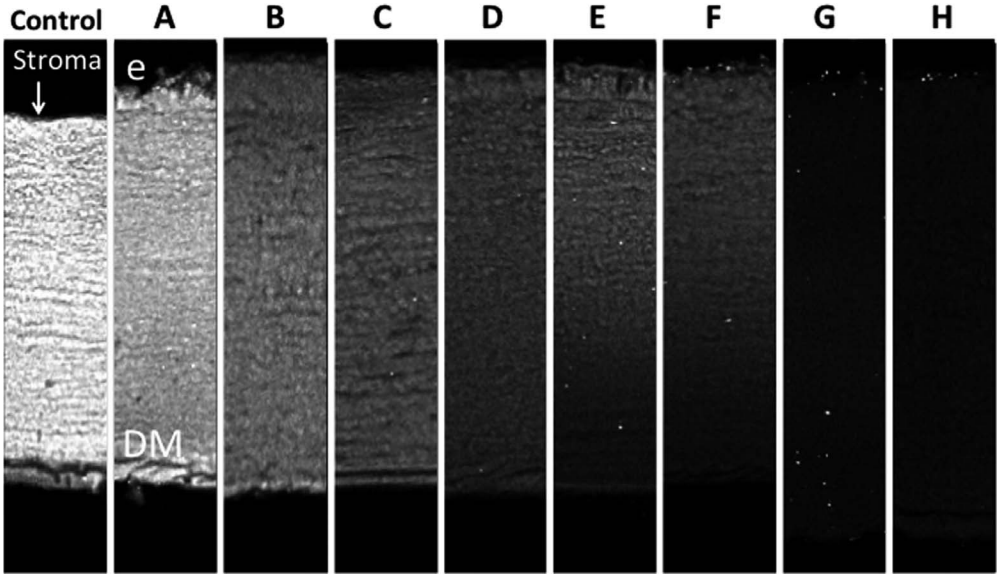

Figure 3. Two-photon fluorescence images of tissue sections (grayscale). Protocols A through H as in Table 2. e, epithelium; DM, Descemet's membrane. All images have been formatted to increase brightness by the same amount to improve view. DM scroll artifact visible in some preparations. Apparent epithelial damage with BAC-containing protocol A should not be inferred as a treatment effect; our TPF methodology was not designed to qualify epithelial structural changes, and a processing-induced artifact cannot ruled out.

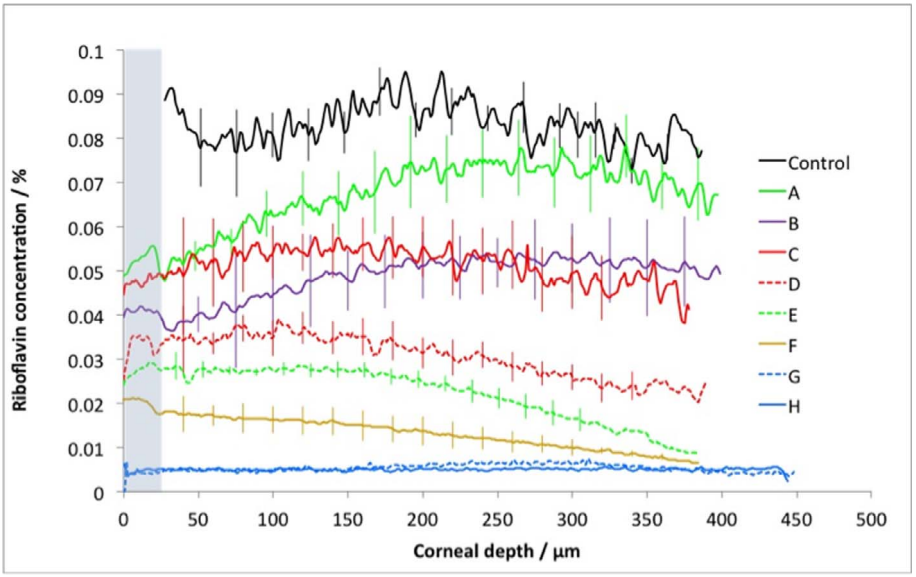

FIGURE 4. Mean concentrations (standard deviation error bars) of riboflavin achieved using different transepithelial protocols, compared with epithelium-off penetration. *Shaded area* denotes epithelium. Protocols A through H as in Table 2. Note the increased concentrations achieved at 300 um with protocols A and C compared to B and D due to the second iontophoresis/soak cycle.

Existing recommendations for iontophoresis in transepithelial CXL utilize 1 mA for 5 minutes with a 0.1% wt/vol riboflavin solution. Improved riboflavin penetration can be obtained by modifying these parameters. Stromal riboflavin concentrations using protocol A here were similar to epithelium-off controls ($P = 0.63$) (Fig. 4; Table 3). This protocol utilized a solution of 0.25% wt/vol riboflavin with BAC and the St. Thomas'/Cardiff Iontophoresis protocol. This protocol was derived in collaborative pilot experimentation at St Thomas' Hospital and Cardiff University using spectrophotometry, and comprises two cycles of iontophoresis each followed by a 5-minute soak period to allow time for riboflavin to diffuse more posteriorly. The use of the cationic surfactant BAC has been shown with percutaneous iontophoresis to have a synergistic effect on the transport of anions.[22] We similarly observed this synergistic effect with higher stromal concentrations achieved with protocol A (0.25% wt/vol riboflavin, BAC) compared to protocol C (0.25% wt/vol riboflavin, no BAC) (Fig. 4; Table 3). A further variable in iontophoresis relates to the zeta potential and electrophoretic mobility of different riboflavin solutions that may, in part, explain our results. Although beyond the scope of this study, this warrants further investigation in the future.

A key advantage of our TPF imaging method, as compared to whole-tissue analysis (high-performance liquid chromatography, HPLC;[19,32–34] spectrophotometry[9,29,30]), is the ability to quantify riboflavin concentration within the epithelium. We have previously demonstrated in transepithelial protocols (without rinsing the cornea) that epithelial riboflavin concentration often exceeds that in the underlying stroma.[13] Analyzing a full-thickness specimen, including a riboflavin-loaded epithelium and precorneal film, will lead to significant overestimation of corneal stromal riboflavin concentrations.[13] Clinically, riboflavin loading in the epithelium may reduce CXL efficacy by attenuating ultraviolet light transmission to the stroma. It may also result in an "arc-eye" type of reaction, particularly where riboflavin preparations including BAC are used, accounting for the epithelial defects seen in up to two-thirds of patients after transepithelial CXL using MedioCross TE.[35] The ideal transepithelial protocol would load the stroma with riboflavin while leaving the epithelium relatively clear. To this end, in this study we rinsed the surface of the globe after iontophoresis in a bid to wash out some of the riboflavin from within the epithelium. Figures 3 and 4 and Table 3 show that this approach did not work, since there was no significant difference in fluorescence between the epithelium and the subjacent stroma. Extended rinsing (5 minutes) of the ocular surface in protocols A and B (MedioCross TE) also reduced corneal stromal concentrations of riboflavin up to an approximate depth of 200 µm. Stromal riboflavin elution was less evident with non-BAC-containing solutions (protocols C and D), where the epithelial tight junctions were still probably largely intact. Although iontophoresis is not commercially promoted for use with BAC-containing solutions, this stromal washout effect with prolonged rinsing of the ocular surface may compromise tissue cross-linking within the anterior cornea.

It is important to note that iontophoresis was ineffective with riboflavin formulations containing saline (protocols G and H) that produced minimal riboflavin penetration and some stromal swelling.

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. Despite steps to minimize this as described in the Methods section, 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, resulting 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 passes through. 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 change in signal between samples should be present. Given these limitations, we are unable to guarantee an absolute concentration from these TPF data alone.

In conclusion, this study confirms that transepithelial riboflavin penetration can be improved by increasing soak time, riboflavin concentration in the soak preparation, and iontophoretic dosage. While the optimum tissue concentration for effective CXL is unknown, protocol C (0.25% wt/vol riboflavin [BAC-free], St. Thomas'/Cardiff Iontophoresis protocol) achieves more than 50% greater stromal penetration compared to the standard iontophoresis protocol, as well as far higher concentrations than we have seen with non-iontophoresis transepithelial protocols.[24] Although less than that achieved epithelium-off, its considerable stromal riboflavin penetration, without relying on epithelial-toxic additives, may represent the best transepithelial technique to date. Clinical trials will determine whether this level of stromal riboflavin penetration produces effective CXL.

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