Evaluating In Vivo Delivery of Riboflavin With Coulomb-Controlled Iontophoresis for Corneal Collagen Cross-Linking: A Pilot Study

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Purpose. To evaluate the efficacy of coulomb-controlled iontophoresis (CCI) for delivery of riboflavin prior to corneal collagen cross-linking (CXL).

Methods. The eyes of 20 8-week-old Lewis rats, subject to epithelium-ON (epi-ON, $n = 20$ eyes) or epithelium-OFF (epi-OFF, $n = 20$ eyes) conditions, were used to evaluate the in vivo delivery of two riboflavin solutions: 0.1% riboflavin-20% dextran T500 solution (riboflavin-dextran) and 0.1% riboflavin 5′-phosphate (riboflavin-phosphate). After systemic intramuscular anesthesia, 0.25 mL of the photosensitizing agent was delivered by either instillation or CCI (2.11 mA/cm^2 for 4 or 10 minutes) into either epithelial condition. The CCI probe on the eye without current served as control. Confocal microscopy of flat-mounted corneas was used to analyze intracorneal penetration and fluorometry was used to quantify riboflavin in the aqueous within 30 minutes of treatment.

Results. Instillation and CCI allowed for uniform delivery of riboflavin-dextran throughout the stroma after epithelial debridement. Transepithelial delivery of riboflavin-dextran was not efficacious. Riboflavin-phosphate was successfully delivered in both epithelium conditions. Complete saturation of the cornea was achieved using CCI after removing the epithelium, the epi-ON case allowed for limited diffusion. Increasing the time from 4 to 10 minutes greatly increased the amount of riboflavin detected in the cornea and aqueous humor.

Conclusions. Coulomb-controlled iontophoresis is an effective technique for transepithelial delivery of riboflavin-phosphate into the cornea. This drug delivery method would allow clinicians to significantly shorten the time required for the CXL procedure, with or without epithelial debridement. Whether efficient crosslinking can be achieved through an intact epithelium remains to be demonstrated.

Keywords: iontophoresis, drug delivery, keratoconus, riboflavin, collagen cross-linking

Keratoconus is a disease characterized by the progressive thinning of the cornea. The cornea develops a cone-like shape,[1] which is primarily the result of reduced interweaving of the collagen fibrils in the stroma.[2] Keratoconus leads to impaired visual acuity, ocular irritation, and increased sensitivity to light. Hard contact lenses can be worn to stabilize the corneal protrusion and to correct the blurry vision; however, in the most severe cases, 15% to 20% of cases, a corneal graft is required.[3] Collagen cross-linking (CXL) is known as a therapeutic modality for stabilization of progressive keratoconus. Collagen cross-linking was introduced by Wollensak et al.[4] in 2003 and was quickly implemented in the clinic. Studies have shown that after the CXL treatment the rigidity of porcine and rabbit corneas increases by approximately 70% and positive clinical results were also achieved in keratoconic patients.[4,5] Collagen cross-linking treatment entails the debridement of the epithelium, the introduction of riboflavin (a photosensitizing agent) into the stroma via instillation (1 drop every 3–5 minutes for 30 minutes), and the irradiation of the cornea for 30 minutes with UV-A (370 nm, 3 mW/cm^2) to induce the strengthening of the stromal collagen.

Although CXL is an effective treatment modality, certain questions such as the treatment time and steps taken are still debated. Much focus has been placed on the effect of UV-A

irradiation on the corneal endothelium.[6,7] Another area of concern is the removal of the epithelial layer in order for the riboflavin to penetrate into the corneal stroma effectively. The scraping and subsequent regeneration of the epithelial layer is painful,[8] and can sometimes cause vision problems.[9] Recently, transepithelial CXL was developed, but studies have shown this modality may not be as effective as the standard treatment.[8,10] A final limitation of CXL is the lengthy treatment time, as drug delivery takes approximately 30 minutes and irradiation takes another 30 minutes, decreasing the procedure time would greatly benefit both patients and clinicians.

Coulomb-controlled iontophoresis (CCI) is a drug delivery modality, which uses an electric field to increase drug penetration.[11] Previous studies in animals and humans have shown CCI to be a safe procedure that can effectively deliver many types of molecules including anti-inflammatory agents,[12,13] antifungal,[14] and other drugs[15–18] through the cornea and into other ocular tissues.[11] Preliminary studies have recently demonstrated the feasibility of applying CCI to deliver a riboflavin-dextran solution for CXL ex vivo in pig corneas and human grafts (Dias J, et al. *IOVS* 2012;53:ARVO E-Abstract 1523) and in vivo in rabbits (Ziebarth N, et al. *IOVS* 2011;52:ARVO E-Abstract 2544). These studies show that compared with the standard treatment, CCI delivery doubled the amount of riboflavin in the corneal stroma, both epithelium-ON (epi-ON) and epithelium-OFF (epi-OFF) cases, demonstrating that riboflavin is a good candidate for iontophoretic delivery.

This pilot study aims to continue this work in order to further evaluate this alternative technique of administration of riboflavin in vivo into the stroma prior to collagen CXL. The efficacy of CCI was evaluated in rat corneas. As the thickness of the rat's cornea with epithelium is only approximately 160 μm compared with 540 μm for a human, the duration of instillation and CCI treatments were reduced accordingly. A custom applicator was used to deliver two formulations of riboflavin, in epi-ON and epi-OFF conditions. Confocal fluorescent microscopy and fluorometry were used to evaluate the amount of the photosensitizing agent that diffused into the cornea and ocular media.

Materials and Methods

Investigations were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study was approved by the ethics committee in Animal Experiment Charles Darwin of Ile-de-France region (approval Ce5/2012/030). Lewis rats (6–8 weeks of age) were purchased from Janvier (Le Gesnet St. Isles, France) and quarantined in our vivarium for 1 week prior to experimentation.

Experimental Plan

Before treatment, the animals were anesthetized using an intramuscular anesthetic consisting of a Ketamine (Clorketam 1000; Virbac, Carros, France)/Xylazine (Rompun 2%; Bayer Healthcare, Lyon, France) mixture solution (Ketamine 87 mg/kg/Xylazine 13 mg/kg). Prior to epithelial debridement topical anesthesia was instilled (Tetracaine 1%, Novartis Pharma, Rueil-Malmaison, France).

The study consisted of two experiments conducted in 20 rats. In each animal, the epithelium of the right (OD) cornea was removed (epi-OFF cornea) and the left (OS) cornea was kept intact (epi-ON cornea).

The first part of the study aimed to compare two formulations of riboflavin, the standard 0.125% riboflavin-20% dextran T500 solution (riboflavin-dextran) used for the Dresden protocol (0.125% riboflavin phosphate sodium dihydrate salt, 0.95% dibasic sodium phosphate dihydrate, 0.22% monobasic sodium phosphate dihydrate, 0.125% sodium chloride, 20% dextran T500, and distilled water Qb) and a 0.1% riboflavin 5′-phosphate (riboflavin-phosphate, R7774; Sigma-Aldrich, Saint-Quentin Fallavier, France) solution.

Eight animals were divided in four groups and treated with the following: (1) riboflavin-dextran or riboflavin-phosphate instillation, (2) riboflavin-dextran CCI (2.11 mA/cm^2 for 5 minutes), (3) riboflavin-phosphate CCI (2.11 mA/cm^2 for 4 minutes), and (4) riboflavin-phosphate CCI probe (no charge for 4 minutes).

The second part of the study aimed to evaluate the effect of increased duration of CCI for the selected riboflavin formulation. Twelve animals were divided into three groups treated with the following: (5) riboflavin-phosphate CCI probe (no charge for 4 minutes), (6) riboflavin-phosphate CCI (2.11 mA/cm^2 for 4 minutes), and (7) riboflavin-phosphate CCI (2.11 mA/cm^2 for 10 minutes).

Within 30 minutes of treatment, the animals were euthanized using an overdose of sodium pentobarbital (Sanofi-Aventis, Paris, France), and the eyes were enucleated. For both experiments, the corneas were flat-mounted on microscope slides without fixation for analysis using confocal microscopy. The ocular media of each eye in the second experiment were subjected to fluorometric analysis.

Epithelial Debridement

Removal of the epithelial layer was performed as described by Hattori et al.[19] A biopsy punch was used to make a 4-mm diameter circular incision at the center of the cornea. A 4-mm diameter filter paper, soaked with 70% ethanol, was applied on the incised area for 5 seconds. After a gentle wash with 5 mL of saline, the epithelium was removed with micro-sponges (Alcon Inc., Schaffhausen, Switzerland).

Delivery of the Photosensitizing Agents

Two riboflavin solutions were used: the standard riboflavin-dextran, used for the Dresden protocol and the riboflavin-phosphate (Sigma-Aldrich) solution. Both solutions were prepared with pH of 5.9 and kept away from the light. The photosensitizing agents were delivered to the rat corneas either by instillation, as per the Dresden protocol,[4] or by CCI. For delivery via instillation, a micropipette was used to instill 30 μL of each riboflavin solution every 5 minutes for 30 minutes resulting in a total volume approximately 0.25 mL of the solution delivered on the cornea. In the other case, CCI was performed using an iontophoresis generator and a transcorneal applicator.

Coulomb-Controlled Iontophoresis (CCI)

The CCI system is comprised of a generator and an iontophoresis probe. The CCI generator (EyeGate model 61-21-EYE; Optis, La Garde, France, now EyeGatePharma, Waltham, MA, USA) has an adjustable "intensity" control from 0 to 2.5 mA (ramped in 1 second after start), in increments of 0.25 mA, "time" from 0 to 5 minutes, in increments of 30 seconds, and polarity of the probe (Fig. 1). The iontophoresis probe was built at the Ophthalmic Biophysics Center (Miami, FL) and consists of a 5.52-mm diameter poly(methylmethacrylate) cup that is both custom-made for rat eyes and identical to the one used by Behar-Cohen et al.[12,17] in previous studies.[18] The probe placed on top of the corneal surface formed a reservoir, which was filled with the photosensitizing agent

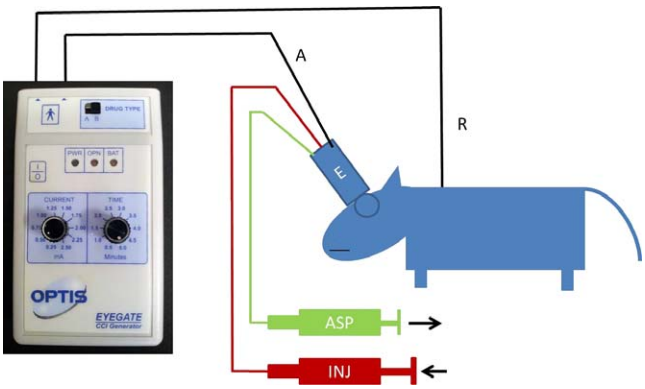

FIGURE 1. Iontophoresis device. A translimbal applicator adapted to the size of the rat cornea is filled with the solution to be administered and connected to the negative electrode. The positive needle electrode is connected to the neck skin as a return electrode. Both electrodes are connected to the CCI generator.

during CCI. Two silicone tubes were used for drug infusion: one tube delivered the solution and the other aspirated air bubbles. This allowed for continuous infusion of the riboflavin solution throughout the procedure. Because riboflavin is a neutral molecule[20] diluted in a saline solution, CCI was performed by connecting the probe to the negative electrode and the positive electrode was connected via a hypodermic needle inserted in the neck skin of the rat. After optimization of the electrical parameters, a current density of 2.11 mA/cm^2 was chosen to deliver the solutions.

Confocal Fluorescence Imaging

A confocal microscope (LSM 710; Zeiss International, Oberkochen, Germany) equipped with a 63× water immersion objective was used to test for the diffusion and depth penetration of riboflavin in the cornea. The photosensitizing agent's emission spectrum was determined with a flat-mounted cornea soaked in riboflavin (Fig. 2A). The microscope was set to excite the riboflavin with an Argon laser emitting in the blue (λ = 458 nm) at 5% power and the emission signal was detected and amplified with a photomultiplier tube (PMT; gain = 1000) set to detect wavelengths between 515 and 565 nm. The microscope settings were tested on an untreated cornea to evaluate the effect of fixation on autofluorescence and it was determined that only unfixed tissue does not exhibit autofluorescence (Fig. 2B). Images were taken with the cornea in the endothelium-up orientation because the fluorescence signal was less attenuated by the light scatter in this orientation. Z-stacks were acquired in the center of the corneas with a step size of 4.3 μm and a depth of view of 8.6 μm. Images of the cornea ranged from 100 to 220 μm. After acquisition, three-dimensional reconstructions and fluorescence intensity profiles were generated from the images using Zen software (Zeiss International).

Fluorometric Analysis

The concentration of riboflavin in the aqueous humor and vitreous was analyzed using a Wallac Victor 1420 multilab counter (PerkinElmer, Waltham, MA, USA; $\lambda_{excitation}$: 450 nm, $\lambda_{emission}$: 535 nm). A standard riboflavin curve was obtained using serial dilutions of the riboflavin-phosphate solution. The concentration of riboflavin in the ocular media was determined for each of the samples collected from the rat eyes using the Beer-Lambert law. The results were expressed as mean ± SD in each group. Data were compared with a nonparametric Mann-Whitney *U* test.

RESULTS

Instillation Runs (Experiment 1)

In the epi-OFF corneas, the riboflavin solutions were instilled for 30 minutes as per the Dresden protocol.[5] The corneal stroma was nearly completely saturated with both solutions (Figs. 3A, 3B), as detected by the confocal microscope.

To evaluate the effect of CCI on the epithelial layer, iontophoresis using saline buffer was conducted prior to riboflavin solutions instillation using a micropipette on the epi-ON eyes of the animals. With riboflavin-dextran, there was very limited diffusion of the solution into the epithelium, and very little to no recordable amount of riboflavin was able to diffuse into the stroma (Fig. 3C). In contrast, transepithelial delivery was successful with the riboflavin-phosphate solution (Fig. 3D).

This result demonstrates that saline-CCI permeabilized the epithelium and allowed for diffusion of the instilled riboflavin into the stroma, only when the riboflavin is not combined with dextran-T500. Due to its high molecular weight (550 kDa), riboflavin-dextran has a high viscosity, which impedes the electrotransfer of riboflavin molecules through the epithelium and into the stroma.

Transcorneal CCI Delivery

Confocal Fluorescence Imaging (Experiment 1). Delivery of the riboflavin solutions using CCI was attempted under both epithelium conditions. After epithelial debridement and CCI delivery, riboflavin was detected in the flat-mounted corneas using the confocal microscope. The three-dimensional reconstructions showed that iontophoresis could deliver both riboflavin solutions into the stroma of the epi-OFF corneas (Figs. 4A, 4B). However, the fluorescence intensity profiles demonstrated that CCI delivery was more efficient with the riboflavin-phosphate solution. Complete saturation of the cornea can be seen in Figure 4B, which is very similar to the images generated after instillation for 30 minutes (Fig. 3B).

After transepithelial CCI delivery, little to no riboflavin-dextran was detected in the corneal stroma (Fig. 3C), whereas riboflavin-phosphate was detected throughout the full thickness of the stroma (Fig. 3D). This result confirms that only riboflavin-phosphate can be delivered effectively through an intact epithelium.

To confirm that CCI delivery is responsible for this effect, two animals were treated using the CCI probe without charge. In the epi-OFF corneas, the CCI probe was placed on the surface of the eye allowing for passive diffusion of the solution. More riboflavin-phosphate in the control run (Fig. 4E) was detected than the riboflavin-dextran (Fig. 4A), but less than the CCI riboflavin-phosphate delivery (Fig. 4B). In the epi-ON case, little to no riboflavin-phosphate was detected in the stroma (Fig. 4F). These observations demonstrated that the charge during the CCI procedure is necessary to permeabilize the epithelium and to increase the amount of riboflavin penetrating into the stroma.

Confocal Fluorescence Imaging (Experiment 2). The effect of increasing the CCI delivery duration was assessed under both epithelium conditions. Fluorescence intensity in the *z*-axis was quantified from the confocal microscope images and the mean of each experimental group was calculated. Increasing the time of delivery from 4 to 10 minutes allowed

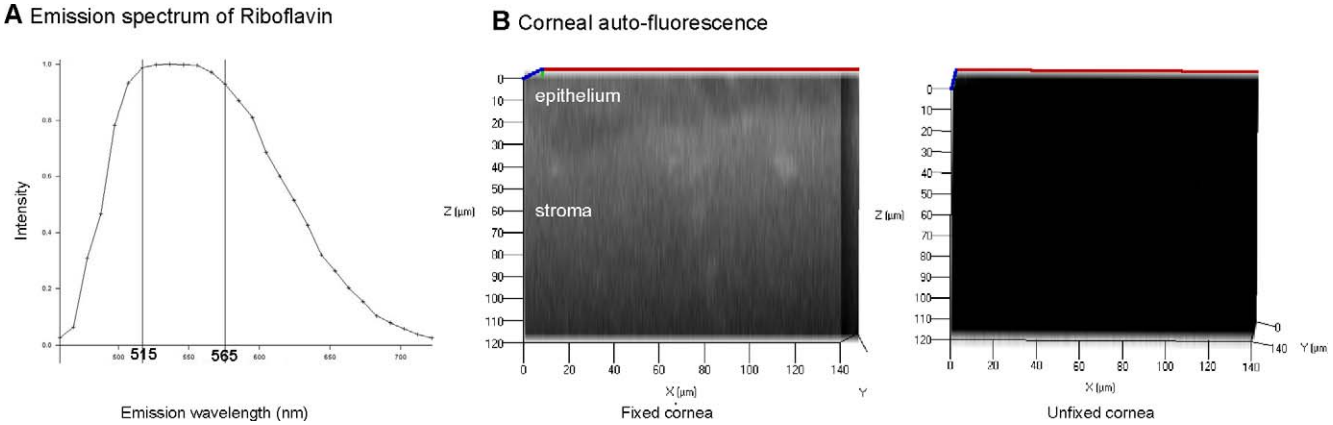

FIGURE 2. (A) Emission spectrum of riboflavin, in an epi-OFF cornea, saturated with the riboflavin solution and observed with the confocal microscope LSM710, a 63× water immersion objective, under an excitation wavelength of 458 nm. (B) Observation of fixed and unfixed flat-mounted corneas.

for more riboflavin-phosphate to be delivered into the cornea. In the epi-OFF case, 10 minutes of drug delivery allowed for nearly 2.5× the amount of riboflavin-phosphate to be delivered into the eye compared with the control group (application of the CCI probe without applying an electrical charge) and the 4 minute CCI delivery group (Fig. 5A). In the epi-ON case, the amount of riboflavin-phosphate detected after 10 minutes of delivery was almost 1.75× the amount of the control group. There is also more riboflavin-phosphate detected in the 10 minute CCI delivery group compared with

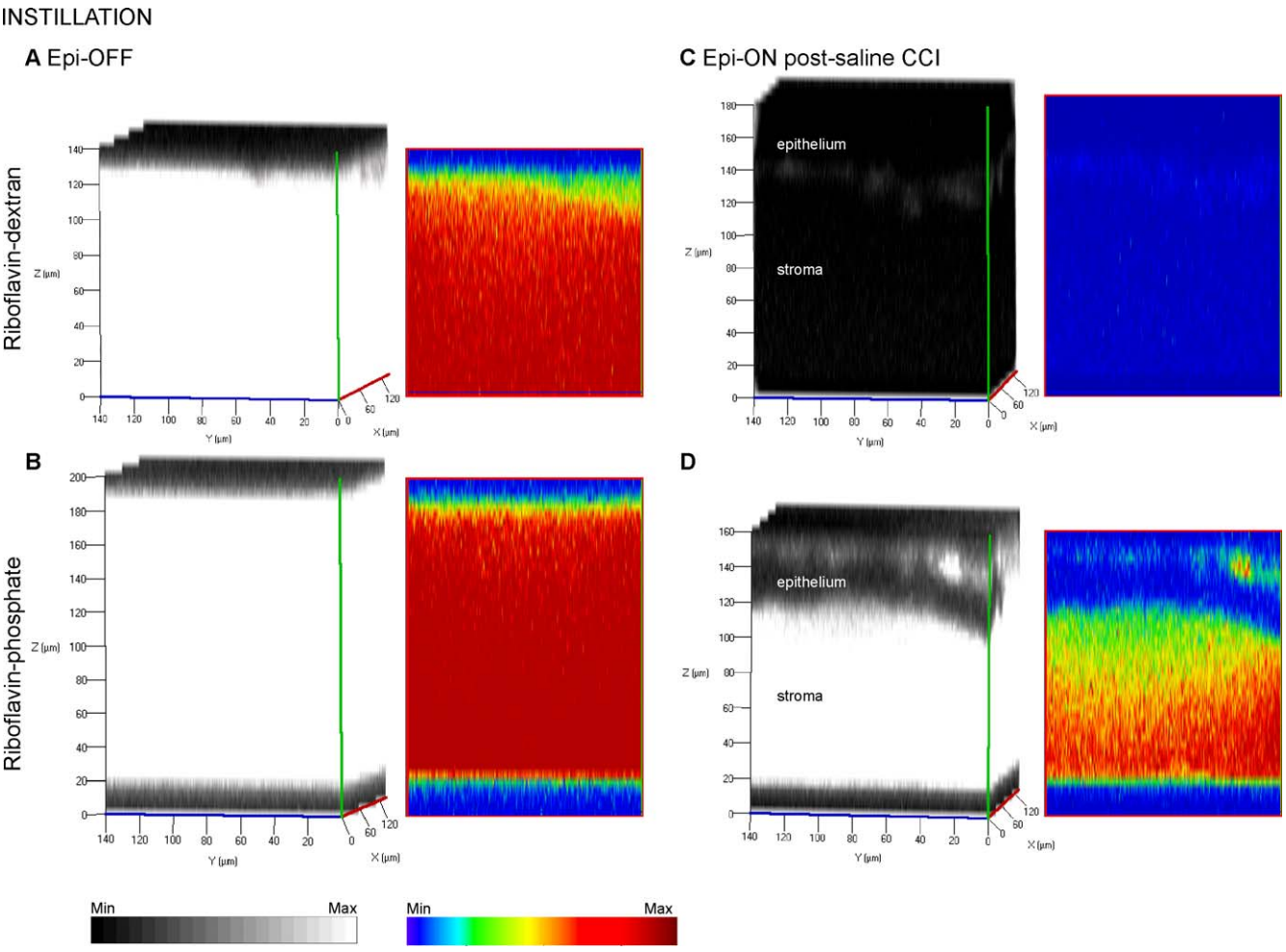

FIGURE 3. Three-dimensional views and intensity profiles of the corneas after the instillation runs using either the 0.1% riboflavin-dextran or 0.1% riboflavin-phosphate solution. (A, B) Instillations on the epi-OFF corneas of the riboflavin-dextran solution (A) or of the riboflavin-phosphate solution (B). (C, D) Saline CCI on the epi-ON corneas, then instillation of the riboflavin-dextran solution (C), or of the riboflavin-phosphate solution (D).

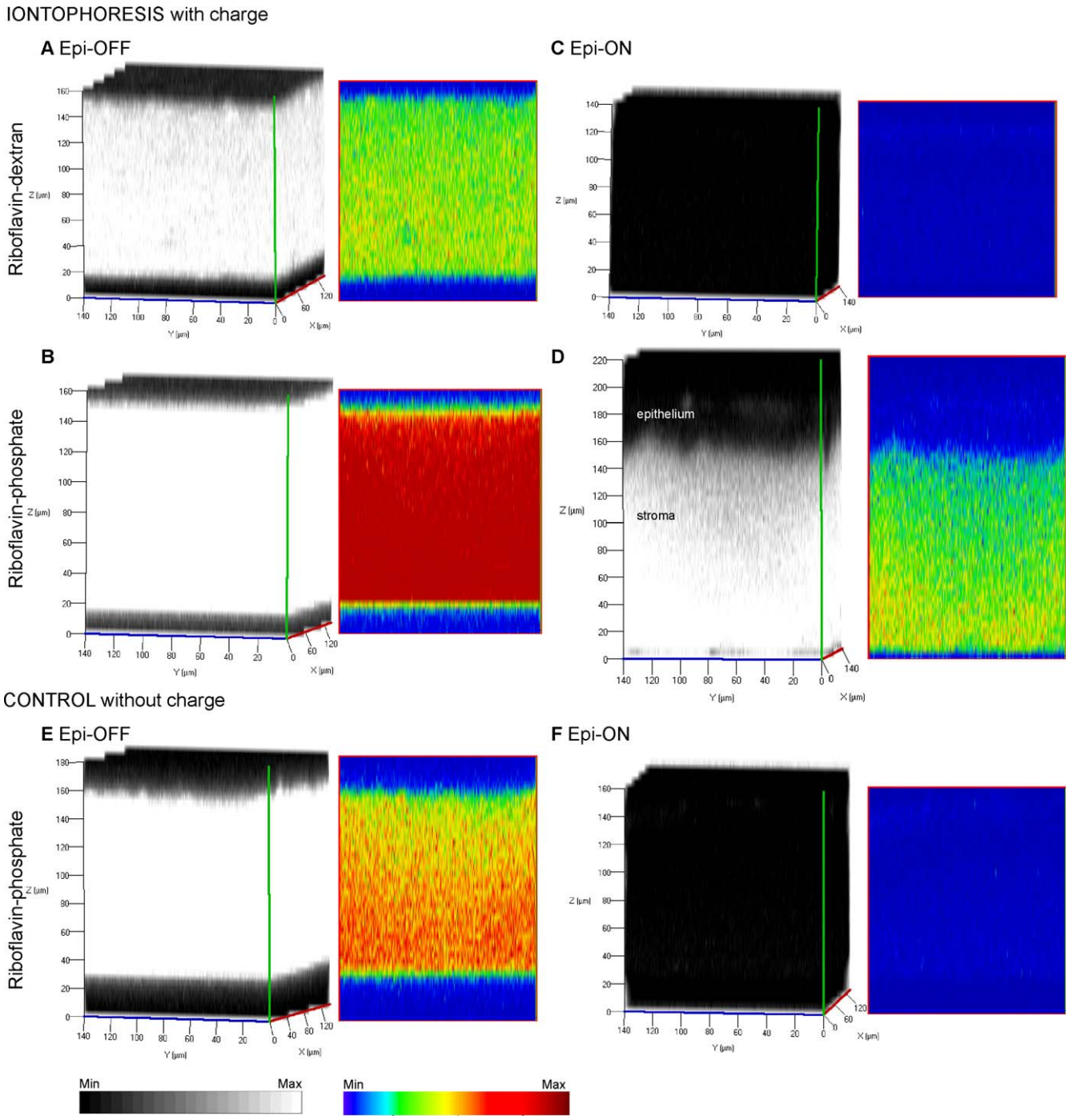

FIGURE 4. Three-dimensional views and intensity profiles of the corneas after delivery with the iontophoresis device using both riboflavin solutions. (**A**, **B**) Coulomb-controlled iontophoresis delivery to the epi-OFF corneas of riboflavin-dextran solution (**A**) or of riboflavin-phosphate solution (**B**). (**C**, **D**) Coulomb-controlled iontophoresis delivery to the epi-ON corneas of riboflavin-dextran solution (**C**), or of riboflavin-phosphate solution (**D**). (**E**, **F**) Control of the riboflavin-phosphate delivery, using CCI probe without charge to an epi-OFF cornea (**E**) and to an epi-ON cornea (**F**).

the 4 minute CCI delivery group, but this difference is not significant (Fig. 5B).

Fluorometry in Ocular Media. The results from the fluorometry of the aqueous humor mirrored the outcome found on the confocal microscope (Fig. 6). After epithelial debridement, CCI for 10 minutes delivered the most riboflavin throughout the cornea and into the aqueous humor (Fig. 6, epi-OFF). The 10 minute CCI group delivered 1.8× the amount of riboflavin when compared with the control group, and 1.64× the amount when compared with the 4 minute CCI delivery group. However, there was no statistical difference between groups. In intact corneas, CCI for 10 minutes delivered the most riboflavin into the aqueous humor, with the concentration of riboflavin being 2.6× higher than the control group ($P = 0.0286$) and 1.7× higher than the 4 minute CCI delivery group ($P = 0.0159$; Fig. 6, Epi-ON). This higher concentration of

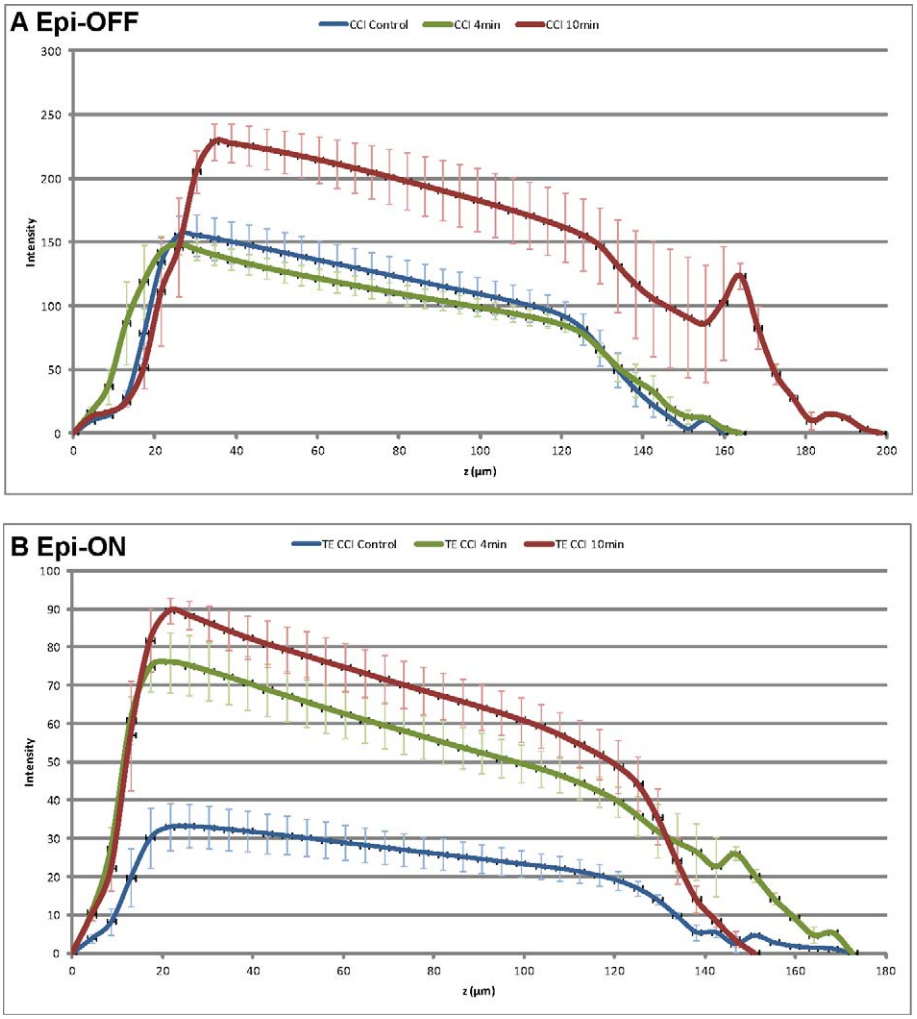

FIGURE 5. Graph showing mean intensity of riboflavin fluorescence detected with the confocal microscope throughout the cornea in groups 5 to 7. (**A**) Riboflavin delivery after epithelial debridement. (**B**) Transcorneal riboflavin delivery. (*Blue:* control; *Green:* 4-minute CCI delivery; *Red:* 10-minute CCI delivery).

riboflavin in the ocular media suggests that when delivering for 10 minutes, the riboflavin-phosphate goes through the cornea and continues traveling down the electric gradient into the aqueous humor.

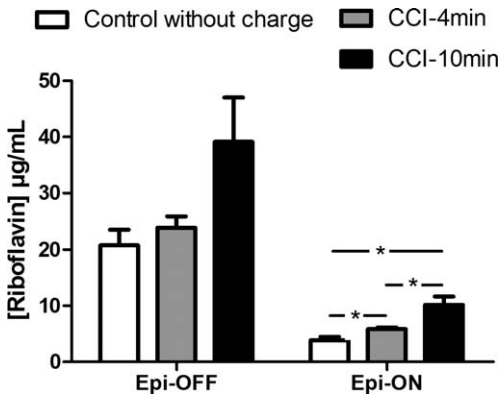

FIGURE 6. Concentration (µg/mL) of riboflavin-phosphate assayed by fluorometry in the ocular media from the groups 5 to 7 for both epithelium conditions. The histogram represents mean ± SD of riboflavin concentration in the control and the experimental groups delivering CCI for 4 and 10 minutes. *$P < 0.05$.

DISCUSSION

Using confocal fluorescence microscopy and fluorometric analysis, this study demonstrates that CCI is a good candidate for riboflavin delivery into the cornea prior to CXL. The intensity profiles from the confocal images and results from the fluorometry also show that riboflavin formulation in saline buffer is a better candidate for iontophoresis when compared with riboflavin-dextran for both epithelium conditions. In the epi-OFF condition, 4 minutes of CCI allowed for the uniform diffusion of riboflavin-phosphate in the stroma, similar to diffusion seen after 30 minutes of instillation. The lipophilic epithelium and its tight junctions are the main barrier to intraocular penetration of hydrophilic molecules, such as riboflavin. Once that barrier is removed, riboflavin can passively diffuse into the rat corneal stroma, which consists of 80% water, and iontophoresis enhances this diffusion. Coulomb-controlled iontophoresis can thus significantly decreases the amount of time needed for this step of the CXL procedure.

Moreover, CCI-assisted transepithelial delivery was only achieved with the riboflavin-phosphate solution. Its instillation after saline-CCI on the epi-ON corneas demonstrated the permeabilizing effect of iontophoresis. However, due to the 500-kDa molecular weight of dextran, the second formulation

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prevented the penetration of riboflavin through the epithelium during the instillation run. We demonstrated that CCI can be used for the transepithelial delivery of riboflavin into the corneal stroma, but that the formulation should be in a saline buffer. The low molecular weight of the riboflavin-phosphate allowed for much more diffusion into the stroma and could also avoid corneal thinning due to the hyperosmotic dextran, an issue commonly seen in CXL.[21] As riboflavin is slightly acidic with a pH of 6.0, the CCI efficiency is most likely due to an electrophoretic effect on the buffer salts, which carries the photosensitizing agent. However, the amount of riboflavin in the cornea was not as much in the transepithelial case than in the epi-OFF case.

This study was performed in an animal model that was geometrically proportional to the human eye. Our experiments showed that the application of an electrical charge to the eye for drug delivery had no adverse effects on the health of the cornea. Analysis on the confocal microscope showed no haze or damage. Also, the IOP, measured with the TonoVet (Icare VET, Vantaa, Finland) tonometer, did not change as a result of iontophoresis and no corneal haze was seen.

Further studies on other animal models whose corneal structure and dimensions are more similar to the human eye should be performed. Proof of the efficacy of transepithelial delivery raises another question regarding CXL: the efficacy of UV-A irradiation on the corneal stroma. Bikbova and Bikbov[22] recently employed iontophoresis for transepithelial corneal CXL and had positive results at the 1-year follow-up. In this study, hypotonic riboflavin was formulated without dextran and the maximum current field applied during CCI was 1.27 mA/cm^2 for 10 minutes. Although they did not look at the diffusion of riboflavin into the stroma, the positive results from the clinical examinations offer support for iontophoresis to be used on patients who require crosslinking.

Spectral-domain optical coherence tomography might be useful to assess cross-linking depth in patients.[23] Vinciguerra et al.[24] reported on a comparison of six patients, three underwent epithelium removal followed by a standard riboflavin application, and three received a transepithelial iontophoresis of riboflavin with a current of 1 mA. All six eyes underwent a 10 mW/cm^2 UV-A treatment lasting 9 minutes. The authors concluded that riboflavin depth penetration was lower with transepithelial iontophoretic treatment. However, the authors did not indicate the current density, a key factor in iontophoretic treatment, nor did they report on the clinical effectiveness in these six patients. To assess riboflavin depth penetration in the cornea stroma, further experimentation needs to be performed with a larger animal model to determine the effect of CCI on the riboflavin concentration gradient within the cornea as a function of the riboflavin pharmaceutical preparation, the current density, and the delivery time.

Studies have employed high-performance liquid chromatography (HPLC) for the analysis of riboflavin diffusion into the cornea. Mastropasqua et al.[25] compared the efficacy of different drug delivery methods (epi-OFF instillation of Ricrolin, epi-ON instillation of Ricrolin TE, and epi-ON iontophoretic delivery of hypotonic riboflavin) in ex vivo human eyes. Their work showed that iontophoresis is an efficient drug delivery method for riboflavin throughout the entire cornea (anterior, intermediate, and posterior stroma). Transcorneal iontophoresis worked to deliver nearly double the amount of riboflavin than in normal transcorneal delivery though the amount was far less than the Dresden protocol. Baiocchi et al.[26] studied the effects of the epithelium on the amount of riboflavin delivered throughout the ex vivo cornea. They showed that the amount of riboflavin in debrided corneas was much higher than the epi-ON group. HPLC can be a useful technique to obtain a more accurate quantification of the riboflavin distribution in the stroma.

Studies should also be performed to determine how much UV-A efficiently reaches the stroma with epi-ON, with and without riboflavin. In the rabbit, UV-B exposure at 280 nm and 12 J/cm^2 did not cause any observable lesions in stromal keratocytes when the epithelium was intact, while keratocyte apoptosis was induced after epithelium removal. This demonstrates that most of UV-B is absorbed by the epithelium.[27] In the human cornea, the stroma has the most significant absorbance for UV filtering. If UV spectra shorter than 310 nm are mostly absorbed by the epithelium and Bowman's membrane, the remainder of the UV irradiation is absorbed more or less equally by these layers.[28] A more recent study has shown that the transmission of UV at 370 nm, which is the wavelength used for CXL, in the stroma is reduced in the periphery of the cornea from 55% to 35% as compared with the center.[29]

In conclusion, CCI serves as an efficient drug delivery method for riboflavin-phosphate prior to corneal CXL. Whether or not the amount of riboflavin-phosphate under these conditions is sufficient and if the CXL induced by the application of UV-A can be as effective through an intact epithelium remains to be determined.

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