All-Optical Method to Assess Stromal Concentration of Riboflavin in Conventional and Accelerated UV-A Irradiation of the Human Cornea

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Purpose. We investigated the concentration of riboflavin in human donor corneas during corneal cross-linking using two-photon optical microscopy and spectrophotometry.

Methods. Eight corneal tissues were de-epithelialized and soaked with 20% dextran-enriched 0.1% riboflavin solution for 30 minutes. After stromal soaking, three tissues were irradiated using a 3 mW/cm^2 UV-A device for 30 minutes and three tissues irradiated using a 10 mW/cm^2 device for 9 minutes. Two additional tissues were used as positive controls. A Ti:sapphire laser at 810 nm was used to perform two-photon emission fluorescence (TPEF) and second harmonic generation axial scanning measurements in all specimens before and after stromal soaking and after UV-A irradiation. In addition, spectrophotometry was used to collect the absorbance spectra of each tissue at the same time intervals. Analysis of the absorbance spectra and TPEF signals provided measures of the concentration depth profile of riboflavin in corneal stroma.

Results. After stromal soaking, the average peak concentration of riboflavin (0.020% ± 0.001%) was found between a stromal depth of 100 and 250 μm; the concentration of riboflavin was almost constant up to 320 ± 53 μm depth, then decreased toward the endothelium, though riboflavin was still enriched in the posterior stroma (0.016%% ± 0.001%). After conventional and accelerated UV-A irradiation, the concentration of riboflavin decreased uniformly 87% ± 2% and 67% ± 3% ($P < 0.001$), respectively.

Conclusions. The combined use of two-photon optical microscopy and spectrophotometry provides relevant information for investigating the concentration of riboflavin in corneal stroma. The method can assist with the assessment of novel riboflavin formulations and different UV-A irradiation protocols.

Keywords: cross-linking, riboflavin, two-photon optical microscopy, spectrophotometry

The clinical outcome of corneal cross-linking by UV-A excitation of stromal riboflavin depends on the intrinsic tissue properties and the homogeneous distribution of riboflavin in the corneal stroma.[1] Although UV-A irradiation of the cornea soaked with riboflavin is an established procedure for halting the progression of keratoconus, to our knowledge there is as yet no structured study that evaluated the concentration depth profile of riboflavin after conventional stromal soaking and UV-A irradiation of the stroma in human corneas. On the other hand, understanding the penetration of riboflavin in the stroma after conventional soaking as well as its consumption after different UV-A irradiation protocols may be valuable to improve and standardize clinical protocols of corneal cross-linking.[2]

Two-photon multimodal imaging of the cornea can offer detailed information about stromal microstructures and diffusion of fluorescent molecules across the stroma.[3] Two-photon emission fluorescence (TPEF) and second harmonic generation (SHG) signals are two nonlinear optical phenomena of interest for in-depth imaging of the cornea, providing both structural and functional information on the tissue.[4]

The intrinsic stromal TPEF signal is weak and can be registered by exciting the tissue between 750 nm and 826 nm; it is based on the grade of cross-links between collagen fibrils and on the nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide content within keratocytes. Second harmonic generation involves nearly simultaneous absorption of two photons of the same wavelength by noncentrosymmetric molecules such as collagen, with subsequent emission of a single photon of high-energy light that is half of the wavelength of the excitation light.[5] The SHG signal originates from collagen fibrils when exciting the corneal tissue at wavelengths between 790 and 860 nm.

The absorption spectrum for riboflavin shows multiple absorption peaks, including 370 and 449 nm and an emission maximum at 530 nm.[1,6,7] In this context, the 810-nm femtosecond (fs)-pulsed excitation wavelength can be effectively used to image the native and riboflavin-doped corneal

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tissue and to simultaneously collect TPEF and SHG signals from the stroma during corneal cross-linking.

Fluorescence microscopy has been already used to measure the three-dimensional distribution of riboflavin in feline, bovine, porcine and rabbit corneas.[8–15] However, several assumptions have been made regarding tissue properties and methodology that made it difficult to directly translate the results to the clinic. In this paper, we present an integrated approach to evaluate the concentration of riboflavin within the human corneal stroma using two optical methods, such as two-photon optical microscopy and spectrophotometry. In each human donor tissue, stromal riboflavin concentration was estimated after conventional soaking using dextran-enriched solutions and after UV-A irradiation of the stroma using either the conventional (3 mW/cm^{2}UV-A device for 30 minutes) or the accelerated (10 mW/cm^2 UV-A device for 9 minutes) cross-linking protocols.

Methods

Corneal Tissues

Eye bank–donor corneoscleral tissues from different donors that are not suitable for transplantation were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy). The tissues were explanted between 3 and 22 hours after death (mean, 12 ± 5 hours) and cultivated at 30°C in corneal storage medium. Donors (mean age, 59 ± 12 years) did not have a history of corneal pathologies or eye surgery. Inclusion criteria included an endothelial cell density of 1700 cells/mm^2 or greater. 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 National Research Council (CNR) research ethics and bioethics advisory committee. Each corneoscleral tissue was kept in 20% dextran-enriched storage solution at room temperature for 30 minutes before commencing each session of experiments.

Corneal Cross-linking

Three tissues (n = 3; conventional group) underwent conventional corneal cross-linking, and three tissues (n = 3; accelerated group) underwent accelerated corneal cross-linking. Each corneoscleral tissue was placed in an artificial anterior chamber (AAC) (Coronet; Network Medical Products Ltd., Ripon, UK), pressurized with the AAC filled with 0.9% sodium chloride using a 5-mm syringe, and treated according to clinical guidelines. After measuring central corneal thickness with an ultrasound corneal pachymeter (Pachmate; DGH, Exton, PA, USA), the epithelium was removed using an Amoils' brush; thereafter, a solution containing 0.1% riboflavin and 20% dextran (Ricrolin; Sooft Italia SpA, Montegiorgio, Italy) was instilled every 3 minutes for 30 minutes before UV-A irradiation. After corneal soaking, the stromal surface of each tissue was gently washed for 20 seconds using sodium chloride 0.9% solution. The tissues were then irradiated with an UV-A lamp (370 ± 8 nm) with an irradiance of 3 mW/cm^2 for 30 minutes (conventional group: Vega 3mW; CSO Opthalmics, Florence, Italy) or 10 mW/cm^2 for 9 minutes (accelerated group: Vega 10mW; CSO Opthalmics). Both UV-A devices were calibrated with a power meter before corneal irradiation, and an irradiation area of 8-mm diameter was used in all cases, with the delivery systems located 56 mm from the cornea.

In addition, one tissue (n = 1, submerged case) underwent submerged soaking with 20% dextran-enriched 0.1% riboflavin solution for 6 hours and then was irradiated using the 10 mW/cm^2 device for 9 minutes. Another tissue (n = 1, drop case), after soaking the corneal stroma with riboflavin for 30 minutes, underwent conventional corneal cross-linking, while adding a drop of the 20% dextran-enriched 0.1% riboflavin solution every 3 minutes over the stromal surface during UV-A irradiation, as usually done in clinical practice.

Spectrophotometry

In each session of experiments, spectrophotometry measurements were performed before two-photon microscopy imaging. The corneoscleral tissue specimen was placed facing upward on a quartz microscope slide and mounted on a custom-designed spectrophotometer optical setup.[1,16] The light beam (200- to 2500-nm range) was generated by a deuterium-halogen lamp (AvaLight-DH-S-BAL; Avantes BV, Apeldoorn, The Netherlands) and collimated using a 6.0-mm diameter lens with 8.7-mm focal length. The light was delivered to the corneal surface using a 600-μm diameter solar-resistant optical fiber with 0.22 numerical aperture (FC-UV-600-2-SR; Avantes BV). The resulting beam was focused at the front surface of the central cornea with a diameter of 2.0 mm. After passing through the cornea, the light beam was collected and focused by a lens, with the same aforementioned characteristics, on an analogous solar-resistant optical fiber to a spectrophotometer (Avaspec 2048L; Avantes BV), which was connected to a computer for data acquisition and processing.

For each tissue, an absorbance spectrum was measured in the 300- to 700-nm range over the central corneal region. Before each session of measurements, the setup was calibrated by acquiring dark and white signals. The dark signal was obtained by turning off the lamp; the white signal was acquired with the light beam passing through the quartz microscope slide. The obtained data were expressed as spectrum of absorbance, also called optical density. The absorbance spectrum acquired for each sample was therefore referenced with respect to the calibration curve, which was obtained using riboflavin dilutions lower than 0.01%. Any potential loss in light transmission due to the quartz microscope slide and to the refractive index mismatch between the corneal tissue and air was minimized because each cornea was measured under the same conditions before and after corneal soaking with riboflavin and after UV-A irradiation.[16]

Two-Photon Microscopy Imaging

The two-photon microscopy setup used in this study was based on an upright microscope (Leica DM6000CS; Leica Microsystems GmbH, Wetzlar, Germany). A Ti:sapphire laser (VISION II; Coherent Inc., Santa Clara, CA, USA) with an integrated proprietary prism-based unit designed to compensate the broadest range of group velocity dispersion was used as excitation source. This laser presents a tunable wavelength range from 680 to 1080 nm, operating with a pulse width of 140 fs at 80-MHz repetition rate. The laser power was attenuated by an electro-optic modulator and then coupled into the scanner (Leica SP8-Spectral Scan-Head; Leica Microsystems GmbH), where it passed through the x-y scanning module, allowing scanning in the x-y focal plane, before being focused by a water-immersion objective with a working distance of 2.5 mm (HCX IRAPO 25x/0.95 NA; Leica Microsystems GmbH).

Each tissue was placed upward on a quartz microscope slide under the microscope and illuminated by the laser tuned to 810 nm. The laser power was 15 mW before entering the water immersion objective; it was measured before and during each session of measurements in order to allow direct comparison of measurements between different sessions of

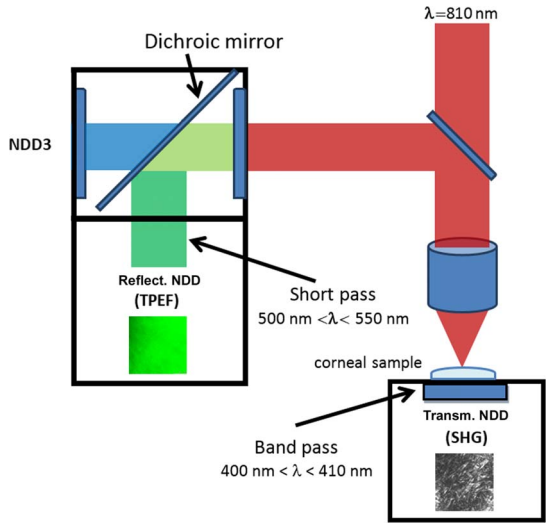

FIGURE 1. Optical setup of the two-photon microscopy used in the present study. Near-infrared femtosecond-pulsed excitation ($\lambda = 810$ nm) of the cornea provides both morphological and structural information on the stroma by SHG signals and on the stromal diffusion of riboflavin by TPEF. The signals are detected simultaneously by using two detection channels in the forward and backward directions.

experiments. The TPEF light was collected in a backward direction by a nondescan detector (NDD) for reflected light, and the SHG signal was collected in a forward direction by a NDD in the transmission path of the microscope. For the transmission NDD unit, the incoming light first goes through a short pass filter (SP680; Leica Microsystems GmbH) ($\lambda < 680$ nm) and then is filtered by a 10-nm full width at half-maximum band-pass filter centered at 405 nm (FF01-405/10-25; Semrock, Inc.) in order to image only the SHG forward signal emitted by the corneal stroma. For the reflection NDD unit, the light is filtered by the infrared filter (SP680; Leica Microsystems GmbH) and then encounters a dichroic beam splitter (Di02R405-25x36; Semrock, Inc.). Therefore, the reflected light path is filtered by a band-pass filter (525/50; Semrock, Inc.) and enters into the reflection NDD unit. The NDD sensitivity settings for collecting the TPEF light were the same for all tissue specimens; this method permitted us to compare data between different sessions of experiments. A schematic of the optical setup is summarized in Figure 1.

For each tissue, two-photon microscopy imaging was performed before and after stromal soaking with 20% dextran-enriched riboflavin and after UV-A irradiation of the corneal stroma. Each sample was scanned over the central 2 mm with a 10-μm step size in the z-axis, extending from above the corneal surface to below the endothelium. Images with 512×512 pixel resolution were recorded of three locations in the central region of each tissue. Image visualization was carried out using proprietary Leica software and an image processing package (Image J software, http://www.imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) using custom-written macros. The TPEF depth profile across the stromal thickness of each tissue, $C(z)$, was retrieved by averaging, for each z-depth, the fluorescence signal within a region of interest with 433×433-μm diameter.

Analysis of Stromal Riboflavin Concentration

At 810-nm excitation, the TPEF signal emitted by native corneal stroma is weak and generated by intracellular and extracellular fluorophores, such as NADPH and collagen, respectively. After stromal soaking with riboflavin, two-photon excitation at 810 nm produces a high TPEF signal in the 500- to 550-nm spectral range, which is generated by riboflavin. By subtracting the fluorescence signal of the corneal tissue collected before and after riboflavin soaking within the stromal depth, we obtained the TPEF profile of the riboflavin penetrated in the stroma. In addition, we retrieved the TPEF profile of riboflavin in the stroma after UV-A irradiation by subtracting the endogenous TPEF from the signal collected at the end of each session of measurements. The SHG signal was used to detect the anterior and posterior stromal interfaces and hence to align the TPEF intensity profile with stromal thickness.

The calibration curve of the TPEF signal was performed by using the same excitation laser power used for the experiments. Different dilutions, or mass concentrations, in water in the 0.0025% to 0.0050% range of the commercial riboflavin solution were imaged by varying the voltage of the reflection NDD unit; this concentration range was chosen because self-quenching phenomena of fluorescence were absent. For each voltage, a linear regression curve was calculated in order to fit the TPEF signals to each known concentration of riboflavin; therefore, the appropriate coefficients were extracted for the calibration. Nevertheless, the evaluation of riboflavin concentration by two-photon fluorescence microscopy could be influenced by various factors, such as the changes of the chemical and physical properties of riboflavin in the corneal matrix, the intrinsic fluctuation of the laser power during the experimental session, the variation of refractive index between the corneal tissue and the solution used for calibration, the nonlinear photon conversion phenomena occurring within corneal stroma, and the fluorescence quenching phenomena (via fluorescence lifetime decrease) within the stromal matrix.[17,18] All these factors contribute to reduce the reliability of the riboflavin mass concentration estimation in the corneal stroma. For this purpose, we developed an integrated approach to accurately assess the riboflavin depth concentration in corneal stroma by taking into account both the optical density, based on the analysis of electronic transition levels, and the TPEF signals collected by each corneal specimen (Fig. 2).

The amount of riboflavin, c_{ribo}, absorbed by the corneal stroma was determined by subtracting the optical density of the cornea measured both after riboflavin soaking and UV-A irradiation to the pretreatment one. The experimental results, $A_p(\lambda)$, were fitted with an analytical expression, $A(\lambda)$, for extracting the stromal concentration of riboflavin:[1,16]

$$A(\lambda) = c_{\text{ribo}} A_w s + a\lambda^{-n} + b \qquad (1)$$

where A_w is the unitary riboflavin absorbance, s is the central corneal thickness, and the quantity $a\lambda^{-n} + b$ takes into account the scattering contribution of the stroma. The unknown parameters c_{ribo}, a, b, and n were retrieved by minimizing the sum of the squares of residual $r(\lambda) = A_p(\lambda) - A(\lambda)$; the riboflavin concentration c_{ribo} was estimated for $\lambda = 445$ nm. For all specimens, the value $n = 2$ was obtained from the minimization. By knowing the concentration of riboflavin achieved by spectrophotometry, it is possible to rescale the profile of TPEF measurements in the corneal stroma, $C(z)$, in order to take into account the deviation between the mean value of $C(z)$ and the absolute riboflavin concentration, c_{ribo}, by finding the factor K, which satisfies the following equation:

$$c_{\text{ribo}} = K \frac{1}{s} \int_0^s C(z)dz \qquad (2)$$

This approach ensured that all factors influencing the assessment of the riboflavin concentration in corneal stroma were taken into account. The stromal riboflavin concentration

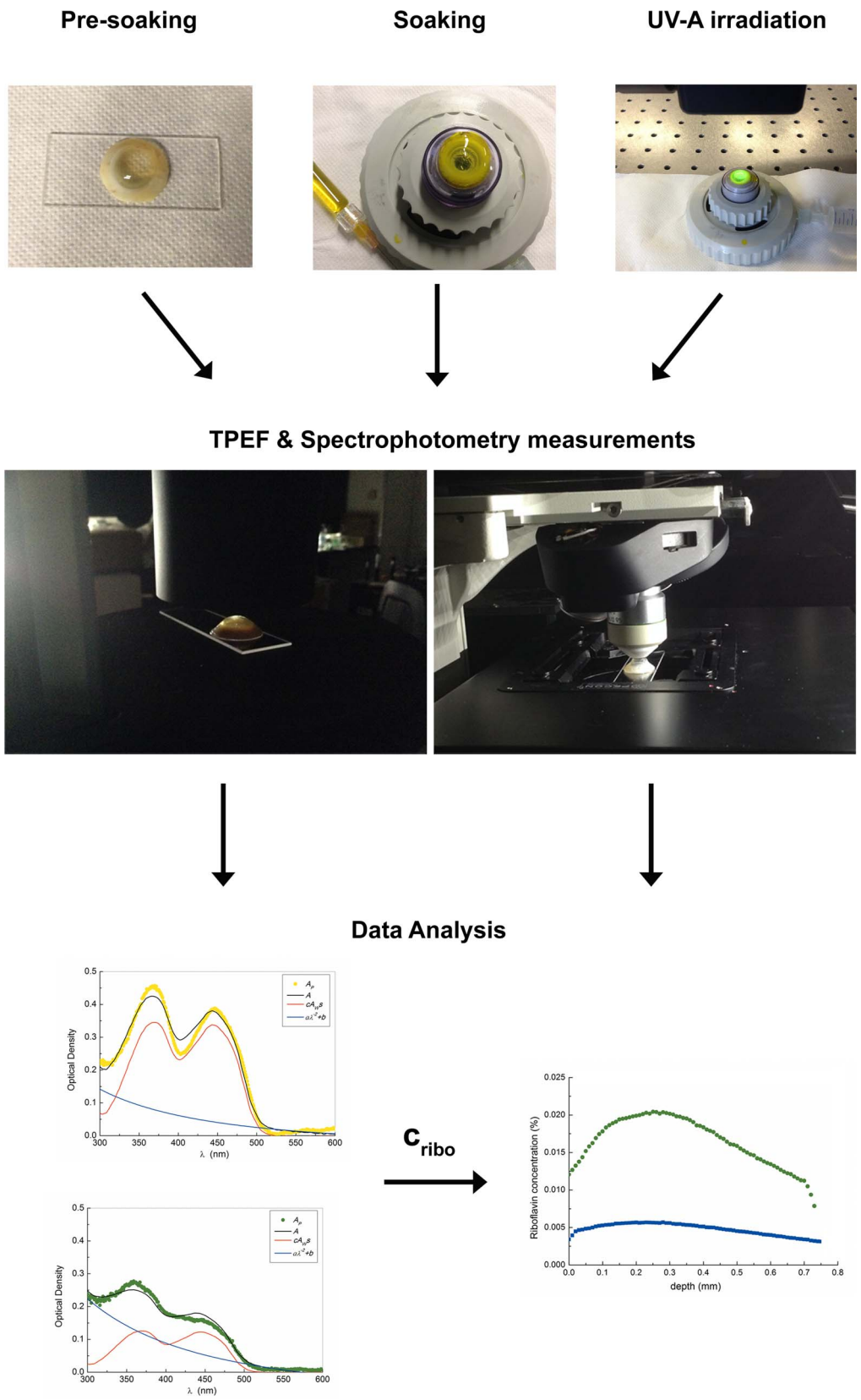

Figure 2. Diagram summarizing the main steps of the experiments performed in the present study. Spectrophotometry and two-photon optical microscopy measurements were done at baseline (presoaking), after stromal soaking with 20% dextran-enriched 0.1% riboflavin solution, and after irradiation of the stroma using either 3 mW/cm^2 or 10 mW/cm2 UV-A device. Data collected by spectrophotometry (optical density) and two-photon optical microscopy (TPEF signal) at each time interval were used for assessing the riboflavin concentration profile in corneal stroma after soaking with riboflavin and UV-A irradiation. The results $A_p(\lambda)$ were fitted with an analytical expression, $A(\lambda) = c_{ribo}A_W s + a\lambda^{-2} + b$, for extracting the riboflavin concentration, c_{ribo}; the profile of TPEF measurements, $C(z)$, was rescaled by using equation 2 in order to take into account the fluorescence quenching phenomena of riboflavin within the stroma (see main text for details).

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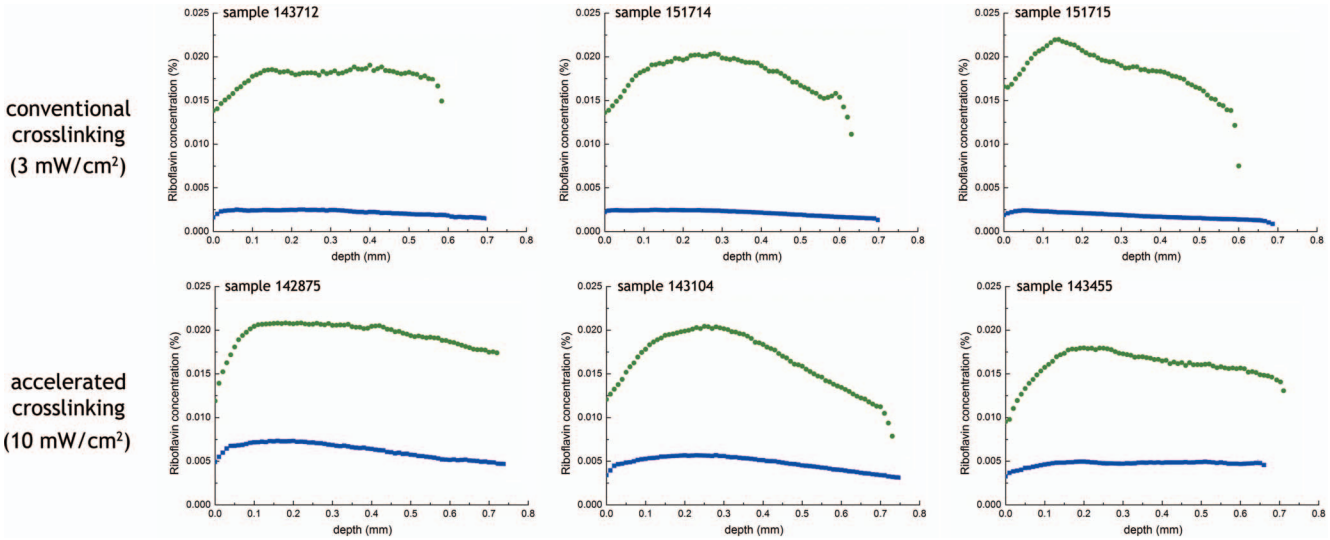

Figure 3. The TPEF intensity signal profiles showing the stromal riboflavin concentration after stromal soaking (*green curve*) and UV-A irradiation (*blue curve*) in all tissue specimens. Immediately after stromal soaking with riboflavin for 30 minutes, there were no differences in stromal concentration of riboflavin between specimens. *Upper row:* After conventional UV-A irradiation (using 3 mW/cm^2 device for 30 minutes), the concentration of riboflavin decreased an average of 87% ± 2% within the corneal stroma. *Lower row:* After accelerated UV-A irradiation (using 10 mW/cm^2 device for 9 minutes), it decreased an average of 67% ± 3% ($P < 0.001$).

provided by the TPEF signal alone was found to be lower, up to a maximum of 20%, than the optical density. Such underestimation was mostly due to the fluorescence-quenching phenomena of riboflavin within the stromal matrix.

Results

After stromal soaking with 20% dextran-enriched 0.1% riboflavin solution, the TPEF intensity profile collected in the central stroma showed highly consistent results between corneal specimens (Fig. 3). On average, stromal riboflavin concentration was 0.017% ± 0.001%, with no differences between tissues, which were subsequently irradiated using the conventional (0.017% ± 0.001%) or the accelerated (0.017% ± 0.001%) UV-A protocol. The mean riboflavin concentration peak (0.020% ± 0.001%) was found in the anterior stroma between depths of 100 and 250 μm (mean, 177 ± 54 μm; 30% stromal depth). The concentration of riboflavin was almost constant up to 250 to 400 μm stromal depth (mean, 320 ± 53 μm; 55% stromal depth), then decreased toward the endothelium, though riboflavin was still enriched in the most posterior stroma (0.016% ± 0.001%).

After UV-A irradiation, stromal concentration of riboflavin decreased in all tissue specimens. We found significant differences between tissues that underwent conventional and accelerated irradiation protocols (Student's *t*-test; $P < 0.001$). After irradiation using a 3 mW/cm^2 UV-A device for 30 minutes, the average consumption of riboflavin was 87% ± 2%, with residual stromal concentration of 0.002% ± 0.0002% (Fig. 4); after irradiation with a 10 mW/cm^2 device for 9 minutes, it was 67% ± 3%, with residual stromal concentration of 0.005% ± 0.0008% (Fig. 5). In all cases, the consumption of riboflavin was constant throughout the stromal depth (Table).

After immerging a corneal specimen in 20% dextran, 0.1% riboflavin solution for 6 hours (submerged case), we found a mean stromal concentration of 0.033%, decreasing to 0.008% after accelerated UV-A irradiation (75% lower concentration of riboflavin). Immediately after immersion, the concentration of riboflavin was almost constant within the stroma, showing two peaks at the anterior and posterior interfaces; after UV-A

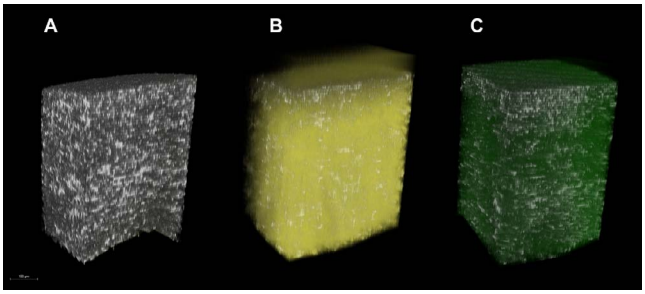

Figure 4. Three-dimensional reconstruction of TPEF intensity signal, with superimposed SHG signal, showing penetration and consumption of riboflavin in the corneal stroma during conventional corneal crosslinking in a representative case. (**A**) Superimposed TPEF and SHG three-dimensional images before stromal soaking, (**B**) after stromal soaking, and (**C**) after UV-A irradiation with a 3 mW/cm^2 lamp for 30 minutes. *Scale bar:* 100 μm. The contrast and intensity of each image (*false colors*) were adjusted in order to enhance details.

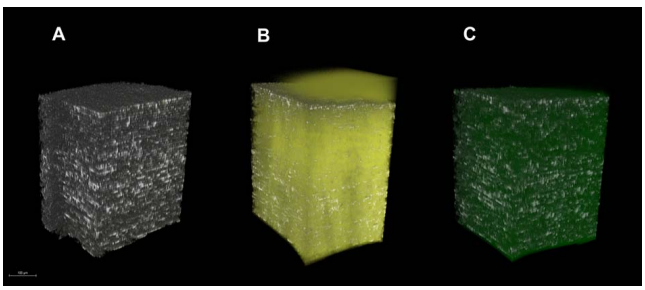

Figure 5. Three-dimensional reconstruction of TPEF intensity signal, with superimposed SHG signal, showing penetration and consumption of riboflavin in the corneal stroma during accelerated corneal crosslinking in a representative case. (**A**) Superimposed TPEF and SHG three-dimensional images before stromal soaking, (**B**) after stromal soaking, and (**C**) after UV-A irradiation with 10 mW/cm^2 lamp for 9 minutes. *Scale bar:* 100 μm. The contrast and intensity of each image (*false colors*) were adjusted in order to enhance details.

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TABLE. Stromal Concentration of Riboflavin (%) in Each Tissue After Stromal Soaking and UV-A Irradiation of the Stroma

Tissue Code	Riboflavin Soaking Protocol	Average Stromal Concentration, %	Anterior 250-μm Stromal Concentration %	Posterior Stromal Concentration %	UV-A Irradiation Protocol	Average Stromal Concentration %	Anterior 250-μm Stromal Concentration %	Posterior Stromal Concentration %	Consumption, % (anterior %–posterior %)
142875	Two drops every 3 min for 30 min	0.018	0.019	0.017	10 mW/cm^2 for 9 min	0.0060	0.0069	0.0055	65% (64%–66%)
143104	Two drops every 3 min for 30 min	0.018	0.020	0.016	10 mW/cm^2 for 9 min	0.0060	0.0067	0.0055	66% (66%–65%)
143455	Two drops every 3 min for 30 min	0.016	0.015	0.016	10 mW/cm^2 for 9 min	0.0047	0.0045	0.0048	70% (70%–70%)
150783	Immersed for 6 h	0.033	0.033	0.030	10 mW/cm^2 for 9 min	0.0085	0.008	0.007	75% (75%–76%)
143712	Two drops every 3 min for 30 min	0.017	0.017	0.016	3 mW/cm^2 for 30 min	0.0022	0.0024	0.0020	86% (85%–87%)
151714	Two drops every 3 min for 30 min	0.016	0.018	0.014	3 mW/cm^2 for 30 min	0.0021	0.0024	0.0019	86% (86%–86%)
151715	Two drops every 3 min for 30 min	0.017	0.020	0.018	3 mW/cm^2 for 30 min	0.0018	0.0021	0.0015	89% (89%–89%)
143882	Two drops every 3 min for 30 min	0.016	0.025	0.011	3 mW/cm^2 for 30 min applying one drop every 3 min	0.0077	0.0120	0.0056	52% (52%–52%)

Data are given for the full stroma and either for the anterior 250 μm or the posterior stroma (>250 μm).

irradiation, the consumption of riboflavin was constant throughout the stromal depth (Fig. 6A).

Application of one drop of 20% dextran, 0.1% riboflavin every 3 minutes during conventional UV-A irradiation (drop case), as usually done in clinical practice, caused a consumption (50%) lower than expected of stromal riboflavin, which decreased on average from 0.016% to 0.008% (Fig. 6B).

DISCUSSION

The conventional soaking protocol for corneal cross-linking provided consistent concentration of riboflavin in the central stroma, with minimal variation between tissues (mean: 0.017%; SD: 0.001%). Riboflavin was enriched also in the posterior stroma, as previously found using either spectrophotometry or two-photon optical microscopy.[14,19] This result provided the evidence that standard stromal soaking with 20% dextran-enriched 0.1% riboflavin solution for 30 minutes attains a unique baseline reference for subsequent UV-A irradiation of the tissue. Previous studies[20,21] have shown that 20 minutes of stromal soaking with 20% dextran-enriched 0.1% riboflavin solution achieves greater than or equal to 85% of concentration measured after 30 minutes; on the other hand, we did not measure the final concentration of riboflavin after UV-A irradiation of the cornea, which would have resulted in total consumption of riboflavin leading to potentially severe damage to endothelial cells. In addition, at 20 minutes we have found variable results (data not published) in riboflavin concentration within the stroma (ranging between 0.012% and 0.016%) between tissues, which would limit standardization of the procedure in the clinic.

The peak concentration of riboflavin was found in the anterior 30% stromal depth (mean, 177 ± 54 μm); in addition, riboflavin was found to permeate mostly the 55% anterior stroma, though riboflavin was found also in the most posterior stroma. The concentration profile of riboflavin in the most anterior stroma was likely related to the washing of the stromal interface with sodium chloride 0.9% solution before acquisition of spectrophotometry and two-photon measurements. We adhered to clinical guidelines in order to replicate as much as possible the methods of in vivo conditions.

One of the tissue specimens was immersed for 6 hours in 20% dextran-enriched 0.1% riboflavin solution and served as positive control; this experiment was valuable to help us understand the maximum threshold of concentration (0.033%) that riboflavin could reach in the human corneal stroma. Keeping the tissue in the riboflavin solution longer did not increase significantly the amount of riboflavin in the stroma (data not published). Previous studies overestimated the stromal concentration of riboflavin (up to 0.09%),[8–10,13] because the calibration procedures were performed using solution at 0.1% riboflavin, for which a fluorescence self-quenching phenomenon occurs.[17,18] In addition, previous fluorescence measurements were done on nonhuman tissue specimens, which further increases ambiguity when translating results to the clinic. We paid particular attention to minimize any uncertainties in the determination of riboflavin concentration in the human corneal stroma and made efforts to directly compare the results between tissues and different treatment protocols with accuracy.

Several laboratory and clinical studies[22–26] have been done with the aim to demonstrate the equivalence between conventional and accelerated UV-A irradiation protocols. The hypothesis of equivalence has been made based on the

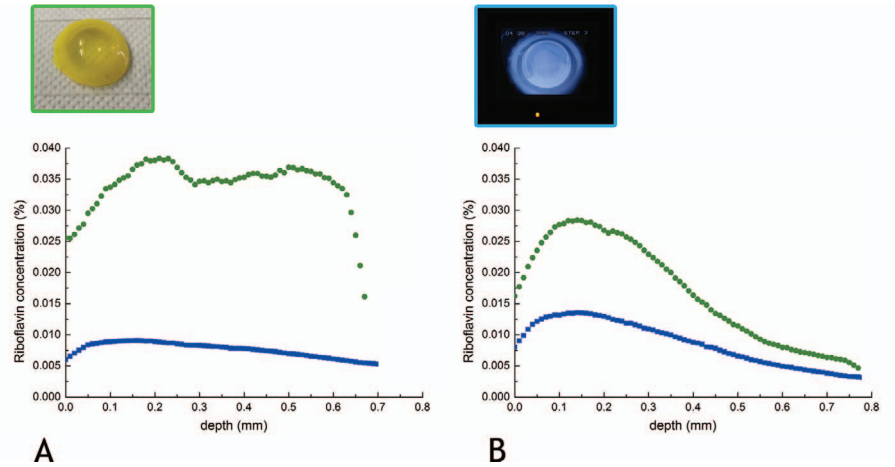

FIGURE 6. The TPEF intensity signal profile showing the stromal riboflavin concentration in two corneal tissue specimens, which were used as positive controls. (**A**) The TPEF intensity signal profiles showing the concentration of riboflavin within the stroma after keeping the tissue immersed for 6 hours in 20% dextran-enriched 0.1% riboflavin solution (*green curve*) and after UV-A irradiation with 10 mW/cm^2 device (*blue curve*). By keeping the corneoscleral tissue immersed in the riboflavin solution for a long time, we determined the maximum threshold of concentration that riboflavin could achieve within the corneal stroma. (**B**) The TPEF intensity signal profiles before (*green curve*) and after (*blue curve*) conventional corneal cross-linking (3 mW/cm^2 device), adding one drop of riboflavin every 3 minutes during UV-A irradiation. Adding drops of 0.1% riboflavin solution over the stromal surface during UV-A irradiation reduces the total amount of riboflavin consumed.

Bunson-Roscoe law of reciprocity.[27,28] On the other hand, this hypothesis has been shown not to be true in biological tissues.[1] Accordingly, Schwarzschild[29] proposed a modification of the reciprocity law, which later became known as Schwarzschild's law. In this study, we found significant differences in riboflavin consumption between tissues that have been irradiated with the conventional and accelerated UV-A protocol. The conventional irradiation of the stroma achieved on average 20% more consumption of riboflavin than the accelerated one. It should be the object of further investigation whether this difference may lead to a different amount of additional cross-linking bonds between stromal proteins and therefore to different biomechanical strengthening of the corneal stroma in patients. So far, to our knowledge, no previous experiment provided evidence of such difference,[22,23,25,30] except for a biomechanical study on porcine eyes by Hammer et al.[31] These authors found 15% lower stiffening effect with increasing UV-A irradiance from 3 mW/cm^2 to 9 mW/cm^2 and decreased time from 30 to 10 minutes, respectively. Several factors, including differences in tissue specimens (humans, nonhumans), soaking protocols, UV-A devices, biomechanical testing, etc., make comparison between different studies challenging. Further work is needed to determine the exact relationship between different UV-A irradiation protocols, riboflavin consumption, and biomechanical strengthening of the human corneal stroma. This knowledge would enhance our understanding of the arrangement and interactions between stromal components, which have been shown to be highly correlated to corneal depth. In previous work,[32] the depth-dependent change of stromal mechanical elasticity from anteriorly high to posteriorly low has been correlated with a gradual change of structural collagen features within the stroma. Since the riboflavin consumption was homogeneous throughout stromal depth, we may expect the stromal stiffening gradient not to be greatly changed after corneal cross-linking; however, there will be an overall increase of the stromal mechanical elasticity, as found in previous inflation testing of human cadaver eyes.[33]

In the present work, we also tested the effect of adding one drop of 20% dextran-enriched riboflavin 0.1% solution every 3 minutes over the stromal surface, as usually done in clinical practice. Adding riboflavin 0.1% drops acted as a masking filter, thus decreasing the total amount of riboflavin consumed during conventional UV-A irradiation of the stroma (from average 87% to 50%). However, we did not correlate the riboflavin consumption with stromal stiffening, and no clinically significant conclusions may be derived from this result. In addition, we should consider how protection of the corneal endothelium is needed in real eyes, since the hyperosmolar effect of dextran-enriched riboflavin solution induces 20% to 30% stromal thinning[34] and accordingly the endothelium can be severely damaged by UV-A irradiation. From the present results, it would be recommended to add drops of diluted 0.1% riboflavin solution (0.025%) over the stromal surface to limit the masking effect of riboflavin and maintain the safety of the procedure during UV-A irradiation.

We did not aim to investigate the changes induced by corneal cross-linking on collagen fibrils. We limited our analysis to the assessment that no alteration of the stroma occurred during experiments. On the other hand, previous work[11] has shown an increase in the packing of the fibrils after corneal cross-linking.

In conclusion, the method disclosed in the present study was valuable for understanding the high consistency of stromal riboflavin concentration values reached after the conventional Dresden soaking protocol. In the central cornea, riboflavin is mostly concentrated in the anterior half of the stroma, though it is enriched in the whole stroma. A constant consumption of riboflavin was found throughout the stromal depth after either conventional or accelerated irradiation protocols; nevertheless, there were differences in the total consumption of riboflavin between protocols, with the conventional UV-A irradiation performing slightly better than the accelerated one. The present method can assist with the assessment of either novel delivery strategy of riboflavin in the stroma or the effect of different UV-A irradiation protocols in order to improve the outcomes of corneal cross-linking in patients with progressive keratoconus.

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