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Iontophoresis transcorneal delivery technique for transepithelial corneal collagen crosslinking with riboflavin in a rabbit model

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ABSTRACT

Purpose

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

Methods

We designed 3 experimental sets using 152 New Zealand rabbits to study riboflavin application by iontophoresis using charged riboflavin solution (Ricrolin+®) with a 1mA current for 5 min. The first set was to compare riboflavin concentration measured by High-Performance Liquid Chromatography (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 two-fold less riboflavin concentration than conventional application (936.2 ± 312.5 ng/ml 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 kPa 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 ±5.28 % and 72.21 ±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.

INTRODUCTION

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 Theo Seiler *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 digestion[6] 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 post-operative days, temporary loss of visual acuity during the first three months[12] and serious complications such as infection[13-15] and stromal opacity due to corneal scarring. These complications are mostly 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 in order 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,Montegiorgio, FM, Italy) which combines two enhancers, amino alcohol TRIS (trometamol) and sodium ethylenediaminetetraacetic acid (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 is still under investigation and no prospective randomized clinical study has proved its efficiency.

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 non-invasive 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 1940's with the administration of antibiotics for the treatment of bacterial endophthalmitis and keratitis.[22, 23] Ocular iontophoresis is still 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 high-performance liquid chromatography (HPLC) system analysis and two-photon microscopy (TP) imaging with 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 with 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 $3mW/cm^2$ 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

One hundred and fifty two female New Zealand White albino rabbits were used 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 anaesthesia was combined with anaesthetic eye drops composed with oxybuprocaine 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, Montegiorgio, FM, Italy) was instilled each minute for 30 minutes. Finally, cornea was irradiated by a UVA light (C.S.O, Florence, Italy) for 3mW/cm^2 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 (1ml), 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,Montegiorgio, FM, Italy). 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-min application time.

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

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 3mW/cm^2 during 30 minutes (conventional UVA procedure). In addition, we performed other I-CXL with an accelerated UVA procedure using a UVA light (C.S.O, Florence, Italy) at 10 mW/cm^2 during 9 minutes.

TREATMENT GROUPS

New Zealand rabbits (n=152) were processed according to 3 set of experiments.

The first set was aimed at investigating whether iontophoresis could result in different riboflavin concentration, compared to conventional application on de-epihtelialized 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 immediately sacrificed 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. 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 with C-CXL (n=9).

Different controls have been used for the CXL procedures: C-UVA therapy after de-epithelialisation 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 with 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 with 9 corneas treated by C-CXL and 10 untreated control corneas.

An additional set aimed at evaluating the iontophoresis corneal toxicity: 6 rabbits were treated by riboflavin application with iontophoresis. They were immediately analysed and compared to 3 untreated control corneas.

BIOCHEMISTRY ANALYSIS STUDIES

All aqueous humors were taken with a 30G 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 µl of PBS -/- at 4°C by tissue lyser (2 minutes, 25batt/s), immediately centrifuged at 3500g for 10 minutes at 4°C. ChromSystem® Reagent Kit (Munich, Germany) for the analysis of vitamin B2 was used for the preparation of 100 µl of corneal homogenates or 100 µl of aqueous humor (extraction, precipitation and stabilisation) and processed using HPLC system (column and mobile phase). The HPLC system was composed of an ICS isocratic pomp and sampler, a FP 1520 JASCO programmable fluorescence detector connected to the CHROMELEON® integrator program by THERMO 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 ChromSystem® Diagnostic. The validation of the method was carried out by internal control Levels I and II from ChromSystem® and external quality Control from INSTAND (Dusseldorf, Germany).

TWO-PHOTON 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 5µm-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 7*MP* (Zeiss, 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 second harmonic generation (SHG) images were collected through a singlepass filter SP 415 (collagen emission). In the second channel, the fluorescence TP signal was detected through a band-pass filter from 500 nm 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 endproducts (AGE) induced by collagen CXL with 435-455 BP filters. Images were acquired using a 40x oil immersion objective (NA = 1.4, Plan-Apochromat; Zeiss). To allow the visualization of the corneal width, for some images, 3x3 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.

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, Glostrup, Denmark) and observed with the two-photon microscope. Z-stack corneal images were processed as video clips using Zen software (Zeiss, Jena, Germany).

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 histopathological examination. At the same time, untreated rabbit corneas were prepared as control specimens (n=3). Three-µm-thick sections were stained with hematoxylin and eosin (H&E). For immunohistochemical examination, 3-µm-thick sections 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, strips of 5 mm wide of the 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 previously described,[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 5N. During the measurement, the load curve was automatically recorded 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 consistently evaluated 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 0.4mM Ca2+ at room temperature as we previously described.[6] Collagenase solution was changed every 12h. We daily monitored digestion of the corneal buttons over 7 days. Buttons were photographed (Nikon P520) and their surface was computed using Image J software. Remaining surface was calculated as a percentage of the initial surface at day 0.

STATISTICAL ANALYSIS

Student t-test was used to compare riboflavin concentrations, by HPLC, after its application by iontophoresis with those after conventional application of riboflavin. Data from HPLC analysis, expressed as average ± standard deviation, were considered as statistically significant for a p value <0.05. Student t-test was also 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 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.

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 1, we observed 45% less riboflavin concentration in corneas treated by iontophoresis (936.2 ± 312.5 ng/ml) compared to corneas soaked with the conventional application (1708 ± 908.3 ng/ml). This difference was statistically significant ($p<0.05$). Moreover, HPLC analysis 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 the figure 2, the histological structure of corneal epithelium, analyzed with H&E, was unchanged after iontophoresis (Fig 2A) as 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 (Fig 2B and 2D).

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

EFFECTS ON CORNEAL STRUCTURES AFTER IONTOPHORESIS-CXL TREATMENT

TWO PHOTON 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 day 0 and at day 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). As shown in figure 3, 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 500-550. 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 anymore riboflavin 14 days after C-CXL as in untreated corneas.

By using SHG imaging, we next investigated the distribution of collagen fibers after I-CXL. 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 1/3 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 the figure 5 (bottom), collagen fibers were more stacked and more linear than in untreated corneas. Fourteen days after I-CXL treatment, Z-stack images (Movie S1) of corneal stroma showed a stronger networking of collagen fibers, with a pronounced lamellar stacking in the anterior stroma, compared with untreated corneas (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 (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 with untreated corneas (335.5 ±34.5 kPa) ($p=0.0001$). Moreover, the stress at 10% strain was similar in I-CXL (631.9 ±91.26 kPa) and in C-CXL (680.3 ±76.52 kPa) treated corneas ($p=0.6888$). The I-CXL group tended towards to be slightly less resistant than 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 both 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. The figure 6B showed that C-CXL and I-CXL treated corneas resisted more against enzymatic digestion at Day 7 (72.21 ±4.32 % and 61.90 ±5.28 % of remaining surface, respectively) than untreated corneas (12.44 ±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 ±5.28 %) and C-CXL (72.21 ±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 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 plays the role of a conductor of current through the tissue. Iontophoresis is suitable for substances that are positively or negatively charged at physiological pH, preferably with low molecular weight.[25] Riboflavin is theoretically 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 the 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 shows 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 two 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 is most probably 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 is also probably 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 show).[35]

As previously used after CXL,[26] we performed TP combined with Metamorph software, in order 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 and coworkers.[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 show) and no more riboflavin was detected by HPLC in corneas 14 days after riboflavin application with or without UVA therapy (data not show). This finding was in agreement with the fact that CXL induces an autofluorescence signal through the formation of advanced glycation endproducts (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 which showed a peak fluorescence emission at 760nm excitation wavelength, whereas a minority was detected with 519-549 BP filters (data not show).

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 confirm not only that C-CXL induces stromal morphological modifications as previously reported.[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 biomechanics[44] and is consequently 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 Wollensak and Lomdina's ones.[46] Most importantly, we show for the first time 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.

In order 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 both 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, morphological 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 authors recently reported that iontophoresis may be an efficient and useful technique for CXL.[47]

I-CXL 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 minutes 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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LEGEND FIGURES

Figure 1:

Diagram of the iontophoresis procedure

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

Figure 2:

Corneal epithelium. Cytological features of a cornea after iontophoresis (Fig 2A; hematoxylin and eosin coloration (H&E), x200) and of a control cornea (Fig 2C; H&E x200). Immunohistochemistry using anti-active caspase 3 antibody in a cornea after iontophoresis (Fig 2B; active caspase 3 antibody, x200) and in a control cornea (Fig 2D; active caspase 3 antibody, x200). Arrows show few active caspase 3 positive epithelial cells in red.

Figure 3:

Two-photon emission fluorescence in the entire cornea after corneal collagen crosslinking (CXL) immediately (Day 0) and 14 days (Day 14) after UVA irradiation.

Two-photon microscopy images of fluorescence emission at 500-550 nm at Day 0 after CXL of: control cornea (Fig 3A), cornea after CXL with iontophoresis (I-CXL)(Fig 3B), and cornea treated by conventional CXL (C-CXL)(Fig 3C). Two-photon microscopy images of

fluorescence emission at 500-550 nm 14 days after CXL of: I-CXL cornea (Fig 3D) and in C-CXL cornea (Fig 3E).

Figure 4

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

Figure 5:

Collagen stromal organization using second harmonic generation in the entire cornea immediately (Day 0) and 14 days (Day 14) after CXL.

Collagen second harmonic generation images of stromal organization at Day 0 after CXL in: control corneas (Fig 5A), I-CXL cornea (Fig 5B), C-CXL cornea (Fig 5C). Collagen second harmonic generation images of stromal organization 14 days after CXL in: cornea I-CXLcorneas (Fig 5D and 5E), C-CXL cornea (Fig 5F). Asterisks show the collagen fibers packing.

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 (Fig 6A). Quantification of remaining surface cornea (% of initial surface) after collagenase digestion in I-CXL, C-CXL and untreated control groups (Fig 6B).

Figure S1:

Examples of 2D-FFT analysis on cornea SHG images. *White lines* show the 2D-FFT–derived fiber direction in C-CXL and I-CXL treated corneas indicating a preferential fiber direction compared to the untreated cornea.

FIGURE 1.

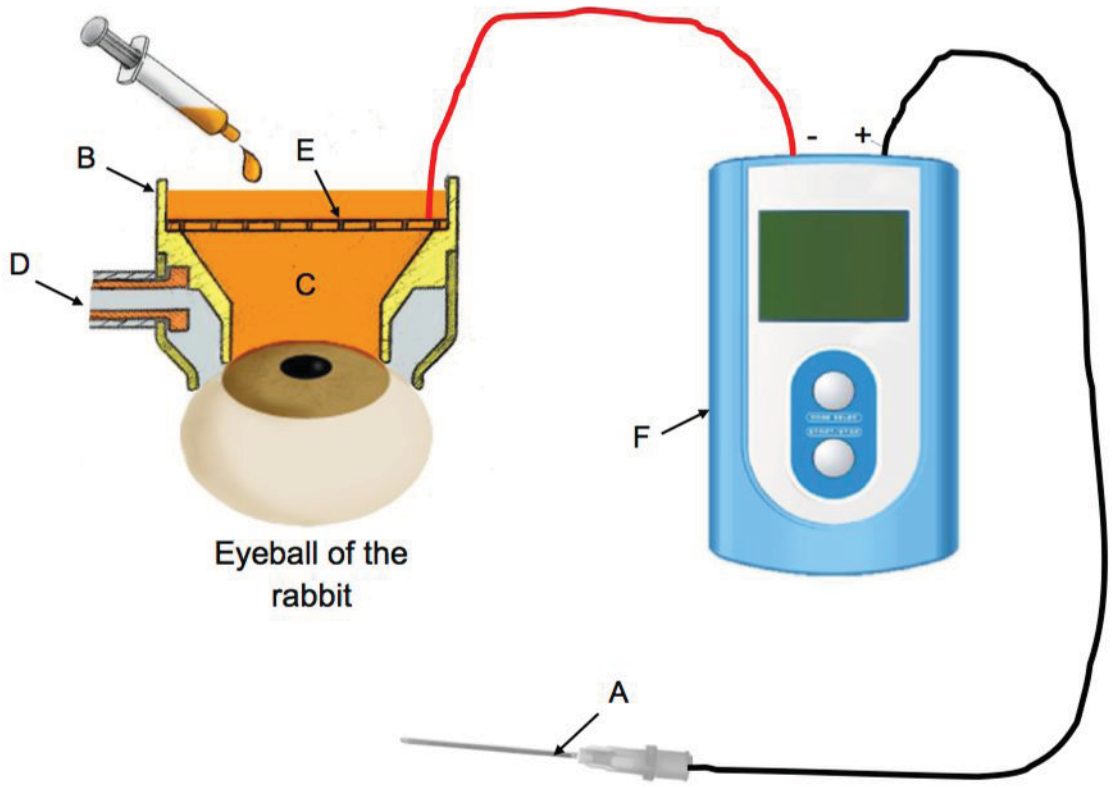
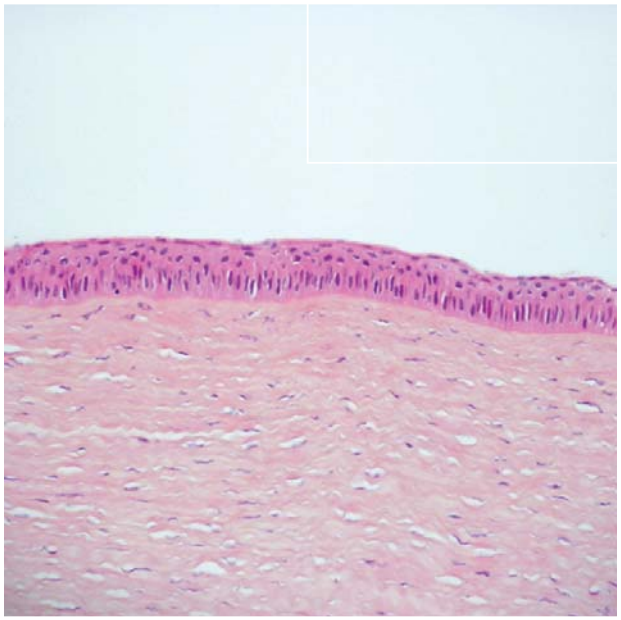
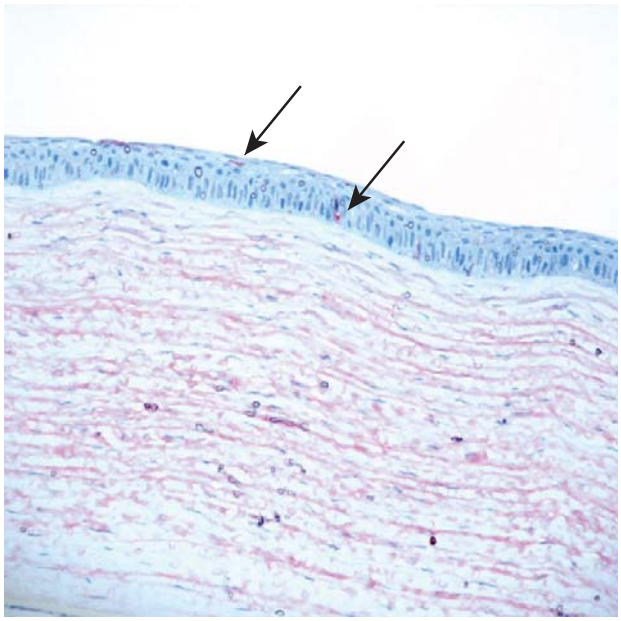


FIGURE 2.

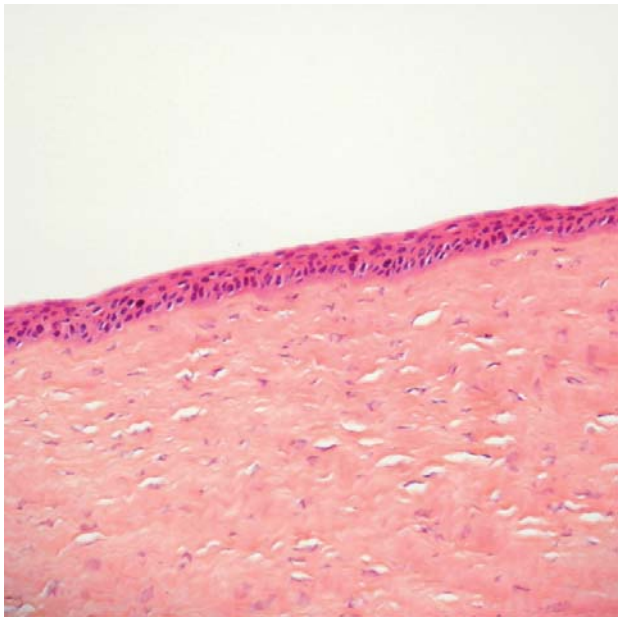
A.



B.

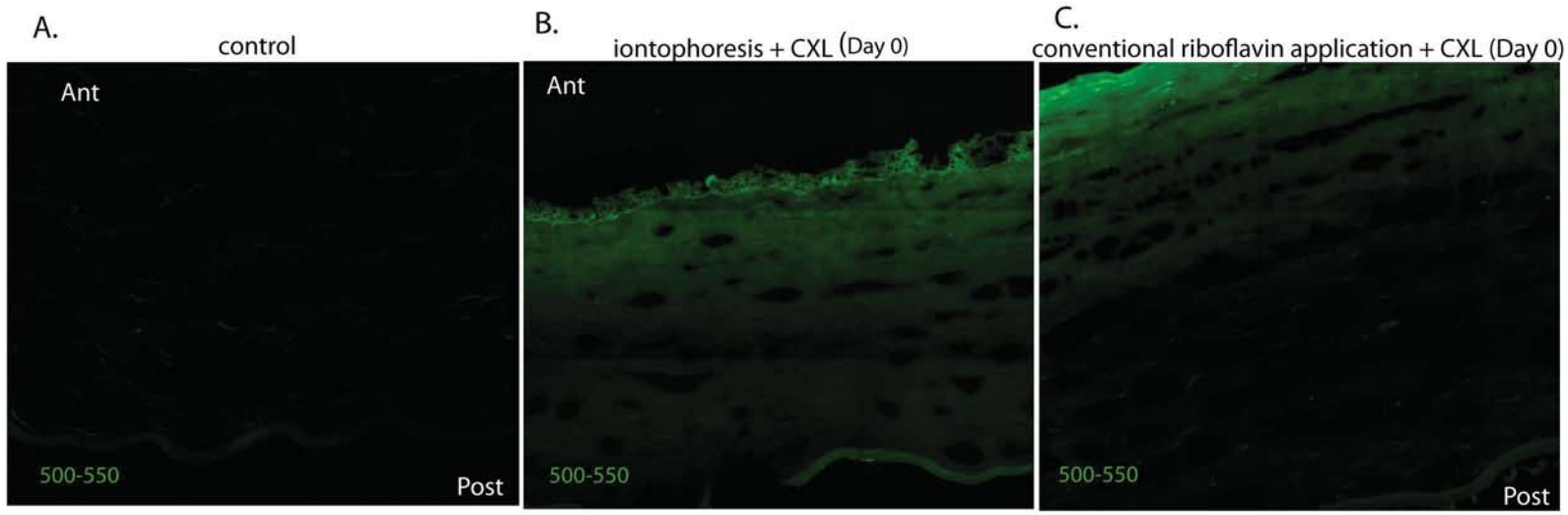


C.



D.

FIGURE 3


A.
control
Ant
500-550
Post
B.
iontophoresis + CXL (Day 0)
Ant
500-550
C.
conventional riboflavin application + CXL (Day 0)
500-550
Post

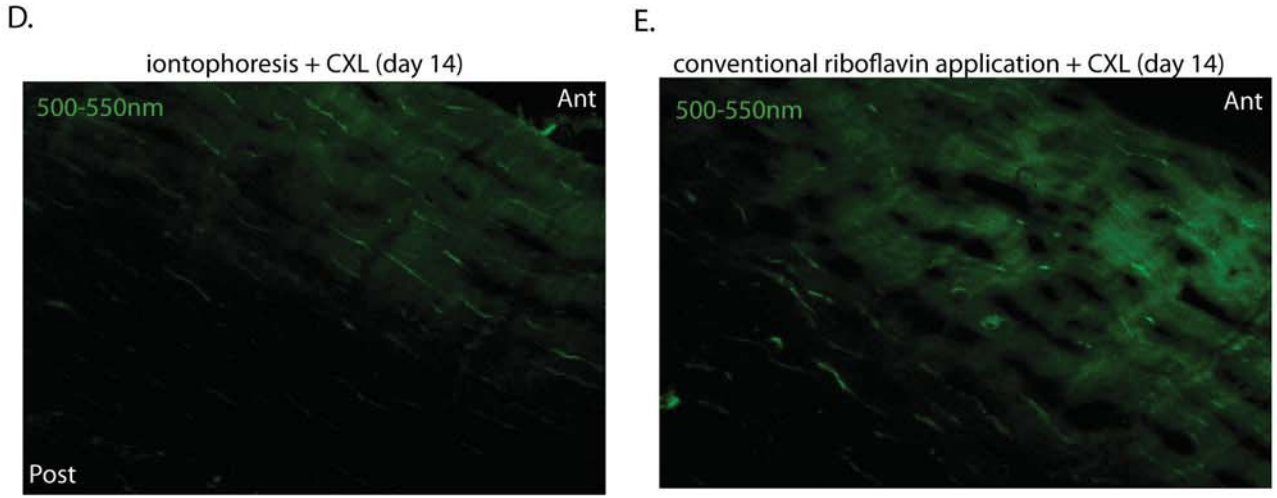

D.
iontophoresis + CXL (day 14)
500-550nm
Ant
Post
E.
conventional riboflavin application + CXL (day 14)
500-550nm
Ant

FIGURE 4.

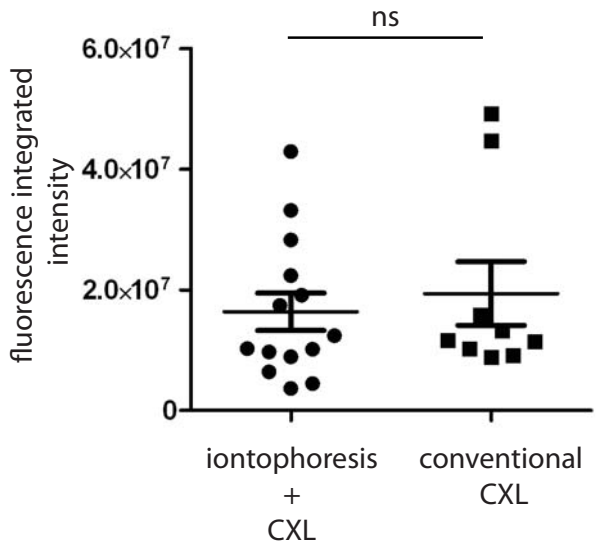

ns
6.0×10^7
4.0×10^7
2.0×10^7
0
fluorescence integrated intensity
iontophoresis + CXL
conventional CXL

FIGURE 5.

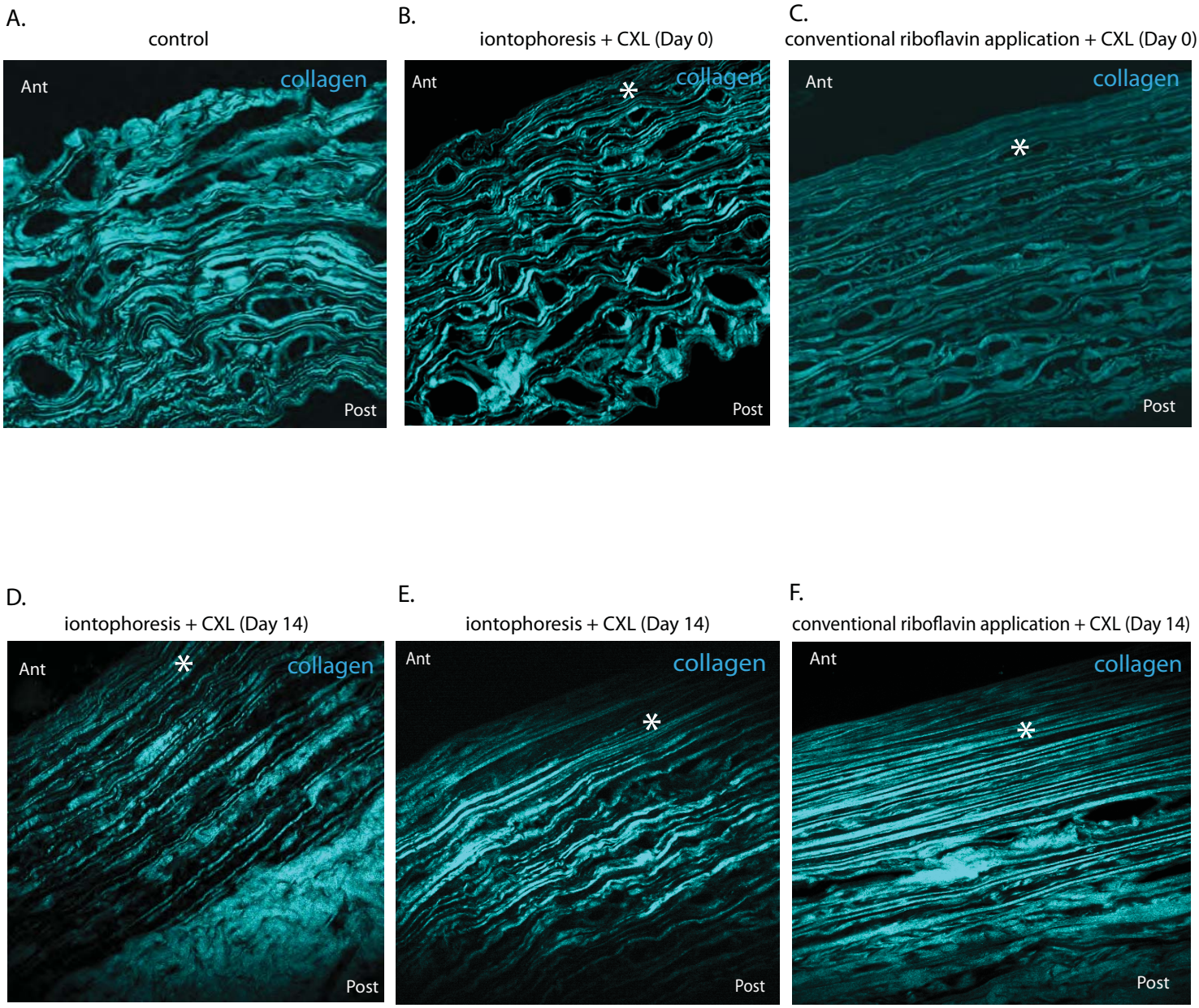
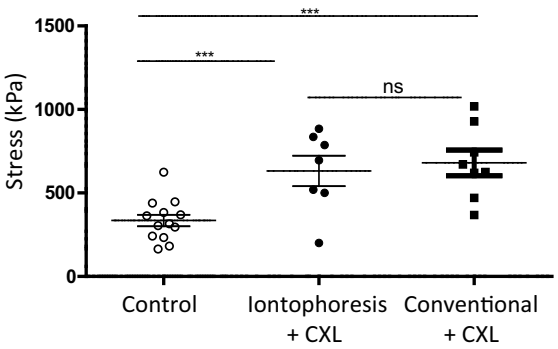

A.
control
Ant
collagen
Post
B.
iontophoresis + CXL (Day 0)
Ant
collagen
Post
C.
conventional riboflavin application + CXL (Day 0)
Ant
collagen
Post
D.
iontophoresis + CXL (Day 14)
Ant
collagen
Post
E.
iontophoresis + CXL (Day 14)
Ant
collagen
Post
F.
conventional riboflavin application + CXL (Day 14)
Ant
collagen
Post

FIGURE 6.

A.


Stress (kPa)
1500
1000
500
0
***
***
ns
Control
Iontophoresis + CXL
Conventional + CXL

B.

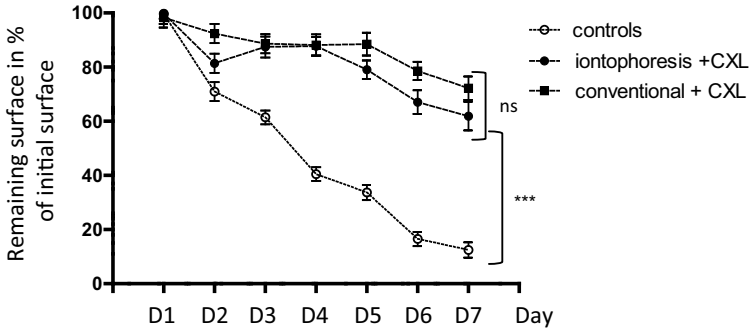
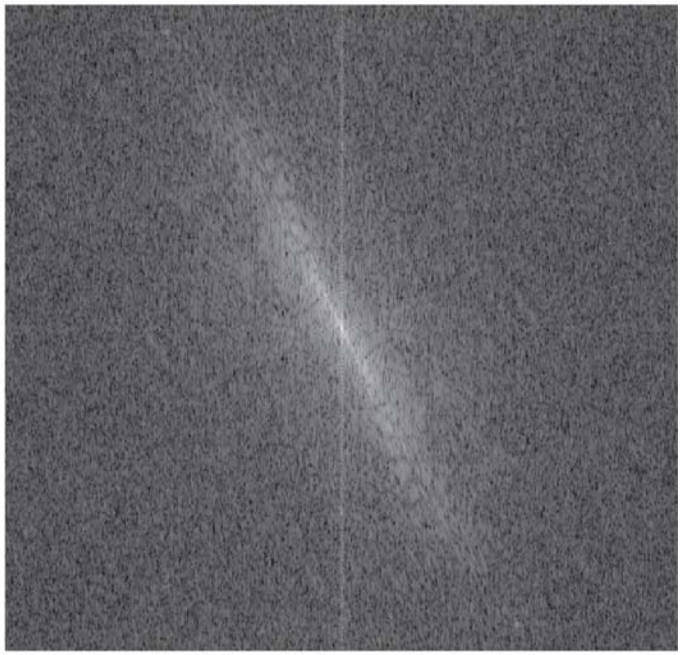

Remaining surface in % of initial surface
100
80
60
40
20
0
D1
D2
D3
D4
D5
D6
D7
Day
controls
iontophoresis +CXL
conventional + CXL
ns
***

FIGURE S1.

Control

ionthophoresis + CXL



conventional CXL

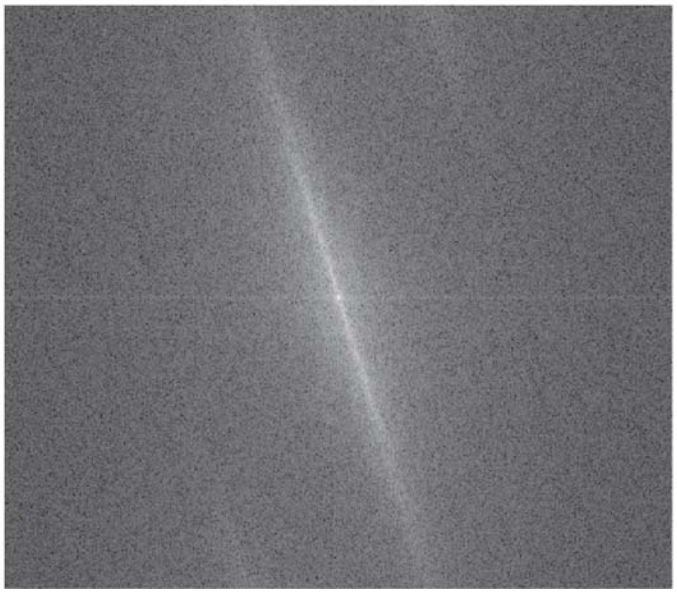


Table 1. Dosage of Riboflavin by HPLC in Aqueous Humor and Cornea

HPLC Dosage of riboflavin (ng/mL)	**APPLICATION BY IONTOPHORESIS (n= 10)**		**CONVENTIONAL APPLICATION (n=10)**		**UNTREATED CONTROLS (n=9)**	
	Aqueous humor	**Cornea**	**Aqueous humor**	**Cornea**	**Aqueous humor**	**Cornea**
	112.83	1 084.30	3 981.79	1701.81	12.09	32.71
	7.65	1 537.56	1 480.00	3 611.05	6.80	27.74
	202.21	827.71	3 119.00	1 531.44	6.08	34.34
	106.79	941.81	1 189.00	530.13	8.32	27.75
	18.97	1 129.62	700.51	2 021.33	7.66	21.13
	21.62	852.71	1 135.32	1 231.88	3.36	18.49
	148.90	1 215.61	421.58	2 229.53	4.82	23.78
	15.51	636.87	1 448.15	1 155.99	5.94	22.46
	42.71	572.66	334.22	2 082.55	6.32	21.28
	3.33	562.60	1 164.72	987.95		
Average ±standard deviation	**68 ±69.8 (p<0.001)**	**936.2 ±312.5 (p<0.05)**	**1497.4 ±1168**	**1708 ±908.3**	**6.82 ±2.46**	**25.52 ±5.46**