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Iontophoresis Transcorneal Delivery Technique for Transepithelial Corneal Collagen Crosslinking With Riboflavin in a Rabbit Model

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MC and CL contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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PURPOSE. We compared an iontophoresis riboflavin delivery technique for transepithelial corneal collagen crosslinking (I-CXL) with a conventional CXL (C-CXL).

METHODS. We designed three experimental sets using 152 New Zealand rabbits to study riboflavin application by iontophoresis using charged riboflavin solution (Ricola+) with a 1-mA current for 5 minutes. The first set was to compare riboflavin concentration measured by HPLC in corneas after iontophoresis or conventional riboflavin application. The second set was to analyze autofluorescence and stromal collagen modification immediately and 14 days after I-CXL or C-CXL, by using nonlinear two-photon microscopy (TP) and second harmonic generation (SHG). In the third set, physical modifications after I-CXL and C-CXL were evaluated by stress-strain measurements and by studying corneal resistance against collagenase digestion.

RESULTS. Based on HPLC analysis, we found that iontophoresis allowed riboflavin diffusion with 2-fold less riboflavin concentration than conventional application (936.2 ± 312.5 and 1708 ± 908.3 ng/mL, respectively, $P < 0.05$). Corneal TP and SHG imaging revealed that I-CXL and C-CXL resulted in a comparable increased anterior and median stromal autofluorescence and collagen packing. The stress at 10% strain showed a similar stiffness of corneas treated by I-CXL or C-CXL (631.9 ± 241.5 and 680.3 ± 216.4 kPa, respectively, $P = 0.908$). Moreover, we observed an increased resistance against corneal collagenase digestion after I-CXL and C-CXL ($61.90\% \pm 5.28\%$ and $72.21\% \pm 4.32\%$ of remaining surface, respectively, $P = 0.154$).

CONCLUSIONS. This experimental study suggests that I-CXL is a promising alternative methodology for riboflavin delivery in crosslinking treatments, preserving the epithelium.

Keywords: crosslinking, collagen, iontophoresis, UVA, riboflavin

Keratoconus is a common bilateral progressive corneal ectatic disease causing visual impairment by inducing irregular astigmatism and paracentral corneal opacities.¹ This disorder typically begins during teenage years, progresses until the age of 30 to 40 years, and, in severe forms, may need a corneal transplantation. Corneal collagen crosslinking (CXL), initially proposed by Wollensak et al,² has changed the natural evolution of keratoconus. It rigidifies the corneal stroma and slows down the progression of keratoconus.³ Stress-strain measurements^{4,5} and increased resistance against enzymatic digestion⁶ have demonstrated the increased crosslinking of corneas after CXL treatment.⁷ It is controversial how CXL actually works, but it is thought to create links between collagen fibrils. The biochemical principle involves developing free oxygen radicals,^{8,9} which leads to the formation of covalent bonds not only between collagen molecules, but also between proteoglycan core proteins.^{10,11}

In the current conventional CXL (C-CXL) treatment method, the corneal stroma is soaked with a riboflavin solution (vitamin B2) before being exposed to ultraviolet-A (UVA) radiation. Since riboflavin cannot penetrate epithelial cell tight junctions to permeate corneal stroma, the central corneal epithelium must be debrided in a diameter of 8.0 mm. Several clinical trials have shown the efficiency of this procedure on progressive keratoconus.³ However, this treatment causes various side effects, such as pain for the first two postoperative days, temporary loss of visual acuity during the first three months,¹² and serious complications, such as infection¹³⁻¹⁵ and stromal opacity due to corneal scarring. These complications mostly are due to epithelium removal, which is indispensable for intrastromal riboflavin penetration.

A procedure preserving the epithelium while keeping the same efficiency as C-CXL would represent a safer therapy for patients suffering from progressive keratoconus. First attempts



consisted in modifying the riboflavin solution formula to facilitate its transepithelial penetration. Several enhancers have been proposed to help riboflavin penetration through epithelium in corneal stroma, while avoiding epithelial debridement.¹⁶ Examples are polyethylene glycol, lysine, and, more recently, NC 1059 peptide.¹⁷ Up to now, the most transepithelial riboflavin studied is Ricrolin TE (Sooft Italia SP, Montegiorgio, Fermo, Italy) which combines two enhancers, amino alcohol TRIS (trometamol) and EDTA. The results of clinical studies on Ricrolin TE are contradictory: Some have shown some effectiveness with less pronounced effects than C-CXL, while others have demonstrated ineffectiveness.^{18–20} To date, the efficacy of this treatment still is under investigation and no prospective randomized clinical study has proved its efficiency to our knowledge.

In the light of these imperfect results, we wanted to evaluate the effect of CXL keeping the corneal epithelium intact by using another strategy, the iontophoresis technique. This is a noninvasive procedure during which a small electric current is applied to enhance the penetration of an ionized substance into a tissue. It has been used in various fields of medicine, for example, in local anesthetics, transdermal anti-inflammatories or analgesics, and transmucosal antiviral administration.²¹

In ophthalmology, the first studies on iontophoresis were performed in the 1940s with the administration of antibiotics for the treatment of bacterial endophthalmitis and keratitis.^{22,23} Ocular iontophoresis still is being investigated as an answer to the low intraocular bioavailability of drugs, in the treatment of several eye disorders of the anterior and posterior segments. It has been proposed for treatment of corneal pathologies, such as paecilomyces keratitis.²⁴

Based on these data, it seemed logical that, as riboflavin is negatively charged and has a low molecular weight,²⁵ the iontophoresis technique could allow intrastromal riboflavin diffusion, while retaining the corneal epithelium, and consequently could be as efficient as the conventional procedure of CXL.

In this study, we evaluated riboflavin diffusion and corneal stromal modifications after CXL using iontophoresis (I-CXL), in a rabbit model, by HPLC system analysis and two-photon microscopy (TP) imaging with second harmonic generation (SHG).²⁶ We next studied the biomechanical effects on corneas after I-CXL using stress-strain measurements^{4,5} and evaluated the impact of I-CXL on the corneal resistance against enzymatic digestion.^{6,27} All results were compared to those obtained with C-CXL and control corneas.

Our study aimed at comparing C-CXL to I-CXL. Both CXL procedures need UVA therapy. The conventional UVA (C-UVA) therapy is 3 mW/cm² during 30 minutes. Consequently, this C-UVA procedure was chosen for our comparative tests. Recent studies show that accelerated UVA (Acc-UVA) therapy could be effective.^{5,28} Although evaluation of the optimal UVA treatment parameters was not our primary objective, we decided to assess the effect of an accelerated UVA therapy with our I-CXL technique as a secondary aim.

MATERIALS AND METHODS

Animals

We used 152 female New Zealand White albino rabbits weighing from 2.2 to 2.9 kg at the beginning of the experiment. All animals were healthy and free of ocular disease. They were anaesthetized with a mixture of ketamine and xylazine hydrochloride. This general anesthesia was combined with anesthetic eye drops composed of oxybupro-

caine without benzalkonium chloride. Only one eye was treated for each rabbit.

Animals were killed with an overdose of pentobarbital immediately after treatment (day 0) or 14 days later (day 14).

All experimental procedures were approved by the Ethical Committee of the CPTP (Centre of Physiopathology Toulouse Purpan), and conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Conventional Crosslinking Procedure

The conventional procedure of CXL was performed in accordance with the standard clinical treatment (according to the Dresden protocol).² Firstly, the epithelium was removed mechanically. Then, riboflavin 0.1% suspended in a dextran T500 20% solution (Ricrolin; Sooft Italia SP) was instilled each minute for 30 minutes. Finally, cornea was irradiated by a UVA light (C.S.O. Srl, Florence, Italy) for 3 mW/cm² during 30 minutes. The solution was instilled every 5 minutes during the UVA treatment. At the end of procedure, a local antibiotic (fusidic acid) was applied in the eye of rabbits kept alive.

Iontophoresis Crosslinking Procedure

The basic setting of iontophoretic experiment involves a constant current source and two electrodes. As shown in Figure 1, the return electrode (anode) was a 30-gauge needle inserted in the nape of the rabbit neck. The main electrode (cathode) was a circular cup with an internal diameter of 8 mm, and a surrounding 1-mm width annular suction ring to affix the device on the cornea, with a small suction (1 mL), during the procedure. The electrode itself is a stainless steel grid, placed into the cup at a minimal distance of 8 mm from the cornea that allows air bubbles, which can disrupt the current supply, to escape. The reservoir is filled with hypoosmolar 0.1% riboflavin solution (Ricrolin+; Sooft Italia SP). It is composed by riboflavin 0.1%, EDTA 0.1%, and Trometanol 0.05%. The solution acts as an electrical contact between the cathode and the rabbit eye. Therefore, riboflavin formulation has been optimized for a penetration by iontophoresis. The large reservoir volume (0.5 mL) prevents pH and concentration shifts during the 5-minute application time.

Before these experiments, an iontophoresis test was conducted applying solutions for 5, 10, and 15 minutes, and pH measurements showed no change. Solutions also were collected after iontophoresis on animals and pH was measured. The generator applies a constant direct current (DC) of 1 mA for a preset period of 5 minutes. Tension was controlled during the experiment by measuring the voltage with a multimeter. It was in the range of 3 to 5 V during all the procedure. Moreover, the generator checked the continuity and a warning signal sounded in case of current disruption (resistance above 40 k Ω).

After the riboflavin administration by iontophoresis, the cornea was washed with balanced salt solution to remove the riboflavin film and irradiated by a UVA light for 3 mW/cm² for 30 minutes (conventional UVA procedure). In addition, we performed other I-CXL with an accelerated UVA procedure using a UVA light (C.S.O. Srl) at 10 mW/cm² for 9 minutes.

Treatment Groups

New Zealand rabbits ($n = 152$) were processed according to three sets of experiments.

The first set was aimed at investigating whether iontophoresis could result in different riboflavin concentration, compared to conventional application on de-epithelialized

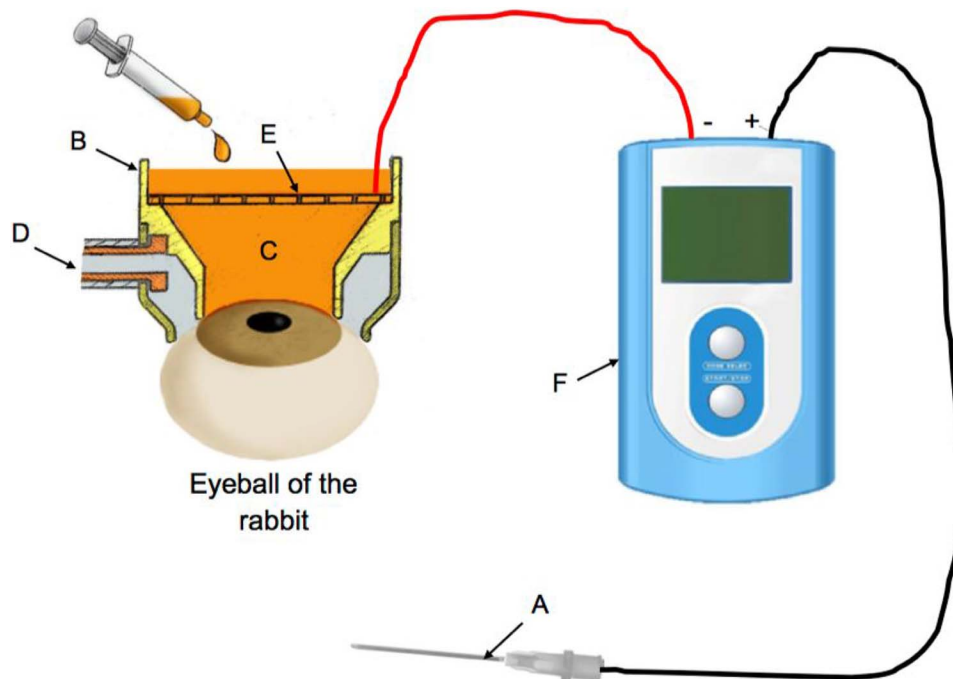


FIGURE 1. Diagram of the iontophoresis procedure experimental setting for iontophoresis experiment: the 1 mA constant current source (F) was connected to the return electrode (A, needle) and to the main electrode device (B, circular cup), which was a reservoir containing riboflavin solution (C) submerging the stainless steel grid (E). A suction tube was connected to an annular suction ring (D) to maintain the device on rabbit cornea during the 5-minute procedure.

corneas. In these experiments, 10 rabbit eyes were soaked with riboflavin by iontophoresis application for 5 minutes and 10 rabbits were treated with conventional riboflavin application for 30 minutes on de-epithelialized corneas. The rabbits were killed immediately and compared to untreated control corneas ($n = 9$) by measuring riboflavin concentration using HPLC.

The second set was aimed at investigating whether I-CXL could result in different autofluorescence collagen diffusion and stromal modifications using TP and SHG, compared to C-CXL. In these experiments, 20 rabbits were treated by I-CXL with C-UVA (3 mW/cm^2 for 30 minutes, $n = 10$) or with Acc-UVA (10 mW/cm^2 for 9 minutes, $n = 10$) therapy. The TPF signal and collagen SHG were measured either at day 0 ($n = 2$) or at day 14 ($n = 18$) after I-CXL. The results were compared to C-CXL ($n = 9$).

Different controls have been used for the CXL procedures: C-UVA therapy after de-epithelialization, but without any riboflavin application ($n = 3$), conventional riboflavin application without UVA therapy ($n = 9$), and untreated controls ($n = 8$).

In this set of experiment, we also measured riboflavin concentration using HPLC analyses in corneas 14 days after C-CXL ($n = 6$) and 14 days after conventional riboflavin application without UVA therapy ($n = 4$). These rabbits were kept alive 14 days in dark cages.

The third set was aimed at investigating the physical corneal effect of I-CXL by using stress-strain measurements and resistance against collagenase digestion analysis, compared to C-CXL. In this experiment, 8 rabbit eyes were treated by I-CXL with Acc-UVA therapy (10 mW/cm^2 for 9 minutes) for stress-strain evaluation, and compared to 8 rabbit eyes treated by C-CXL and with 10 untreated control eyes. In parallel, 10 rabbit eyes were treated by I-CXL with Acc-UVA to evaluate their corneal resistance against enzymatic digestion, and compared

to 9 corneas treated by C-CXL and 10 untreated control corneas.

An additional set aimed at evaluating the iontophoresis corneal toxicity: six rabbits were treated by riboflavin application with iontophoresis. They were immediately analyzed and compared to three untreated control corneas.

Biochemistry Analysis Studies

All aqueous humors were taken with a 30-gauge needle, kept immediately in an opaque tube, and frozen at -80°C . Rabbit 8-mm central corneas, excised with surgical instruments under the microscope and sterile conditions, were finely chopped and then homogenized in $200 \mu\text{L}$ of PBS^{-/-} at 4°C by tissue lyser (2 minutes, 25 beats/s), immediately centrifuged at $3500g$ for 10 minutes at 4°C . ChromSystems Reagent Kit (ChromSystems Instruments and Reagents GmbH, Munich, Germany) for the analysis of vitamin B2 was used for the preparation of $100 \mu\text{L}$ corneal homogenates or $100 \mu\text{L}$ aqueous humor (extraction, precipitation, and stabilization) and processed using an HPLC system (column and mobile phase). The HPLC system was composed of an ICS isocratic pump and sampler, a FP 1520 JASCO programmable fluorescence detector connected to the Chromeleon integrator program by Thermo Fisher Scientific (Courtaboeuf, France). The spectrofluorimeter was set at 465-nm excitation and 525-nm emission wavelengths. The detection limit of riboflavin was 4.16 ng/mL . Calibration curves were obtained by plotting the riboflavin peak area as a function of concentration observed using calibration standard from ChromSystems Instruments and Reagents GmbH. The validation of the method was carried out by internal control Levels I and II from ChromSystems Instruments and Reagents GmbH, and external quality Control from Instand (Dusseldorf, Germany).

TP Microscopy Studies

Corneas were excised with surgical instruments, under the microscope and sterile conditions. They were embedded in tissue freezing medium (OCT) and were cut on a cryostat at -20°C to obtain $5\text{-}\mu\text{m}$ corneal sections. The slides were mounted in Fluorescence Mounting Medium (Dako, Glostrup, Denmark) and were read blind.

Imaging was performed using an upright two-photon microscope LSM 7MP (Carl Zeiss AG, Jena, Germany). Excitation light was provided by a Chameleon Ultra II Ti:Sapphire laser (Chameleon Ultra; Coherent, Inc., Palo Alto, CA, USA) tuned to 830 nm. Emitted fluorescence was split into two channels using a 760-nm dichroic mirror. In the first channel, the SHG images were collected through a single pass filter SP 415 (collagen emission). In the second channel, the fluorescence TP signal was detected through a band-pass (BP) filter from 500 to 550 nm (BP 500-550). Additional acquisitions at 760 nm excitation wavelength have been performed to detect specifically collagen SHG (BP 370-410) and the formation of advanced glycation end products (AGE) induced by collagen CXL with 435 to 455 BP filters. Images were acquired using a $\times 40$ oil immersion objective (numerical aperture = 1.4, Plan-Apochromat; Carl Zeiss AG). To allow the visualization of the corneal width, for some images, 3×3 tile scans were performed. In all cases, images were acquired with standardized conditions for gain and offset (brightness and contrast). Tile scan images were not perfectly overlapped and needed correction. Overlap correction was performed using the plugin MosaicJ on Fiji software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

To evaluate the measurement of fluorescence intensity (MFI) of corneas, unprocessed images showing the entire cornea were analyzed using the Region Measurements function of Metamorph software (Universal Imaging, Downingtown, PA, USA). This software calculates the integrated fluorescence intensity for the entire image.

To study the orientation of the collagen fibers, we applied a 2D-Fast Fourier transform (2D-FFT) algorithm with Fiji software to the collagen second harmonic generation images.^{29,30} To realize Z-stack acquisition, other corneas were entirely and freshly mounted in Fluorescence Mounting Medium (Dako) and observed with the two-photon microscope. Z-stack corneal images were processed as video clips using Zen software (Carl Zeiss AG).

Evaluation of Toxicity After Iontophoresis Treatment on Corneal Epithelium

To evaluate the toxicity of iontophoresis on epithelial cells, we used an active caspase 3 antibody, which is specific for late phase of apoptosis and generally considered as a relevant marker of programmed cell death.^{31,32}

Specimens were fixed, immediately after iontophoresis, in 10% buffered formalin ($n = 6$). They were embedded in paraffin and processed for routine histopathologic examination. At the same time, untreated rabbit corneas were prepared as control specimens ($n = 3$). Sections $3\ \mu\text{m}$ thick were stained with hematoxylin and eosin (H&E). For immunohistochemical examination, sections $3\ \mu\text{m}$ thick were tested using a Ventana Benchmark XT immunostainer (Ventana, Tucson, AZ, USA) with active caspase 3 antibody (a-CASP3; rabbit polyclonal, dilution 1:1000; Abcam, Cambridge, UK). For each sample, we scored the number of a-CASP3-positive cells in all cornea epithelium sections. We used a secondary antibody without a-CASP3 as control.

Analysis of Physical Corneal Properties

For stress-strain measurement, 5-mm wide strips of cornea were cut from superior to inferior corneal axis immediately after I-CXL or C-CXL treatments. Corneal stiffness was determined by uniaxial stress-strain measurements as we described previously,^{4,5} using a material testing machine (MINIMAT; Polymer Laboratories, Stretton Shropshire, UK).

Briefly, after proper alignment in the testing machine with a clamp-to-clamp distance of 6 mm, the sample was fixed by tightening two screws. After a complete relaxation of the sample tissue, the clamps were moved apart until a preload of 20 mN was reached. The initial length of the sample was recorded as reference for the stress-strain curve. Then, the sample was stretched with a velocity of 2 mm/min up to a maximum force of 5 N. During the measurement, the load curve was recorded automatically up to 18% strain and loads were converted to stress by dividing it by the cross-sectional area (the sample's width times its thickness). The stiffness (Young's modulus) as a derivative of the stress-strain curve was determined. For the statistical analysis, Young's modulus was evaluated consistently at 10% strain.

To evaluate the resistance of I-CXL cornea against enzymatic digestion, central corneas were excised with a corneal trephine of 8 mm 14 days after I-CXL or C-CXL treatment. Untreated control corneas were excised with a trephine of the same diameter. All these corneal buttons were placed into a 0.1% bacterial collagenase A solution (0.1 U/mL per cornea) in PBS at pH 7.5 (EC 3.4.24.3 from *Clostridium histolyticum*; Sigma-Aldrich, Saint-Quentin Fallavier, France) with 0.4 mM Ca^{2+} at room temperature as we described previously.⁶ Collagenase solution was changed every 12 hours. We daily monitored digestion of the corneal buttons over 7 days. Buttons were photographed (Nikon P520; Nikon, Tokyo, Japan) and their surface was computed using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Remaining surface was calculated as a percentage of the initial surface at day 0.

Statistical Analysis

Student's *t*-test was used to compare riboflavin concentrations, by HPLC, after its application by iontophoresis to those after conventional application of riboflavin. Data from HPLC analysis, expressed as average (Av) \pm SD, were considered as statistically significant for a *P* value of <0.05 . Student's *t*-test also was used to compare measurements of integrated fluorescence intensity for the entire corneal sections, obtained from TP analysis, after I-CXL (with C-UVA therapy) treated corneas versus C-CXL treated corneas. Student's *t*-test allowed comparing statistically the measurement of corneal stiffness of I-CXL with C-CXL after stress-strain and after collagenase digestion experiments.

Statistical analyses from MFI data and physical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

Intracorneal Riboflavin Diffusion Using Iontophoresis

By using HPLC analyses we investigated whether iontophoresis modifies the riboflavin concentration compared to the conventional application. As shown in the Table, we observed 45% less riboflavin concentration in corneas treated by iontophoresis ($936.2 \pm 312.5\ \text{ng/mL}$) compared to corneas soaked with the conventional application ($1708 \pm 908.3\ \text{ng/mL}$). This difference was statistically significant ($P < 0.05$). Moreover, HPLC analysis

TABLE. Dosage of Riboflavin by HPLC in Aqueous Humor and Cornea

HPLC Dosage of Riboflavin, ng/mL	Application by Iontophoresis, <i>n</i> = 10		Conventional Application, <i>n</i> = 10		Untreated Controls, <i>n</i> = 9	
	Aqueous Humor	Cornea	Aqueous Humor	Cornea	Aqueous Humor	Cornea
	112.83	1084.30	3981.79	1701.81	12.09	32.71
	7.65	1537.56	1480.00	3611.05	6.80	27.74
	202.21	827.71	3119.00	1531.44	6.08	34.34
	106.79	941.81	1189.00	530.13	8.32	27.75
	18.97	1129.62	700.51	2021.33	7.66	21.13
	21.62	852.71	1135.32	1231.88	3.36	18.49
	148.90	1215.61	421.58	2229.53	4.82	23.78
	15.51	636.87	1448.15	1155.99	5.94	22.46
	42.71	572.66	334.22	2082.55	6.32	21.28
	3.33	562.60	1164.72	987.95		
Av ± SD	68 ± 69.8,	936.2 ± 312.5,	1497.4 ± 1168	1708 ± 908.3	6.82 ± 2.46	25.52 ± 5.46
	<i>P</i> < 0.001	<i>P</i> < 0.05				

has shown a significant difference ($P < 0.001$) as regards the level of riboflavin in aqueous humor, which was very low in the iontophoresis group (68 ± 69.8 ng/mL) compared to the conventionally treated group (1497.4 ± 1168 ng/mL).

We next have studied the toxicity of iontophoresis application on corneal epithelium compared to untreated corneas. As shown in Figure 2, the histologic structure of corneal epithelium, analyzed with H&E, was unchanged after

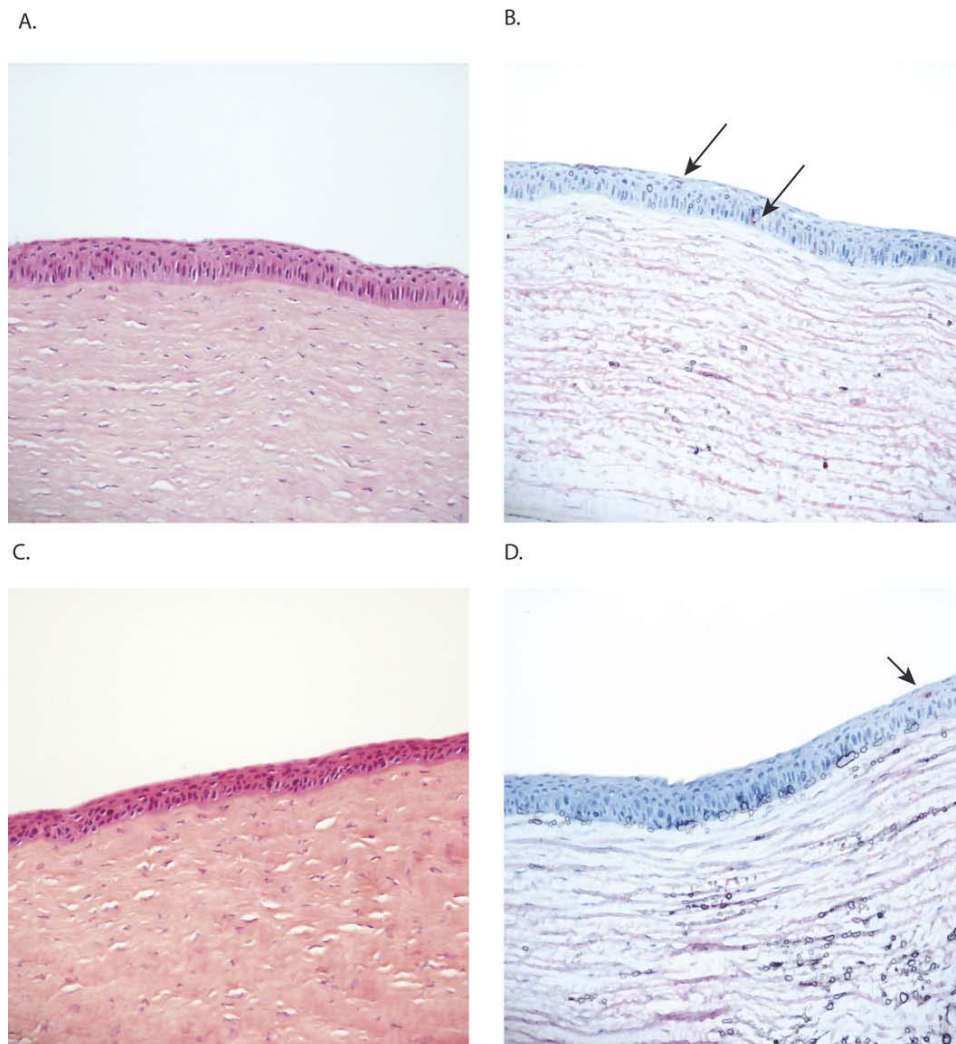


FIGURE 2. Corneal epithelium. Cytologic features of (A) a cornea after iontophoresis and (C) of a control cornea. H&E, magnification: $\times 200$. Immunohistochemistry using antiactive caspase 3 antibody in (B) a cornea after iontophoresis and (D) in a control cornea. Active caspase 3 antibody, magnification: $\times 200$. Arrows show few active caspase 3-positive epithelial cells in red.

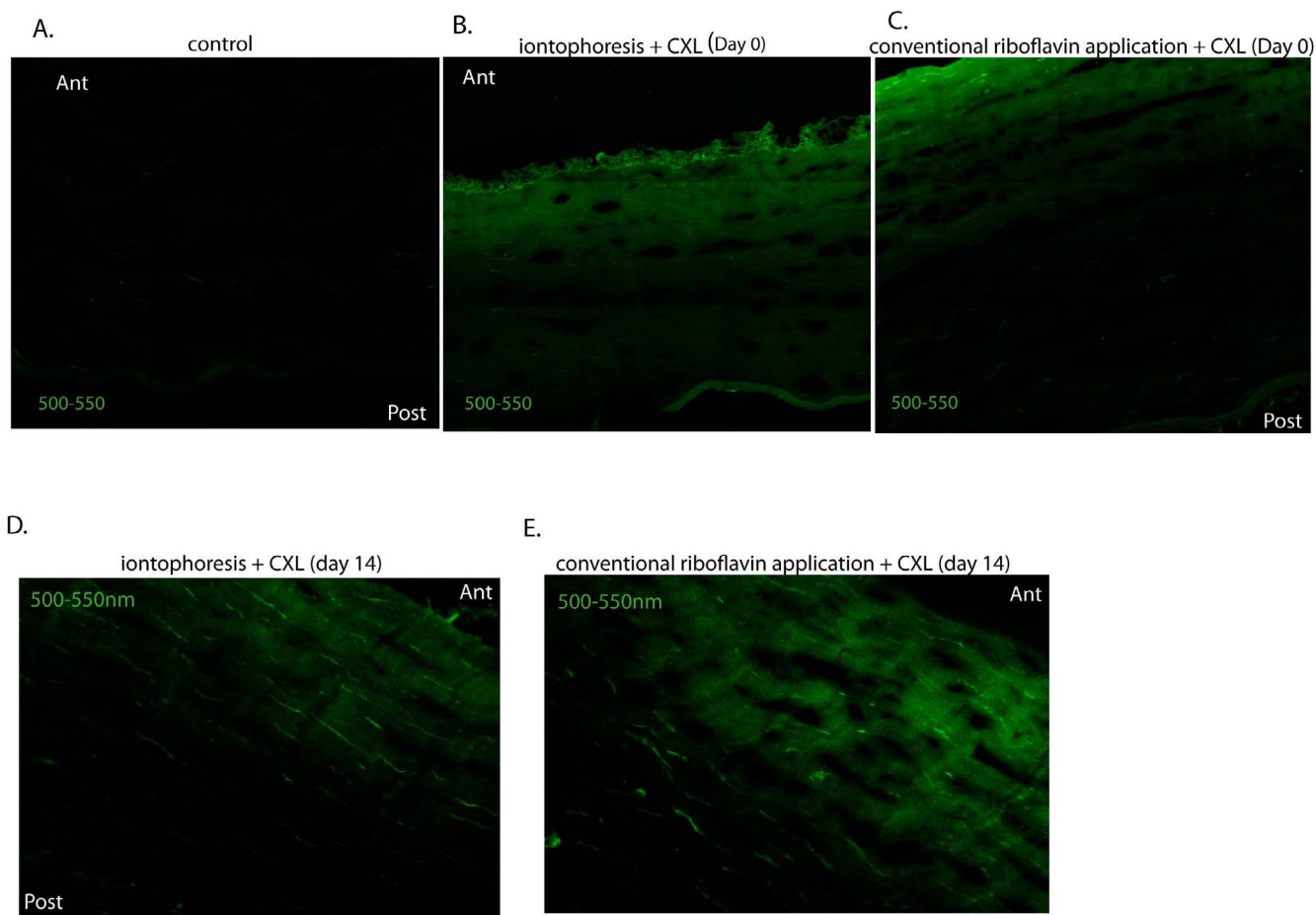


FIGURE 3. Two-photon emission fluorescence in the entire cornea after CXL immediately (day 0) and 14 days (day 14) after UVA irradiation. Two-photon microscopy images of fluorescence emission at 500 to 550 nm at day 0 after CXL of control cornea (A), cornea after I-CXL (B), and C-CXL (C). Two-photon microscopy images of fluorescence emission at 500 to 550 nm 14 days after CXL of I-CXL cornea (D) and in C-CXL cornea (E).

iontophoresis (Fig. 2A) compared to control corneal epithelium (Fig. 2C). By scoring the number of a-CASP 3+ epithelial cells in corneal sections after iontophoresis compared to untreated corneas, we found also no significant difference ($P = 0.698$) between the two groups with a median of a-CASP 3+ at 3.3 cells (± 1.33) for the entire epithelium after I-CXL and a median 2.6 (± 0.88) in untreated controls (Figs. 2B, 2D).

Additionally, pH measurements of solution after iontophoresis showed no change (data not shown).

Effects on Corneal Structures After Iontophoresis-CXL Treatment

TP Microscopy Analyses. By using TP microscopy and SHG imaging, we analyzed the TP fluorescence (TPF) signal diffusion (BP 500–550) and collagen orientation of I-CXL treated corneas at days 0 and 14. As shown in Figure 3, tile-scan images of whole corneal sections analyzed immediately after I-CXL (Fig. 3B) or after C-CXL (Fig. 3C), showed a stronger fluorescent signal in the anterior stroma than in control corneas, which displayed no fluorescence (Fig. 3A). At 14 days after I-CXL (Fig. 3D) or C-CXL (Fig. 3E), corneal sections showed a strong autofluorescence signal from corneal stroma in the anterior zone by using the BP 500 to 550. This fluorescence was similar after I-CXL with Acc-UVA or C-UVA application (data not shown). Moreover, we compared the integrated fluorescence intensity of the entire images from corneal sections after I-CXL and C-CXL treatments. As depicted in Figure 4, no significant difference in the measurement of integrated intensity of entire corneal section was found between I-CXL ($n = 13$) and C-CXL ($n = 9$, $P > 0.7$). Furthermore, untreated corneas, and corneas 14 days after C-UVA alone or 14 days after conventional riboflavin application without UVA irradiation did not show any fluorescence (data not shown). Interestingly, HPLC did not detect any more riboflavin 14 days after C-CXL as in untreated corneas.

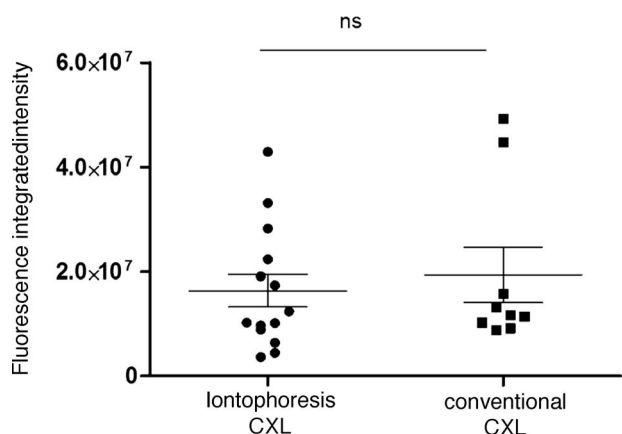


FIGURE 4. Quantification of integrated intensity of 500 to 550 nm fluorescence 14 days after I-CXL- or C-CXL-treated corneas.

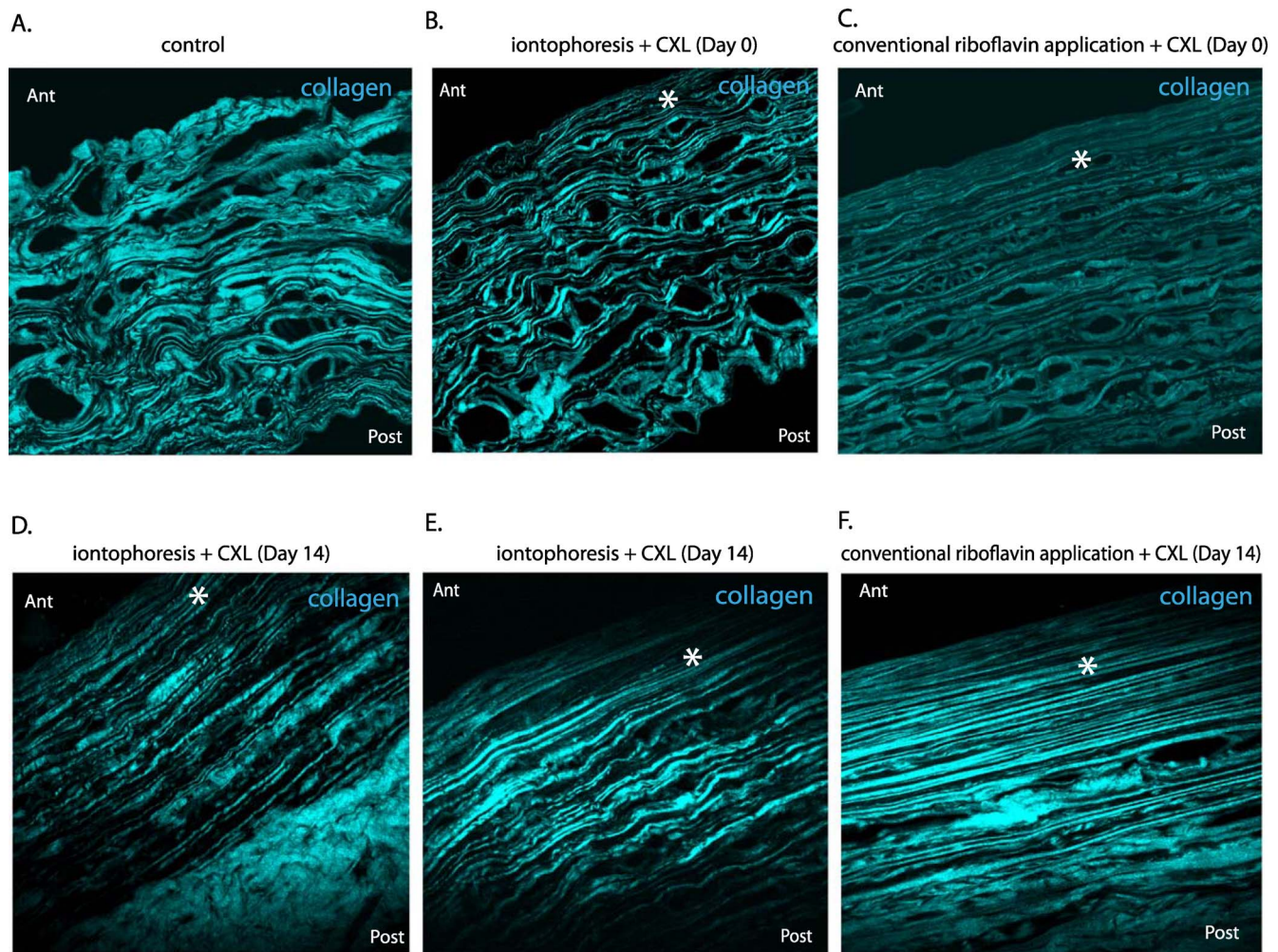


FIGURE 5. Collagen stromal organization using SHG in the entire cornea immediately (day 0) and 14 days (day 14) after CXL. Collagen SHG images of stromal organization at day 0 after CXL in control corneas (A), I-CXL cornea (B), C-CXL cornea (C). Collagen SHG images of stromal organization 14 days after CXL in I-CXL corneas (D, E), C-CXL cornea (F). Asterisks show the collagen fibers packing.

By using SHG imaging, we next investigated the distribution of collagen fibers after I-CXL. The SHG images showed that the “packing” of the collagen fibers was different in CXL-treated eyes and in control eyes. As illustrated in Figures 5B and 5C, immediately after CXL, collagen SHG of I-CXL or C-CXL showed stromal modifications in the third anterior of corneas reflecting a tendency of collagen fibers to stack. These modifications were much more pronounced 14 days after I-CXL (or C-CXL). Indeed, as shown in Figure 5 (bottom), collagen fibers were more stacked and more linear than in untreated corneas. At 14 days after I-CXL treatment, Z-stack images (Supplementary Movie S1) of corneal stroma showed a stronger networking of collagen fibers, with a pronounced lamellar stacking in the anterior stroma, compared to untreated corneas (Supplementary Movie S2). In these conditions, we also observed a predominant collagen orientation by applying 2D-FFT algorithm to the Z-stack images of collagen SHG compared to the untreated cornea (Supplementary Fig. S1).

Physical Corneal Properties Analysis. To analyze the physical corneal modification after CXL, we studied the biomechanical effects of CXL with the stress-strain experiment and corneal capacity to resist enzymatic digestion.

As depicted in Figure 6A, we showed a higher median value of stress at 10% strain (657.7 ± 57.17 kPa) in CXL-treated corneas compared to untreated corneas (335.5 ± 34.5 kPa) (P

$= 0.0001$). Moreover, the stress at 10% strain was similar in I-CXL-treated (631.9 ± 91.26 kPa) and in C-CXL-treated (680.3 ± 76.52 kPa) corneas ($P = 0.6888$). The I-CXL group tended to be slightly less resistant than the C-CXL group, but this difference was not statistically significant ($P = 0.6888$). The measurement of the corresponding Young's modulus confirmed an increased corneal stiffness in the I-CXL (17.2 ± 8.2 MPa) and C-CXL (18.8 ± 7.2 MPa) groups compared to untreated control (8.6 ± 3.5 MPa, $P = 0.6888$).

We next studied the effect of CXL on the resistance of treated corneas against digestion by collagenase solution. Figure 6B showed that C-CXL- and I-CXL-treated corneas resisted more against enzymatic digestion at day 7 ($72.21\% \pm 4.32\%$ and $61.90\% \pm 5.28\%$ of remaining surface, respectively) than untreated corneas ($12.44\% \pm 2.82\%$ of remaining surface, $P < 0.0001$). Moreover, we observed no significant difference in the measurement of the remaining surface between I-CXL ($61.90\% \pm 5.28\%$) and C-CXL ($72.21\% \pm 4.32\%$, $P = 0.154$).

DISCUSSION

We hypothesized that iontophoresis could be a suitable strategy for performing CXL without damaging epithelium. In iontophoresis, the substance is applied with an electrode

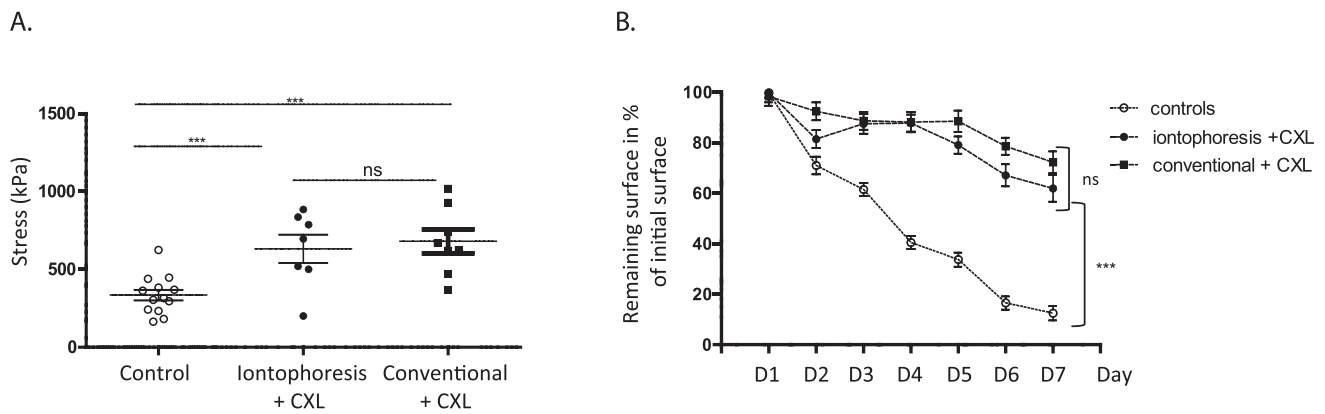


FIGURE 6. Physical corneal properties analysis using stress-strain measurements and collagenase digestion analyses. Quantification of stress at 10% strain after I-CXL- or C-CXL-treated corneas and in untreated control corneas (A). Quantification of remaining surface cornea (% of initial surface) after collagenase digestion in I-CXL, C-CXL, and untreated control groups (B).

carrying the same charge as the substance, and the return electrode, which is of the opposite charge, is placed elsewhere in the body to complete the circuit. The substance has the role of a conductor of current through the tissue. Iontophoresis is suitable for substances that are positively or negatively charged at physiologic pH, preferably with low molecular weight.²⁵ Riboflavin theoretically is a perfect candidate for ocular iontophoresis due to its negatively charged structure and low molecular weight. Other parameters that determine the product penetration are the current density, product concentration, and application time. Our preliminary HPLC studies on the transepithelial penetration of a 0.1% riboflavin preparation, applied by iontophoresis for different times, showed that a 5-minute application at a safe current level of 1 mA should be able to achieve the desired riboflavin intrastromal concentration for crosslinking of collagen fibers with UVA (data not shown).

The present study showed that iontophoresis is an alternative riboflavin delivery technique for transepithelial CXL, allowing riboflavin diffusion and physical corneal modifications.

By using HPLC analysis, we investigated whether iontophoresis modifies the riboflavin concentration in the corneal stroma compared to the conventional application. This biochemical experiment showed that iontophoresis facilitates the diffusion of riboflavin into the cornea compared to untreated control corneas. However, the riboflavin concentration was 2-fold lower after iontophoresis compared to conventional riboflavin application. Our results are in accordance with the data of Mastropasqua et al.³³ in human donor corneas.

In addition, our biochemical analysis showed that riboflavin does not diffuse into the aqueous humor after iontophoresis application. This most probably is due to, on the one hand, the strict transcorneal passage from the iontophoresis applicator, without any transscleral or limbal passage, and the relatively short application time (5 minutes). On the other hand, it also probably is a consequence of the riboflavin administration method by iontophoresis, during which riboflavin penetrates into the eye with a migration front. However, for CXL the diffusion into the aqueous humor is not necessary.³⁴ Indeed, at the beginning of the development of the CXL technique, it was recommended that the clinician check the presence of riboflavin in the anterior chamber, searching for a yellow Tyndall before UVA application. The aim was to ensure the penetration of riboflavin inside the corneal stroma. In addition, the diffusion of riboflavin into the aqueous humor does not seem essential for the eye tissue protection (i.e., lens and retina) against UVA.³⁴

In parallel, by using TP on corneal samples, we also found riboflavin fluorescence after iontophoresis or conventional riboflavin application (without UVA therapy) and observed riboflavin diffusion across the entire section as reported before (data not shown).³⁵

As previously used after CXL,²⁶ we performed TP combined with Metamorph software, to evaluate the impact of I-CXL. We noticed that CXL increased TPF signal in the anterior part of the cornea as reported by Chai et al.³⁶ Interestingly, we noticed that this strongly-enhanced autofluorescence of the anterior stroma lasts 14 days after CXL. This seems to be the result of a combined effect of riboflavin and UVA therapy because, in our experiments, UVA therapy alone did not induce this collagen fluorescence. This fluorescence seems not to be related to the persistence of riboflavin in the stroma 14 days after application. Indeed, we did not observe any abnormal fluorescence 14 days after riboflavin application without UVA therapy (data not shown) and no more riboflavin was detected by HPLC in corneas 14 days after riboflavin application with or without UVA therapy (data not shown). This finding was in agreement with the fact that CXL induces an autofluorescence signal through the formation of AGE, suggesting that the TPF signal may be due to the AGE fluorescence emission. It could be supported by the detection of AGE fluorescence 14 days after I-CXL or C-CXL by using specific 335-455 BP filters that showed a peak fluorescence emission at 760 nm excitation wavelength, whereas a minority was detected with 519 to 549 BP filters (data not shown).

In addition, we performed SHG experiments that have been shown to be a useful microscopic technique, which allows the imaging of the structural organization of collagen and which is applicable to corneal tissue.^{26,37-40} Our SHG results confirmed not only that C-CXL induces stromal morphologic modifications as reported previously,^{41,42} but also that I-CXL leads to similar stromal effects. We observed that collagen packing is more pronounced at day 14. Moreover, in our study as in others,⁴³ SHG revealed that CXL was predominant in the anterior stroma and decreased over depth. This observation may have some clinical implications, since the third anterior part of the stroma is known to be important for corneal biomechanics⁴⁴ and, consequently, is the area where the CXL should take effect.⁴⁵

To validate this idea, we provided comparative data on biomechanics of the cornea with two types of experiments. Firstly, by using stress-strain measurement, we found, in C-CXL-treated corneas, Young's modulus values comparable with those of Wollensak and Iomdina's.⁴⁶ Most importantly, we showed for the first time to our knowledge that I-CXL and C-

CXL had a similar increase in tissue stiffening. Secondly, we observed that I-CXL and C-CXL had a comparable effect on the resistance of tissue resorption against enzymatic digestion. Although these two experiments showed that physical modifications after I-CXL tended to be slightly lower than after C-CXL, the difference was not statistically significant. Consequently, we can assume that I-CXL and C-CXL have a similar biomechanical efficacy in rabbit corneas.

To shorten even more the CXL treatment, recent techniques based on accelerated UVA exposure have been proposed. They are based on the principle of equivalent energy dosing, the amount of corneal strengthening being energy-dependent and not power-dependent. Higher power, delivered over a shorter time, theoretically provides the same corneal strengthening as the conventional treatment. Therefore, in our study, we combined iontophoresis with the conventional UVA irradiation but also with the new UVA “accelerated” procedure. The results were similar in the two groups with respect to fluorescence generated and linearization of stromal collagen.

In conclusion, iontophoresis allows a satisfactory diffusion of riboflavin, which is sufficient after UVA therapy to induce, in the rabbits, morphologic and biomechanical stromal modifications similar to the C-CXL. Stress-strain measurements and collagenase digestion experiments showed that I-CXL appears as efficient as C-CXL for increasing the resistance of the corneal tissue. These findings are in accordance with the first clinical study about iontophoresis in which the investigators recently reported that iontophoresis may be an efficient and useful technique for CXL.⁴⁷

Transepithelial corneal collagen crosslinking has the major advantage of avoiding de-epithelialization and its secondary complications. Furthermore, it has the advantage of shortening the application time of riboflavin from 30 to 5 minutes. This preclinical study has provided evidence that I-CXL is a new administration strategy and a novel concept for treatment generating CXL. These results are similar to those observed in the C-CXL treatment of rabbit eyes. The I-CXL procedure now merits further long-term studies in a clinical trial.

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