LABORATORY SCIENCE

Assessment of stromal riboflavin concentration–depth profile in nanotechnology-based transepithelial corneal crosslinking

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Purpose: To determine the intrastromal concentration of riboflavin in nanotechnology-based transepithelial corneal crosslinking.

Setting: Consiglio Nazionale delle Ricerche, Messina, Italy.

Design: Experimental study.

Methods: Six human donor sclerocorneal tissues were used to evaluate penetration of nanotechnology-based riboflavin 0.1% solution in the stroma through the intact epithelium. Three additional tissues were deepithelialized and soaked with dextran 20.0%–enriched riboflavin 0.1% solution for 30 minutes. After corneal soaking with riboflavin, all tissues were irradiated using a 10 mW/cm^2 device for 9 minutes. Two-photon emission fluorescence (TPEF) axial scanning measurements were collected in all specimens before treatment and immediately after corneal soaking with riboflavin and ultraviolet-A (UVA) irradiation of the cornea. The absorbance spectra of each tissue were collected at the same time intervals. The TPEF signals and absorbance spectra were used to calculate the concentration-depth profile of riboflavin in the corneal stroma during treatments.

Results: The mean stromal riboflavin concentration was 0.008% ± 0.003% (SD) and 0.017% ± 0.001% after transepithelial soaking with the nanotechnology-based solution and standard soaking, respectively (P = .001). After UVA irradiation of the cornea, the mean consumption of riboflavin was 52% ± 13% and 67% ± 2% in the study group and control group, respectively (P < .01).

Conclusions: The nanotechnology-based platform was effective in enriching the anterior stroma with riboflavin through the intact epithelium, although the riboflavin concentration–depth profile rapidly decreased across the mid and posterior stroma. The treatment-induced stiffening effect on the corneal stroma was not assessed in this study.

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Riboflavin–ultraviolet-A (UVA) corneal crosslinking (CXL) consists of permeating the corneal stroma with riboflavin, which acts both as photosensitizer and UV absorber, and irradiating the cornea with a UVA device for generating additional crosslinking bonds between stromal proteins.[1] The procedure ultimately aims at improving the biomechanical stability of the corneal tissue in patients' eyes that have progressive keratoconus or secondary corneal ectasia.[2–5]

The conventional riboflavin–UVA CXL treatment (ie, the Dresden protocol) requires the removal of epithelium before riboflavin administration and UV irradiation of the corneal stroma.[6,7] However, epithelial removal has been associated with major treatment complications including pain, corneal haze and edema, and risk for infection.[8,9] For this reason, transepithelial riboflavin–UVA CXL is gaining increasing interest among corneal specialists. Based on laboratory and clinical studies showing the inefficacy of transepithelial protocols using dextran-enriched riboflavin solutions,[10–17] investigators and manufacturers are maximizing their efforts to evaluate new strategies to deliver riboflavin efficiently through the intact epithelium.

The corneal epithelium has decreasing permeability to compounds with a molecular weight greater than 180 Da

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(riboflavin has 340 Da molecular weight).[18] Strategies to deliver riboflavin through the intact epithelium have included the use of hypotonic ophthalmic solutions without viscous substances (ie, dextran) enriched by enhancers and/or delivered by iontophoresis.[19–26] The enhancers, such as benzalkonium chloride (BAK), ethylenediaminetetraacetic acid (ETDA), trometamol, d-α-tocopheryl poly(ethylene glycol) 1000 succinate, and cyclodextrins, loosen the epithelial cells' tight junctions and facilitate the penetration of riboflavin into the stroma. Iontophoresis is a minimally invasive procedure that has been shown to efficiently deliver dextran-free hypotonic riboflavin solution enriched with ETDA and trometamol through the intact epithelium.

The aim of this study was to determine the efficacy of a new nanotechnology-based platform to deliver riboflavin into the stroma of human donor corneas through the intact epithelium. The riboflavin stromal concentration–depth profile was investigated using a standardized all-optical method with 2-photon optical microscopy and spectrophotometry,[27] and the results were compared with those of conventional stromal soaking with dextran 20%–enriched riboflavin solution.

MATERIALS AND METHODS

Corneal Tissues

Eye-bank donor corneoscleral tissues from different donors and not suitable for transplantation were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino). The tissues were explanted within 20 hours and cultivated at 30°C in corneal storage medium. Inclusion criteria included an endothelial cell density (ECD) of 1800 cells/mm^2 or more. Exclusion criteria included any corneal or ocular surgery. All human tissues were used in compliance with the guidelines of the Declaration of Helsinki for research involving the use of human tissue, and the experimental protocol was approved by the Research Ethics and Bioethics Advisory Committee of the National Research Council.

Nine sclerocorneal tissues with intact epithelium were used in this study. The mean age of the donors was 67 years ± 7 (SD), the mean postmortem interval (from death to culture medium) was 12 ± 6 hours, and the mean ECD was 2070 ± 206 cells/mm^2.

All tissues were cultivated at 30°C in corneal storage medium (Minimum Essential Medium 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid modified solution enriched with nutrients and antibiotics/antimycotic in purified water) for 15 to 20 days before shipment to the laboratory in dextran 20%-enriched storage medium. Tissues were used within 48 hours from shipment.

Each corneoscleral tissue was placed in an artificial anterior chamber (Coronet, Network Medical Products Ltd.) and pressurized with the artificial anterior chamber filled with sodium chloride 0.9% using a 5.0 mm syringe. After the central corneal thickness (CCT) was measured with an ultrasound (US) corneal pachymeter (Pachmate, DGH Technology, Inc.), 6 sclerocorneal tissues with intact epithelium (study group) were soaked with the new hypotonic ophthalmic solution, which consisted of phosphate-free riboflavin 0.1% that was kept in solution by 2.6% 2-hydroxypropyl-β-cyclodextrin nanoparticles; the solution also included EDTA 0.2% and trometamol 0.27% as enhancers. The solution (Nanotech, Sooft Italia SpA) was instilled over the corneal epithelial surface every 20 to 30 seconds for 20 minutes using a silicone ring. Before UVA irradiation, the epithelium was gently washed with sodium chloride 0.9% solution. The cornea was then irradiated with a 10 mW/cm^2 UVA device (Vega 10 mW, Costruzione Strumenti Oftalmici) for 9 minutes, delivering a total energy dose of 5.4 J/cm^2 to the corneal tissue. No riboflavin drop was applied during UVA irradiation.

Three sclerocorneal tissues were treated according to a slightly modified Dresden protocol and were used as the control group.[27] After the CCT was measured with the US corneal pachymeter, the epithelium was removed using an Amoils brush (Innovative Excimer Solutions, Inc.); thereafter, a solution containing riboflavin 0.1% and dextran 20.0% (Ricrolin, Sooft Italia SpA) was instilled every 3 minutes for 30 minutes before UVA irradiation was administered. After corneal soaking, the stromal surface of each tissue was gently washed using sodium chloride 0.9% solution. The tissues were then irradiated with the 10 mW/cm^2 UVA device for 9 minutes. No riboflavin drop was applied during UVA irradiation to avoid the variable window-filtering effect of riboflavin 0.1% over the stroma. Data from control specimens were collected in previous work.[27] The UVA device was calibrated with a power meter before treatment, and an irradiation area of 8.0 mm diameter was used in all cases.

Analysis of Stromal Concentration–Depth Profile of Riboflavin

The methodology to determine the concentration-depth profile of riboflavin in corneal stroma has been fully described.[27] It consists of acquiring the absorbance spectra and the 2-photon emission fluorescence (TPEF) signals from each sample. Measurements were performed before and immediately after corneal soaking with riboflavin (in the latter case, after gentle washing of the stromal surface) and UVA irradiation of the cornea. In control tissues, baseline measurements were collected after the corneal epithelium was removed.

Spectrophotometry measurements were performed before 2-photon optical microscopy imaging by using a purpose-developed spectrophotometry setup. The setup included a deuterium halogen lamp (Avalight-DH-S-BAL) and a spectrophotometer (Avaspec 2048L) (both Avantes BV) connected to a computer for data acquisition and processing. The light beam was focused on the central corneal area with a diameter of 2.0 mm. The absorbance spectrum, $A_p(\lambda)$, also called optical density, was registered in the 300 to 700 nm range over the central cornea. Before each measurement, the system was carefully calibrated according to standardized study protocol.[17,27,28] The riboflavin concentration was expressed as a percentage, where 0.1% = 0.001 g/cm^3.

A commercial 2-photon optical microscopy (Leica DM6000CS, Leica Microsystems GmbH) based on an upright microscope equipped with a Ti:sapphire laser (Vision II, Coherent, Inc.) was used for simultaneously collecting the TPEF signals in the backward direction and the second harmonic generation (SHG) signal in the forward direction. The laser beam was focused on the sample that was placed upward on a quartz microscope slide under the microscope using a HCX IRAPO 25×/0.95 NA water immersion objective with a working distance of 2.5 mm (Leica Microsystems GmbH). The laser was tuned to 810 nm, and the laser power was 15 mW before entering the water immersion objective; it was measured before and during each session of measurements to allow direct comparison of measurements between different sessions of experiments. The forward SHG signal originating from the corneal stroma was filtered by a 10 nm full width at half maximum band-pass filter centered at 405 nm (FF01-405/10-25, Semrock, Inc.); the reflected TPEF signal was filtered by a 525/50 band-pass filter (Semrock Inc.). The nondescaned detector sensitivity settings for collecting the TPEF light were the same for all tissue specimens; this method permitted comparison of data between different sessions of experiments.

Each tissue was scanned over the central 2.0 mm with a 10 μm step size in the z-axis, extending from above the corneal surface to below the endothelium. Images with 512 pixel × 512 pixel resolution were recorded on 3 locations in the central region of each tissue. Image visualization was performed using proprietary Leica software. The TPEF depth profile across the stroma of

each tissue, $C(z)$, was retrieved by averaging, for each z-depth, the fluorescence signal within a region of interest with 433 μm × 433 μm diameter. The SHG signal was used to detect the anterior and posterior stromal interfaces and hence to align the TPEF intensity profile with stromal thickness.

Supplement A (available at http://jcrsjournal.org) and a previous paper[27] show the details of the calibration procedures and data analysis.

Statistical Analysis

Data are given as the mean ± SD. The Student t test was used to statistically compare the riboflavin stromal concentration values achieved before and after UVA irradiation in each group (paired data) and the difference in riboflavin consumption caused by transepithelial and stromal UVA irradiation between groups (unpaired data). Sample size was calculated to determine a mean difference of 11% ± 10% between groups at a statistical significance of 5% and a power of 81%. A commercial software program (KyPlot, KyensLab Inc.) was used for statistical testing.

RESULTS

Before the experiments, the mean CCT was 568 ± 13 μm (intact epithelium) and 547 ± 12 μm (intact epithelium) in the study group and control group, respectively. No differences in any other baseline characteristic were found in samples between the 2 groups. After transepithelial and conventional CXL treatments, the mean CCT was 601 ± 21 μm (intact epithelium) and 516 ± 18 μm (epithelium removed), respectively.

In the study group, the mean stromal riboflavin concentration was 0.008% ± 0.003% and 0.004% ± 0.002% (P = .001) immediately after corneal soaking and UVA irradiation of the cornea, respectively (Table 1). In all cases, the highest concentration of stromal riboflavin was found in the most anterior 50 μm stromal depth, with a maximum of 0.038%; the concentration profile of riboflavin rapidly decreased in the mid stroma, showing a mean value of 0.005% ± 0.003% in the posterior stroma. After transepithelial UVA irradiation of the cornea, the riboflavin concentration decreased almost homogeneously across stromal depth. Figure 1, *A* and *B*, shows the stromal riboflavin concentration–depth profiles during transepithelial CXL with the nanotechnology-based riboflavin in 2 representative cases.

After stromal soaking with dextran 20.0%-enriched riboflavin solution 0.1%, the riboflavin concentration–depth profile showed highly consistent results in all corneal specimens (Table 2 and Figure 1, *C*). The mean stromal riboflavin concentration was 0.017% ± 0.001%, which was significantly greater than that found in the study group (P = .001). The riboflavin peak concentration (0.020% ± 0.001%) was found in the anterior stroma between 100 μm and 250 μm depth. The concentration of riboflavin was almost constant up to the mid stroma, then slightly decreased toward the endothelium, although riboflavin was still enriched in the most posterior stroma (0.015% ± 0.001%). After UVA irradiation of the cornea, the mean stromal riboflavin concentration dropped to 0.006% ± 0.000% (P < .001) and the consumption of riboflavin was constant throughout the stromal depth in all cases.

There were significant differences in consumption of stromal riboflavin between tissues that had transepithelial and stromal UVA irradiation with the same density energy of 5.4 J/cm^2 (P < .01). After irradiation of the corneal stroma using the 10 mW/cm^2 UVA device for 9 minutes, the mean consumption of riboflavin was 67% ± 2%; it was 52% ± 13% after irradiation of the cornea through the epithelium with the same UVA device (Table 1 and Table 2).

The 3-dimensional reconstruction of the riboflavin concentration–depth profile during the transepithelial nanotechnology-based and standard CXL protocols in 2 representative cases are shown in Figure 2 and Figure 3, respectively.

DISCUSSION

In this study, we evaluated a new nanotechnology-based platform to deliver riboflavin in the human corneal stroma through the intact epithelium. The solution consisted of dextran-free hypotonic riboflavin 0.1% base solution with biodegradable polymeric nanoparticles of 2-hydroxypropyl-β-cyclodextrin plus EDTA and trometamol. The nanoparticles of 2-hydroxypropyl-β-cyclodextrin were added as carrier molecules to enhance the solubility of riboflavin; EDTA and trometamol were added to the solution as enhancers to loosen the epithelial tight junctions and further improve the penetration of riboflavin in the stroma through the intact epithelium.[28] Two-photon optical microscopy and spectrophotometry were

Table 1. Riboflavin stromal concentration during transepithelial CXL using nanotechnology-based 0.1% riboflavin solution and 10 mW/cm^2 UVA device for 9 minutes.

Corneal Tissue (Code)	Riboflavin Concentration				Riboflavin Consumption (%)
	After Soaking		After UVA Irradiation		
	%	g/cm^3	%	(g/cm^3)	
143022	0.0051	5.1^{-5}	0.0024	2.4^{-5}	52
143470	0.0067	6.7^{-5}	0.0014	1.4^{-5}	79
143471	0.0085	8.5^{-5}	0.0045	4.5^{-5}	47
143777	0.0058	5.8^{-5}	0.0039	3.5^{-5}	40
150962	0.0075	7.5^{-5}	0.0038	3.8^{-5}	49
150938	0.013	1.3^{-4}	0.0071	7.1^{-5}	45

UVA = ultraviolet A

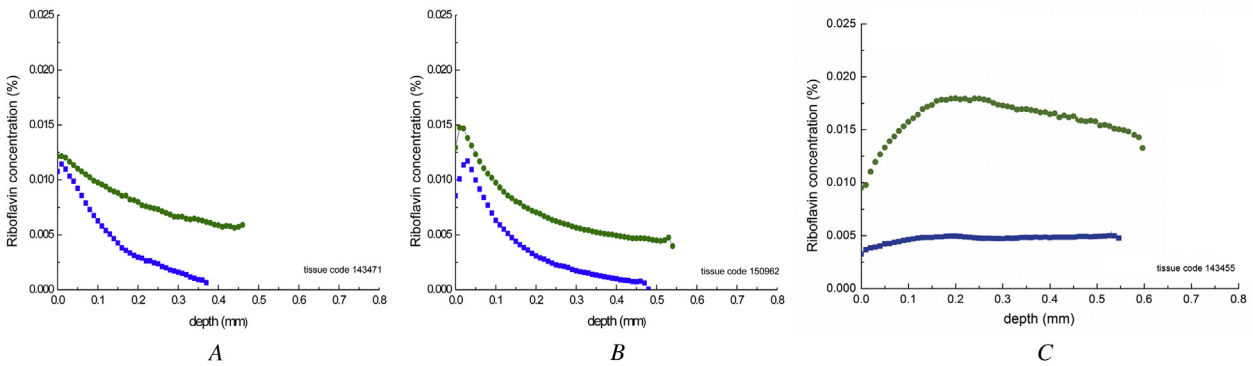

Figure 1. Riboflavin concentration-depth profile calculated by the present all-optical method immediately after corneal soaking (*green curve*) and UVA irradiation of the cornea (*blue curve*). *A* and *B*: Two representative cases that had transepithelial corneal crosslinking with nanotechnology-based riboflavin. *C*: One representative case of standard corneal crosslinking. The *x*-axis shows the stromal penetration depth of riboflavin calculated by the integrated all-optical method. (It does not show the CCT.)

used to measure the stromal concentration of riboflavin and its distribution profile across stromal depth. The results were compared with those of control samples, which were treated using the conventional stromal soaking protocol for CXL.

In the study group, riboflavin was primarily enriched in the most anterior stroma, and its concentration–depth profile rapidly decreased across the mid and posterior stroma. The average stromal concentration of riboflavin was 50% lower than the conventional soaking protocol, and the consumption of riboflavin was on average 23% lower than direct UVA irradiation of the stroma. These data are in accordance with the notion that epithelium filters 20% of UVA light across the central corneal area.[28] The samples in the study group and control group were treated using the same UVA device that delivered the same energy dose (5.4 J/cm^2) to the cornea. The new procedure was performed according to the instructions of the manufacturer; therefore, it was not the aim of this study to compensate for the UVA filtering effect of the epithelium. The stromal riboflavin consumption in the study group could be greater than found in the present study if the irradiation protocol for transepithelial CXL had been enhanced according to current knowledge.[28]

The conventional soaking protocol for CXL provided consistent concentration of riboflavin in the central stroma with minimal variation between tissues, in accordance with findings in previous studies.[17,29–31] Immediately after 30 minutes of stromal soaking, the highest concentration of riboflavin was in the anterior 30% stromal depth, although riboflavin was found to permeate the posterior stroma as well. The variable concentration profile of riboflavin in the most anterior stroma before UVA irradiation was likely related to the washing of the stromal interface with sodium chloride 0.9% solution before measurement recordings. This approach was chosen to adhere to clinical guidelines and to replicate, as much as possible, in vivo conditions. In addition, the corneal stroma was gently washed before UVA irradiation was applied and no riboflavin drop was applied during UVA irradiation to minimize the variable window-filtering effect of riboflavin 0.1% and to avoid the additional confounding effect between groups (eg, riboflavin creates UV filtering window-film over the stroma).[28,32]

Using 2-photon microscopy, Cui et al.[30] analyzed the stromal diffusion of a noncommercial riboflavin solution (0.1%; 290 mOsm/mL) dissolved in 0.02% BAK and 100% deuterium oxide in feline corneas with intact epithelium. After 30 minutes of topical application to the corneal surface, they found the mean peak concentration across the most anterior stroma to be 0.03% $\pm$ 0.002%. Bottos et al.[24] evaluated the stromal penetration of a riboflavin 0.5%–5-phosphate nanoemulsion in rabbit corneas with intact epithelium. The authors found that the nanoemulsion was able to penetrate the intact corneal epithelium, achieving stromal concentrations similar to those with the standard soaking protocol, although 2 hours of instillation were required, which made the protocol unsuitable for clinical use. Gore et al.[33] compared several

Table 2. Riboflavin stroma concentration during standard corneal CXL using 20% dextran-enriched 0.1% riboflavin solution and 10 mW/cm^2 UVA device for 9 minutes.

Corneal Tissue (Code)	Riboflavin Concentration				Riboflavin Consumption (%)
	After Soaking		After UVA Irradiation		
	%	g/cm^3	%	(g/cm^3)	
142875	0.018	1.8^{-4}	0.006	6.0^{-5}	66
143104	0.018	1.8^{-4}	0.006	6.0^{-5}	66
143455	0.016	1.6^{-4}	0.006	4.7^{-5}	70

UVA = ultraviolet A

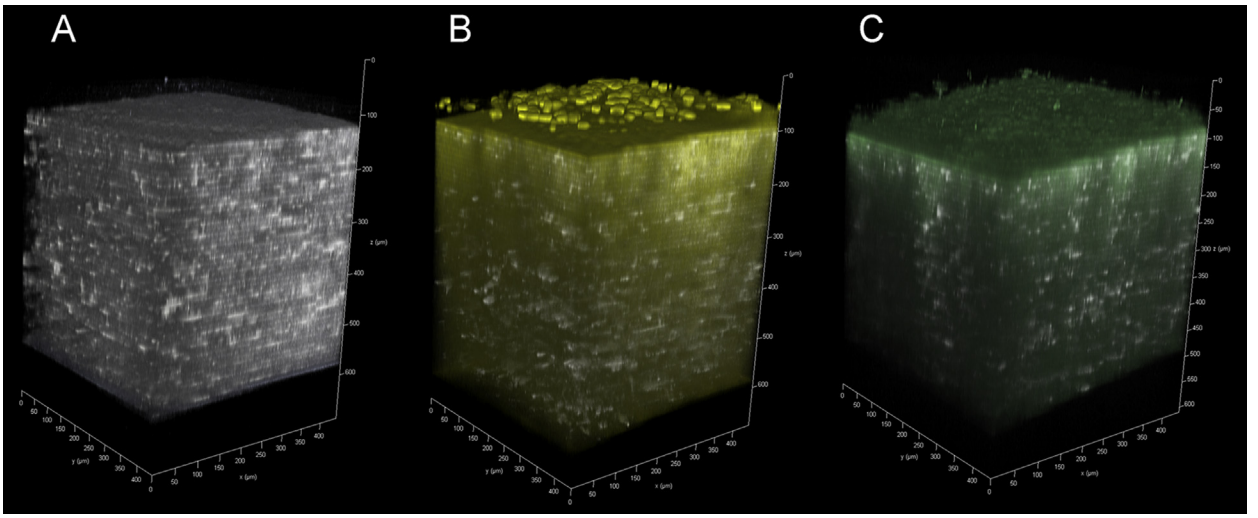

Figure 2. Three-dimensional reconstruction of TPEF intensity signal with superimposed SHG signal showing penetration and consumption of riboflavin in the corneal stroma during nanotechnology-based transepithelial corneal crosslinking in a representative case (*false color*). *A*: Superimposed TPEF and SHG 3-dimensional images before treatment. *B*: After corneal soaking with riboflavin; some riboflavin drops remain in the epithelial cells immediately after corneal soaking. *C*: After UVA irradiation with a 10 mW/cm^2 device for 9 minutes.

commercially available transepithelial CXL protocols using 2-photon microscopy in rabbit eyes. These authors found a significantly lower intrastromal concentration of riboflavin for all transepithelial protocols than found with the gold-standard epithelium-off protocol.

In this study, we used human donor corneal tissues that were processed according to standardized protocol[17,34–36]; no damage to the corneal epithelium was observed in any specimen during transepithelial CXL, as previously shown.[36] The use of dextran 20% solution was also effective in maintaining corneal hydration during experimentation of riboflavin–UVA CXL, as previously discussed.[36–38] Tissues with intact epithelium for which the new nanotechnological protocol was used had a 5% average increase in CCT, which was likely caused by the hypoosmolarity of the riboflavin solution.

In our previous work,[36] we evaluated the stiffening effect of the present transepithelial protocol on the most anterior stroma of human donor corneas with atomic force microscopy; the average Young's modulus was 2.5 times greater than untreated controls and 1.4 times greater than the conventional CXL treatment. On the other hand, these biomechanical results were limited to the most 3 μm anterior stromal depth; therefore, they did not provide a full representation of the effect of this new transepithelial protocol to strengthen the biomechanical properties of the

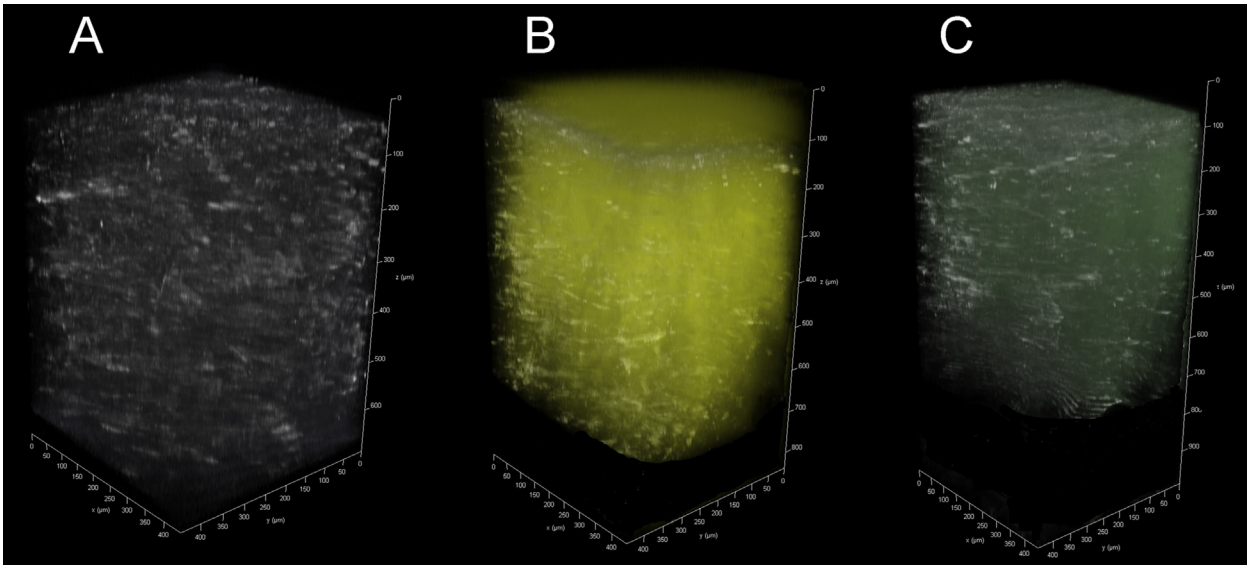

Figure 3. Three-dimensional (3-D) reconstruction of TPEF intensity signal with superimposed SHG signal showing penetration and consumption of riboflavin in the corneal stroma during conventional corneal crosslinking in a representative case (*false color*). *A*: Superimposed TPEF and SHG 3-D images before stromal soaking. *B*: After stromal soaking with riboflavin. *C*: After UVA irradiation with a 10 mW/cm^2 device for 9 minutes.

whole corneal stroma. Because the present nanotechnological-based transepithelial protocol achieved 50% of the riboflavin concentration found in control tissues, further studies are needed to assess its stiffening effect across the whole stroma. Previous experimental work evaluating transepithelial CXL with iontophoresis[25,26,39,40] found a 40% to 50% lower stromal concentration of riboflavin compared with the gold-standard protocol; these measurements were associated with the comparable stiffening effect in the anterior stroma, although the effect on the whole stroma was on average 20% to 30% lower than that with the standard protocol.

We did not aim to assess the changes induced by riboflavin–UVA CXL protocols on collagen fibrils through the analysis of SHG signals. We limited our analysis to the assessment of the alteration of the stroma during experiments, which did not occur. Previous studies[41] have found an increase in the packing of the fibrils after CXL.

In conclusion, the present all-optical study protocol was confirmed to be valuable for assessing the riboflavin concentration–depth profile in the human cornea.[27] It provided a reliable method for comparing a new drug-delivery strategy of riboflavin in the stroma with the standard clinical approach. The method can assist manufacturers and investigators in the development of more efficient transepithelial riboflavin–UVA CXL protocols.

WHAT WAS KNOWN

- Formulation is the most important factor in facilitating the penetration of riboflavin into the corneal stroma through the epithelium.
- Hypotonic dextran-free aqueous solutions enriched with enhancers improve transepithelial diffusion of riboflavin.

WHAT THIS PAPER ADDS

- A riboflavin-base solution with biodegradable polymeric nanoparticles of 2-hydroxypropyl-β-cyclodextrin plus EDTA and trometamol efficiently delivered riboflavin through the intact epithelium in the anterior corneal stroma in 20 minutes.

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