LABORATORY SCIENCE

Noninvasive real-time assessment of riboflavin consumption in standard and accelerated corneal crosslinking

Marco Lombardo, MD, PhD, Giuseppe Lombardo, MEng, PhD

Purpose: To estimate the noninvasive riboflavin concentration in the corneal stroma using a new ultraviolet-A (UVA) theranostic device for corneal crosslinking (CXL).

Setting: Vision Engineering Italy srl, Rome, Italy.

Design: Experimental study.

Methods: Fourteen human donor corneas were treated according to conventional (UVA irradiance of 3 mW/cm^2 for 30 minutes) and rapid (10 mW/cm^2 for 9 minutes) riboflavin–UVA CXL protocols using a theranostic UVA device. Five additional samples were treated by 0.5 mW/cm^2 for 9 minutes and used as positive controls to determine riboflavin photodegradation under near ambient lighting conditions. A 20% dextran–enriched 0.1% riboflavin solution was used in all cases. The device consisted of a UVA light source; a red–green–blue camera, which acquires the fluorescence images of the cornea during treatment; and a single-board computer for managing the overall operations and the raw data processing.

Results: Preirradiation stromal soaking for 30 minutes achieved highly consistent intrastromal riboflavin concentration in all tissues (mean 0.015% ± 0.003% [SD]). There were no differences in the kinetics curves of riboflavin consumption between the 2 UVA irradiation protocols; the intrastromal riboflavin concentration decreased exponentially, with a mean constant energy rate of 2.8 ± 0.2 J/cm^2. In the control group, the intrastromal riboflavin concentration decreased quasilinearly.

Conclusions: The theranostic device provided estimates of the intrastromal concentration of riboflavin noninvasively during treatment. In the 3 to 10 mW/cm^2 range of power densities, the consumption of riboflavin in the stroma by UVA irradiation was only energy dependent in accordance with the Bunsen-Roscoe law.

J Cataract Refract Surg 2019; 45:80–86 © 2018 ASCRS and ESCRS

Riboflavin with ultraviolet-A (UVA) corneal crosslinking (CXL) is the most endorsed treatment option for slowing or halting the progression of keratoconus, which is the most common degenerative disease affecting the cornea.[1–4] At present, the standard CXL protocol (ie, the Dresden protocol) is the only treatment protocol with enough clinical evidence to be effective for the management of progressive keratoconus. The treatment protocol consists of removing the corneal epithelium, soaking the corneal stroma with 20% dextran-enriched riboflavin 0.1% solution for 30 minutes, and then irradiating the tissue with a 3 mW/cm^2 UVA device for 30 minutes. To reduce overall treatment time, several treatment protocols have been developed in which the cornea is irradiated with higher UVA energy power.[5–8] The rule of thumb was to maintain the same total dose of energy delivered to the tissue according to the Bunsen-Roscoe law of reciprocity, regardless of the irradiance or exposure time of UVA illumination. On the other hand, this hypothesis has been shown not to be true in biologic tissues and for illumination intensities higher than 18 mW/cm^2.[6] Uncertainties also exist on whether the conventional energy density of 5.4 J/cm^2 might be effective for high-irradiance (or accelerated) CXL protocols[9]; thus, the use of 7.2 J/cm^2 energy density for "accelerated" UVA protocols is under study at present.[10]

Although some studies[8,11] have shown the clinical equivalence in the clinical management of progressive keratoconus between conventional UVA illumination of the cornea (ie, 3 mW/cm^2 for 30 minutes) and the most used of the

Submitted: April 5, 2018 | Final revision submitted: July 7, 2018 | Accepted: July 30, 2018

From Vision Engineering Italy srl (M. Lombardo, G. Lombardo), Rome, and CNR-IPCF (G. Lombardo), Istituto per i Processi Chimico-Fisici, Messina, Italy.

Domenico Arigò, Roberto Caruso, Giuseppe Lupò, and Giuseppe Spinella (Consiglio Nazionale delle Ricerche, Istituto per i processi Chimico Fisici, Messina, Italy) helped in assembling the prototype device and to Chiara Mustarelli (Consiglio Nazionale delle Ricerche, Istituto Nazionale di Ottica) for assistance in preparing documents for ethics committee approval. Sooft Italia Spa – Fidia Pharma group provided the riboflavin solutions used in this work.

Supported by POR FESR LAZIO 2014-2020 (grant A0114E0087).

Corresponding author: Marco Lombardo, MD, PhD, Vision Engineering Italy srl, Via Livenza 3, 00198 Rome, Italy. Email: mlombardo@visioeng.it.

0886-3350/$ - see frontmatter
https://doi.org/10.1016/j.jcrs.2018.07.062

accelerated protocols (ie, 10 mW/cm^2 for 9 minutes), the findings vary in laboratory studies of the corneal mechanical stiffening induced by these 2 irradiation protocols.[5–7] Part of these discrepancies might arise from the lack of objective monitoring methodologies of the riboflavin concentration in the corneal stroma during treatment.

We developed a theranostic UVA prototype device with a red–green–blue (RGB) camera for measuring noninvasively the intrastromal concentration of riboflavin during CXL.[12] Theranostics is a new strategy of precision and personalized medicine for delivering therapy and examining the effect in real time, mainly through in vivo imaging of tissues. The scope of this work was to compare the intrastromal riboflavin concentration between 2 CXL protocols; that is, with UVA irradiance of 3 mW/cm^2 for 30 minutes and with UVA irradiance of 10 mW/cm^2 for 9 minutes. We further evaluated the energy dependence of intrastromal riboflavin consumption from the 2 irradiation protocols.

MATERIALS AND METHODS

Human Donor Tissue

Nineteen total human donor sclerocorneal tissues from different donors were obtained from the Veneto Eye Bank Foundation, Venezia Zelarino, Italy. Inclusion criteria included an endothelial cell density (ECD) greater than 1800 cells/mm.[2] Exclusion criteria included any corneal or ocular surgery. The study adhered to the tenets of Declaration of Helsinki for the use of human tissues and was approved by the National Research Council research ethics and bioethics advisory committee.

Ultraviolet-A Device Optical Setup

The optical setup of the prototype device consists of a UVA light-emitting diode (365 ± 10 nm [SD]); a photodiode, which monitors the UVA power density; and an RGB camera, which acquires the fluorescence images emitted by the cornea under treatment.[13,14] Details of the optical setup and overall device's operation have been described.[12] In brief, the device provides real-time measurement of the intracorneal riboflavin concentration during riboflavin with UVA CXL by processing the green signals of the RGB camera; the main settings of the electro-optical components are managed by a single-board computer. The UVA device sensitivity is 5×10^{-6} g/cm^3. In this study, the concentration of riboflavin was expressed as g/cm^3 or as percentage (%), where 1×10^{-4} g/cm^3 = 0.01%.

Corneal Crosslinking Treatment and Ultraviolet-A Device Operation

Donors (mean 65.2 ± 6.8 years) did not have a history of corneal pathologies or eye surgery. The tissues were explanted between 3 hours and 20 hours postmortem and cultivated at 30°C for 2 weeks in corneal storage medium enriched with 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (25 mL/L), sodium pyruvate (10 mL/L), L-glutamine (10 mL/L), newborn calf serum (20 mL/L), and antibiotics/antimycotics (1:100). The mean cadaver time was 11.3 ± 6.9 hours. The ECD (mean 2044 ± 353 cells/mm^2) was measured using an inverted optical microscopy (Axiovert 25, Carl Zeiss Meditec AG). The sclerocorneal samples were shipped to the laboratory in 6% dextran-enriched corneal storage medium and were used for the experiment within 24 hours.

Each sclerocorneal tissue was placed in an artificial anterior chamber (Coronet, Network Medical Products Ltd.) pressurized by filling it with sodium chloride 0.9% using a 5.0 mm syringe; the tissue was treated according to clinical guidelines. After the corneal epithelium was removed with an Amoils brush (Innovative Excimer Solutions Inc.), the central corneal thickness (mean 502 ± 89 μm) was measured with an ultrasound corneal pachymeter (Pachmate, DGH Technology, Inc.); thereafter, a solution containing riboflavin 0.1% and 20% dextran (Ricrolin, Sooft Italia SpA) was instilled every 2 to 3 minutes for 30 minutes before UVA irradiation, according to the Dresden protocol.[2,3] The tissues were then irradiated using the theranostic UVA device. In this study, 2 different UVA irradiation protocols were used; however, the 2 protocols delivered the same total UVA energy density (5.4 J/cm^2) to the cornea.[15,16] Seven tissues (conventional group) had conventional CXL with UVA irradiance of 3 mW/cm^2 for 30 minutes, and 7 tissues (rapid group) had rapid CXL with UVA irradiance of 10 mW/cm^2 for 9 minutes. Five tissues were treated with 0.5 mW/cm^2 UVA power density for 9 minutes and used as positive controls (control group). The low, not clinically therapeutic UVA energy dose was used to determine the kinetics of riboflavin photodegradation in the human cornea under near ambient lighting conditions. An irradiation area of 9.0 mm diameter was used in all cases.

Before treatment, the device acquired, processed, and averaged the green signals of the RGB camera of the cornea illuminated by UVA light at very low density (<0.6 mW/cm^2) so as not to induce a photodynamic polymerization process of the corneal stroma; this pretreatment value corresponded to the native corneal fluorescence, B_G. This value was subtracted from the acquired averaged fluorescence image value, I_G, emitted by the same cornea during treatment (after soaking or during UVA irradiation). After 30 minutes of corneal soaking with riboflavin, the excess riboflavin was gently washed from the stromal surface of the cornea using sodium chloride 0.9% solution to allow measurement of only the riboflavin in the stroma. At the end of soaking phase, the intrastromal riboflavin concentration was evaluated from the calibration curve of the device by measuring I_G when the cornea was illuminated with less than 0.6 mW/cm^2 UVA power density. The tissues were then irradiated according to the conventional or rapid UVA irradiation protocol. During UVA irradiation, the intrastromal riboflavin concentration was determined from the value acquired of I_G through the calibration curve. This procedure permitted measurement of the intrastromal riboflavin consumption in real time (Figure 1). No riboflavin was applied over the stromal surface during irradiation to prevent bias caused by the variable masking-filtering effect of 0.1% riboflavin drops during UVA irradiation of the cornea, as shown previously.[17,18]

The riboflavin concentration in the corneal stroma after the soaking phase (C) as well as after UVA irradiation ($C_{\text{UVA-3 mW}}$ or $C_{\text{UVA-10 mW}}$) was also assessed independently from the absorbance using a spectrophotometer (Avaspec 2048L, Avantes BV). The measurements and analysis of absorbance spectra have been fully described.[12,17,19]

The energy dependence of the riboflavin consumption for both CXL protocols was evaluated by fitting with an exponential function the intrastromal riboflavin concentration curve normalized with respect to the riboflavin concentration measured at the end of soaking phase as follows: $C_{\text{norm}}(E) = \exp(-E/E_{\text{rate}})$, where E_{rate} is the energy constant. A linear fit on the normalized curve up to 0.5 J/cm^2 energy density was also performed, and the slope of this linear portion of the curve was calculated for all tissues.

Statistical Analysis

Data were given as the mean ± SD. Sample size was calculated to detect a difference of 5% (±5% SD) between the mean riboflavin consumption for the conventional and rapid study groups (allocation ratio 1:1) at a significance level of 5% and a power of 81%. The differences between the 2 UVA irradiance protocols were calculated using the 1-way analysis of variance. The intraclass correlation coefficient (ICC) (2-way random effects model) was calculated to estimate the absolute agreement between the intrastromal concentration of riboflavin calculated with the 2 optical

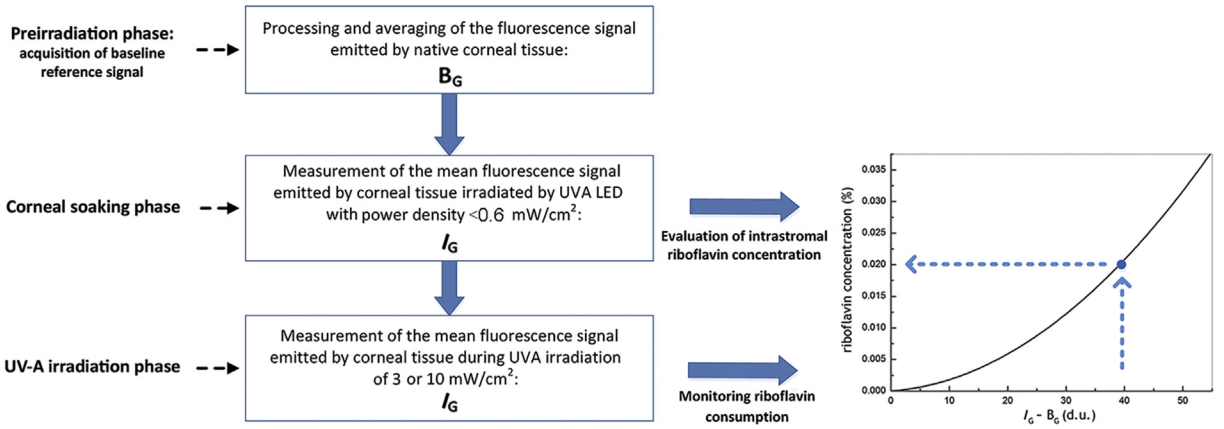

Figure 1. Overall procedure for estimating the corneal intrastromal riboflavin concentration using the theranostic UVA device. The plot shows the calibration curve used to correlate I_G with the intrastromal riboflavin concentration (B_G = native corneal fluorescence acquired at baseline before CXL treatment; d.u. = dimensionless unit; CXL = corneal crosslinking; I_G = mean value of green pixels of the red–green–blue camera, which provides a measure of the whole fluorescence contribution emitted from the cornea enriched with riboflavin during the soaking and irradiation phases; LED = light-emitting diode; UVA = ultraviolet-A).

methods. In addition, the slope of the linear portion of the riboflavin concentration curve during UVA irradiation was calculated for evaluating differences between the 2 protocols.

RESULTS

Corneal soaking for 30 minutes before irradiation achieved highly consistent values of intrastromal riboflavin concentration (mean 0.015% ± 0.003%) between tissues. After UVA irradiation of the cornea, the mean intrastromal riboflavin concentration was 0.003% ± 0.001%, with no significant difference between the 2 UVA irradiation protocols (P = .40).

During UVA irradiation, the stromal riboflavin concentration decreased nonlinearly in all tissues (Figure 2). To assess potential differences between the 2 UVA irradiation protocols, the riboflavin concentration measured from all tissues was normalized to the value obtained at the end of soaking phase (C) and plotted as a function of the UVA energy density (E) delivered to the cornea. The linear slope at the origin of the curves (up to 0.5 J/cm^2) was significantly steeper in the 10 mW/cm^2 protocol than in the 3 mW/cm^2 irradiation protocol (P = .02); thereafter, the shape of the curves did not differ between protocols. The mean energy constant, E_{rate}, was 2.7 ± 0.2 J/cm^2 and 2.9 ± 0.2 J/cm^2 for the 3 mW/cm^2 protocol and for the 10 mW/cm^2 irradiation protocol, respectively (Figure 3). By delivering the same total energy to the corneal tissue, regardless of the radiated light power and exposure time, the 2 UVA irradiation protocols achieved comparable consumption of riboflavin in the corneal stroma (80% ± 6%).

Tables 1 and 2 show the intrastromal riboflavin concentration determined by the prototype device and the spectrophotometer for the 2 treatment protocols. After stromal soaking, the results obtained with the 2 optical methods showed good agreement (ICC = 0.66; P = .04); the mean difference between the 2 measurements was 13%. The absolute agreement between the 2 methods decreased after UVA irradiation of the cornea (ICC = 0.12), likely

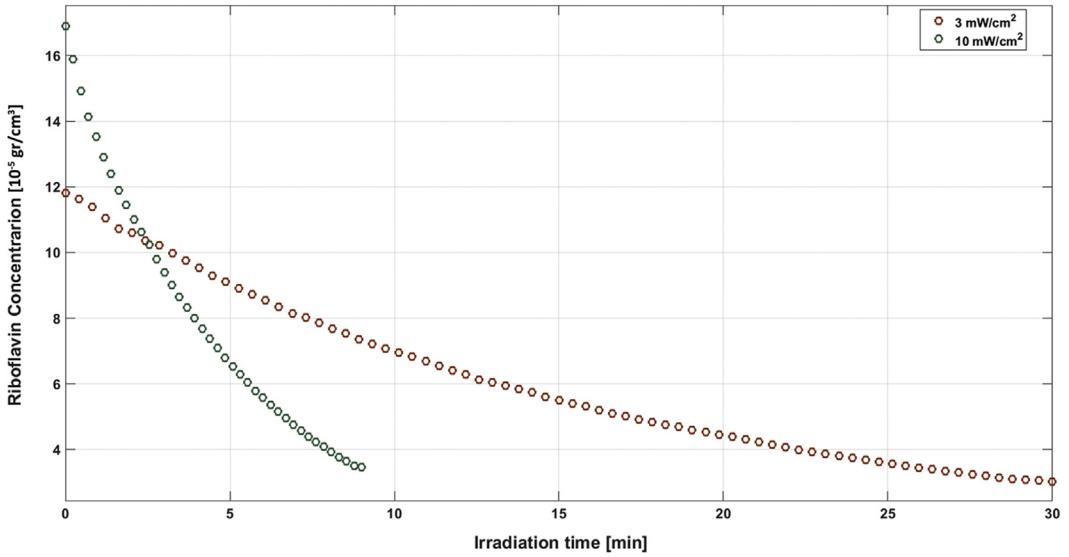

Figure 2. Consumption of intrastromal riboflavin during UVA illumination at 3 mW/cm^2 for 30 minutes (*red dots*) and 10 mW/cm^2 for 9 minutes (*green dots*) for 3 cases (UVA = ultraviolet-A).

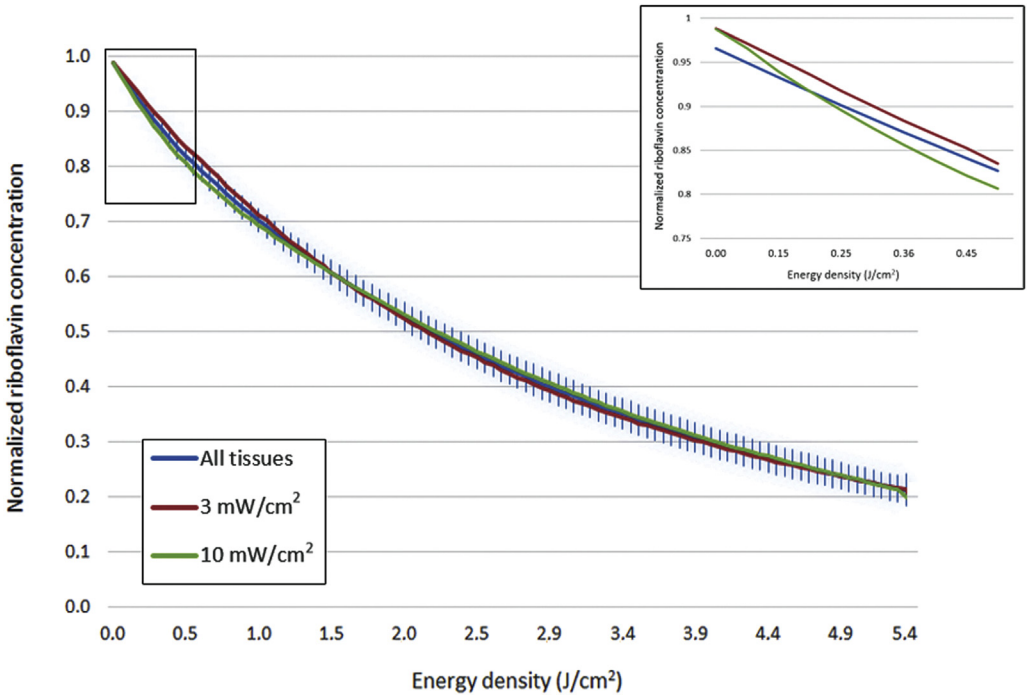

Figure 3. Normalized riboflavin consumption in the corneal stroma during UVA irradiation as a function of the UVA energy density. The blue curve represents an exponential function [$C_{norm}(E) = \exp(-E/E_{rate})$] fitted to the mean values for all tissues (bars indicate ±SD). The red curve and green curve show the mean values for the 3 mW/cm^2 and 10 mW/cm^2 UVA irradiance protocols, respectively. The 2 UVA irradiation protocols were equivalent in terms of riboflavin consumption in the corneal stroma. However, the rapid UVA irradiation protocol (mean −0.0004 ± 0.000) had a statistically significant steeper slope at the origin than the conventional protocol (mean −0.0003 ± 0.000) (UVA = ultraviolet-A) ($P = .02$) (*inset*).

because of tissue shrinking (mean corneal stroma thinning 29% after treatment) and increased back-scattered light from the tissue, which made the absorption measurements less reliable than fluorescence measurements.

In control tissues, 0.5 mW/cm^2 UVA power density for 9 minutes (total energy density was 0.26 J/cm^2) caused low riboflavin consumption (mean 26% ± 5%); at low energy density (ie, under near ambient lighting conditions), the decrease in intrastromal riboflavin concentration was quasilinear (Figure 4). Table 3 shows the riboflavin concentration data in the control group.

DISCUSSION

In this study, for a total energy density of 5.4 J/cm^2, the intrastromal riboflavin decreased exponentially during UVA irradiation of the human cornea. This decrease is consistent with the knowledge that the consumption of riboflavin in the stroma is spatially and temporally homogeneous.[17,20] Because the intrastromal riboflavin concentration is relatively low (≤0.02%), the overall photochemical process can be considered pseudo-first order under anaerobic conditions[12,21]; as a consequence, an exponential kinetic consumption of riboflavin is expected. This implies that in an

Table 1. Riboflavin concentration in the corneal stroma immediately after soaking phase (C) and after UVA irradiation of the cornea using 3 mW/cm^2 for 30 minutes ($C_{UV\text{-}A3\ mW}$) determined by the theranostic UVA device (fluorescence) and by spectrophotometry (absorbance).

	Fluorescence		Absorbance	
Corneal Tissue	C (10^{-4} gr/cm^3)	$C_{UVA\text{-}3\ mW}$ (10^{-4} gr/cm^3)	C (10^{-4} gr/cm^3)	$C_{UVA\text{-}3\ mW}$ (10^{-4} gr/cm^3)
153420	1.2	0.3	1.8	0.8
153769	1.2	0.3	1.5	0.7
151978	1.6	0.3	1.3	0.5
151979	1.4	0.3	1.5	0.7
153865	1.1	0.2	1.3	0.7
160227	1.8	0.4	2.7	0.9
160289	1.4	0.3	1.7	0.6
Mean ± SD	1.4 ± 0.3	0.3 ± 0.1	1.7 ± 0.4	0.7 ± 0.2

UVA = ultraviolet

Table 2. Riboflavin concentration of the corneal stroma immediately after soaking phase (*C*) and after UVA irradiation of the cornea using 10 mW/cm^2 for 9 minutes ($C_{UV\text{-}A10\ mW}$) determined by the theranostic UVA device (fluorescence) and by spectrophotometry (absorbance).

	Fluorescence		Absorbance	
Corneal Tissue	C (10^{-4} gr/cm^3)	$C_{UVA\text{-}10\ mW}$ (10^{-4} gr/cm^3)	C (10^{-4} gr/cm^3)	$C_{UVA\text{-}10\ mW}$ (10^{-4} gr/cm^3)
153099	2.5	0.8	1.8	0.8
153076	1.7	0.5	2.0	0.7
153664	1.7	0.2	2.0	1.5
153665	1.4	0.2	1.6	0.8
153866	1.2	0.3	1.5	0.1
160288	1.5	0.3	1.9	1.0
153768	1.5	0.2	2.0	1.5
Mean ± SD	1.6 ± 0.3	0.4 ± 0.2	1.8 ± 0.2	0.9 ± 0.4

UVA = ultraviolet

ambient environment (ie, 21% partial pressure of oxygen), the role of the type I mechanism is predominant for the formation of additional chemical bonds between stromal proteins in riboflavin with UVA CXL with 30 minutes of stromal soaking with dextran-enriched riboflavin and total UVA energy density of 5.4 J/cm^2 using a 3 mW/cm^2 or 10 mW/cm^2 power density.[22–25]

The conventional and rapid UVA irradiation protocols were equivalent in terms of consumption of intrastromal riboflavin. This means that in this range of power densities, riboflavin consumption in the corneal stroma is only energy dependent in accordance with the Bunsen-Roscoe law.[9,15,16] On the other hand, we found that the accelerated protocol showed a steeper slope of riboflavin consumption

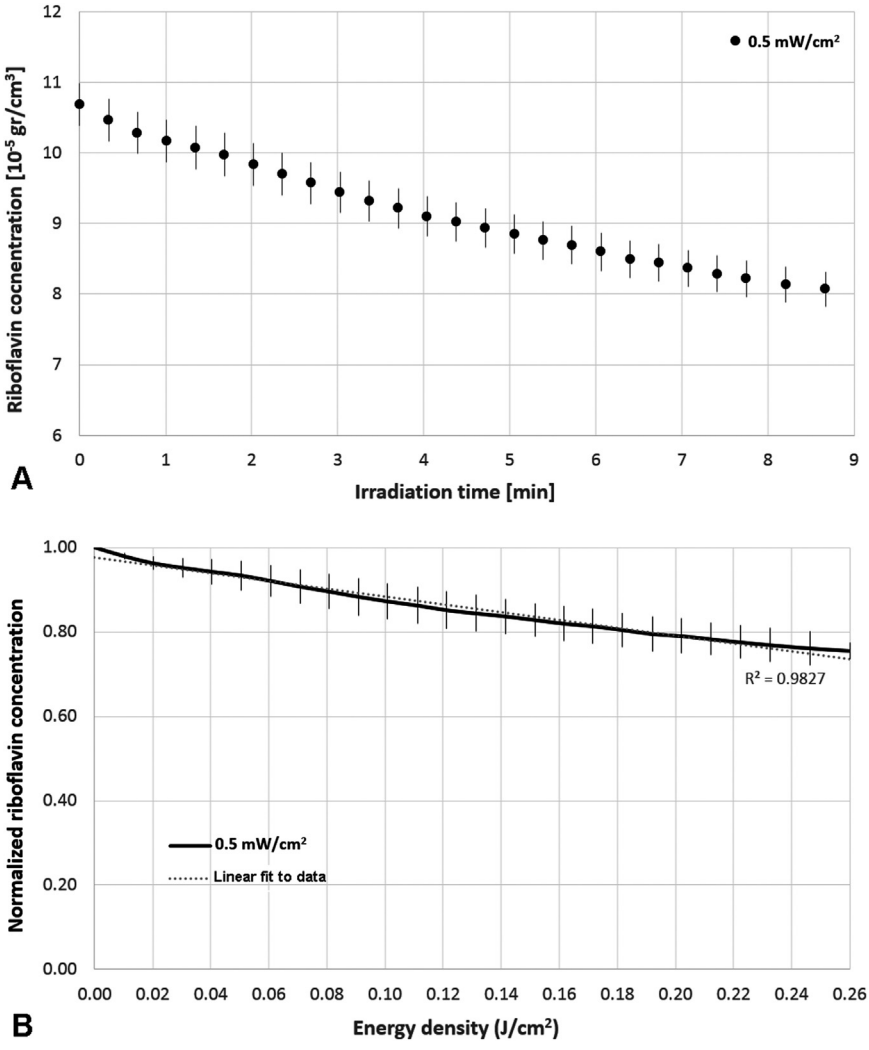

Figure 4. *A*: Mean consumption of intrastromal riboflavin during UVA illumination at 0.5 mW/cm^2 for 9 minutes (*black dots*). The bars represent ±SD. *B*: The black curve represents the mean riboflavin concentration in controls as function of the UVA energy delivered fitted with a linear function. The bars represent ±SD. The tiny dots represent R^2 = 0.98 (UVA = ultraviolet-A).

Table 3. Riboflavin concentration of the corneal stroma immediately after soaking phase (*C*) and after UVA irradiation of the cornea using 0.5 mW/cm^2 for 9 minutes ($C_{UVA05\ mW}$) determined by the theranostic UVA device (fluorescence). No absorbance data were taken in controls.

	Fluorescence	
Corneal Tissue	*C* (10^{-4} gr/cm^3)	$C_{UVA\text{-}05\ mW}$ (10^{-4} gr/cm^3)
173862	0.8	0.6
173899	0.9	0.7
173900	1.4	1.1
173893	1.0	0.7
173862	1.4	1.1
Mean ± SD	1.1 ± 0.3	0.8 ± 0.3

UVA = ultraviolet

in the stroma at the beginning of UVA irradiation, which means that the high irradiance consumes riboflavin faster than conventional irradiance when the stroma is fully enriched with the therapeutic molecule. For a lower concentration of riboflavin in the corneal stroma, as found later during treatment, the consumption kinetics was the same between 3 mW/cm^2 and 10 mW/cm^2 UVA irradiation protocols.

In this study, a positive control group was treated with 0.5 mW/cm^2 UVA irradiation for 9 minutes, delivering a total energy density of 0.26 J/cm^2 to the cornea (ie, 20 times lower than the generally accepted therapeutic dose). We found a quasilinear decrease in intrastromal riboflavin concentration over time, determining for the first time what the amount of riboflavin photodegradation in the human cornea would be under near ambient lighting conditions. With the current device, the rate of riboflavin degradation cannot be accurately determined without involving a minimal photochemical reaction by UVA light. Overall, the findings in our study and control groups are consistent with those in previous studies,[26–28] in which higher UVA light intensity resulted in a higher rate of riboflavin photodegradation.

The performance of the theranostic UVA device was evaluated by comparing the results with those obtained by absorption measurements; this represents the state-of-the-art optical technology for measuring the UV and blue-light absorption of riboflavin.[17–20,29,30] The absorption spectrum allows for the determination of the intrastromal riboflavin concentration from the known value of the molar extinction coefficient of riboflavin[17–19]; however, it cannot be used directly in patients because its invasiveness. In addition, the absorption spectrum is affected by the tissue's structure and optical properties, which can lead to unreliable measurements, as found in this study after UVA irradiation of the cornea. Our UVA prototype device does not have these limitations because it is based on direct acquisition of the green signal of a RGB camera, which overlaps with the fluorescence spectra emitted by the cornea soaked with riboflavin.[9,12,17]

The aim of future studies will be to understand the effect of the epithelium or other rapid UVA irradiation protocols (ie, > 10 mW/cm^2 with 5.4 J/cm^2 or higher energy density) and continuous or intermittent UVA light illumination on the kinetics of CXL with riboflavin.[9]

In conclusion, the data acquired by the theranostic device were reliable in noninvasively determining the intrastromal concentration of riboflavin during CXL and are consistent findings in previous studies, which used different optical techniques.[17–20] Several other techniques have been used to estimate the intrastromal riboflavin concentration in the laboratory, such as high-performance liquid chromatography, confocal fluorescence microscopy, and 2-photon optical microscopy.[17,31–34] Nevertheless, all these techniques are invasive and cannot be translated to the clinic. The UVA theranostic device represents an innovative image-guided solution for noninvasively measuring the intrastromal riboflavin concentration in real time and will be soon translated for use in humans.

WHAT WAS KNOWN

- There are several UVA irradiation protocols for CXL that deliver the same total dose of energy to the cornea according to the Bunsen-Roscoe law of reciprocity, regardless of the irradiance or exposure time of UVA illumination.

WHAT THE PAPER ADDS

- The 3 mW/cm^2 (for 30 minutes) and the 10 mW/cm^2 (for 9 minutes) UVA irradiation protocols were equivalent in terms of consumption of intrastromal riboflavin. Thus, in this range of power densities, riboflavin consumption in the corneal stroma is only energy dependent in accordance with the Bunsen-Roscoe law. On the other hand, the high irradiance protocol consumed riboflavin faster than conventional irradiance when the stroma was fully enriched with the therapeutic molecule.

REFERENCES

1. Chan E, Snibson GR. Current status of corneal collagen cross-linking for keratoconus: a review. Clin Exp Optom 2013; 96:155–164
2. Meek KM, Hayes S. Corneal cross-linking – a review. Ophthalmic Physiol Opt 2013; 33:78–93
3. Raiskup-Wolf F, Hoyer A, Spoerl E, Pillunat LE. Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: long-term results. J Cataract Refract Surg 2008; 34:796–801
4. Chunyu T, Xiujun P, Zhengjun F, Xia Z, Feihu Z. Corneal collagen cross-linking in keratoconus: a systematic review and meta-analysis. Sci Rep 2014; 4:5652
5. Hammer A, Richoz O, Arba Mosquera S, Tabibian D, Hoogewoud F, Hafezi F. Corneal biomechanical properties at different corneal cross-linking (CXL) irradiances. Invest Ophthalmol Vis Sci 2014; 55:2881–2884
6. Schumacher S, Oeftiger L, Mrochen M. Equivalence of biomechanical changes induced by rapid and standard corneal cross-linking, using riboflavin and ultraviolet radiation. Invest Ophthalmol Vis Sci 2011; 52:9048–9052
7. Shetty R, Pahuja NK, Nuijts RMMA, Ajani A, Jayadev C, Sharma C, Nagaraja H. Current protocols of corneal collagen cross-linking: visual, refractive, and tomographic outcomes. Am J Ophthalmol 2015; 160:243–249
8. Kymionis GD, Tsoulnaras KI, Grentzelos MA, Liakopoulos DA, Tsakalis NG, Blazaki SV, Paraskevopoulos TA, Tsilimbaris MK. Evaluation of corneal stromal demarcation line depth following standard and a modified-accelerated collagen cross-linking protocol. Am J Ophthalmol 2014; 158:671–675
9. Lombardo M, Pucci G, Barberi R, Lombardo G. Interaction of ultraviolet light with the cornea: clinical implications for corneal crosslinking. J Cataract Refract Surg 2015; 41:446–459

10. Peyman A, Nouralishahi A, Hafezi F, Kling S, Peyman M. Stromal demarcation line in pulsed versus continuous light accelerated corneal cross-linking for keratoconus. J Refract Surg 2016; 32:206–208
11. Konstantopoulos A, Mehta JS. Conventional versus accelerated collagen cross-linking for keratoconus. Eye Contact Lens 2015; 41:65–71
12. Lombardo G, Villari V, Micali NL, Leone N, Labate C, De Santo MP, Lombardo M. Non-invasive optical method for real-time assessment of intracorneal riboflavin concentration and efficacy of corneal cross-linking. J Biophotonics 2018; 11 (7); e201800028
13. Angelini N, Micali N, Villari V, Mineo P, Vitalini D, Scamporrino E. Interactions between water soluble porphyrin-based star polymer and amino acids: spectroscopic evidence of molecular binding. Phys Rev E Stat Nonlin Soft Matter Phys 2005; 71 (2 pt 1):02191
14. Mazzaglia A, Valerio A, Villari V, Rencurosi A, Lay L, Spadaro S, Scolaro LM, Micali N. Probing specific protein recognition by size-controlled glycosylated cyclodextrin nanoassemblies. New J Chem 2006; 30:1662–1668
15. Bunsen RW, Roscoe HE. Photochemische Untersuchungen [Photochemical studies]. Ann Phys 1859; 184:193–273
16. Schwarzschild K. On the deviations from the law of reciprocity for bromide of silver gelatine. Astrophys J 1900; 11:89–91
17. Lombardo G, Micali NL, Villari V, Serrao S, Lombardo M. All-optical method to assess stromal concentration of riboflavin in conventional and accelerated UV-A irradiation of the human cornea. Invest Ophthalmol Vis Sci 2016; 57:476–483
18. Lombardo G, Micali NL, Villari V, Leone N, Serrao S, Rusciano D, Lombardo M. Assessment of stromal riboflavin concentration–depth profile in nanotechnology-based transepithelial corneal cross-linking. J Cataract Refract Surg 2017; 43:680–686
19. Lombardo M, Micali N, Villari V, Serrao S, Pucci G, Barberi R, Lombardo G. Ultraviolet A: visible spectral absorbance of the human cornea after transepithelial soaking with dextran-enriched and dextran-free riboflavin 0.1% ophthalmic solutions. J Cataract Refract Surg 2015; 41:2283–2290
20. Spoerl E, Raiskup F, Kampik D, Geerling G. Correlation between UV absorption and riboflavin concentration in different depths of the corneal and CXL [letter]. Curr Eye Res 2010; 35:1040–1041; reply by AP Søndergaard, J Hjortdal, T Breitenbach, A Ivarsen, 1042–1043
21. Kamaev P, Friedman MD, Sherr E, Muller D. Photochemical kinetics of corneal cross-linking with riboflavin. Invest Ophthalmol Vis Sci 2012; 53:2360–2367
22. Reiser K, McCormick RJ, Rucker RB. Enzymatic and nonenzymatic cross-linking of collagen and elastin. FASEB J 1992; 6:2439–2449
23. McCall AS, Kraft S, Edelhauser HF, Kidder GW, Lundquist RR, Bradshaw HE, Dedeic Z, Dionne MJC, Clement EM, Conrad GW. Mechanisms of corneal tissue cross-linking in response to treatment with topical riboflavin and long-wavelength ultraviolet radiation (UVA). Invest Ophthalmol Vis Sci 2010; 51:129–138
24. Lin J-T, Cheng D-C. Modeling the efficacy profiles of UV-light activated corneal collagen crosslinking. PLoS One 2017; 12 (4):e0175002
25. Lin J-t. A proposed concentration-controlled new protocol for optimal corneal crosslinking efficacy in the anterior stroma [letter]. Invest Ophthalmol Vis Sci 2018; 59:431–432
26. Ahmad I, Fasihullah Q, Noor A, Ansari IA, Nawab Manzar Ali QNM. Photolysis of riboflavin in aqueous solution: a kinetic study. Int J Pharm 2004; 280:199–208
27. Sheraz MA, Kazi SH, Ahmed S, Anwar Z, Ahmad I. Photo, thermal and chemical degradation of riboflavin. Beilstein J Org Chem 2014; 10:1999–2012
28. Ahmad I, Anwar Z, Ahmed S, Sheraz MA, Bano R, Hafeez A. Solvent effect on the photolysis of riboflavin. AAPS PharmSciTech 2015; 16:1122–1128
29. Iseli HP, Popp M, Seiler T, Spoerl E, Mrochen M. Laboratory measurement of the absorption coefficient of riboflavin for ultraviolet light (365 nm). J Refract Surg 2010; 27:195–201
30. Hayes S, Morgan SR, O'Brart DP, O'Brart N, Meek KM. A study of stromal riboflavin absorption in ex vivo porcine corneas using new and existing delivery protocols for corneal cross-linking. Acta Ophthalmol 2016; 94:e109–e117
31. Alhamad TA, O'Brart DPS, O'Brart NAL, Meek KM. Evaluation of transepithelial stromal riboflavin absorption with enhanced riboflavin solution using spectrophotometry. J Cataract Refract Surg 2012; 38:884–889
32. Mastropasqua L, Nubile M, Calienno R, Mattei PA, Pedrotti E, Salgari N, Mastropasqua R, Lanzini M. Corneal cross-linking: intrastromal riboflavin concentration in iontophoresis-assisted imbibition versus traditional and transepithelial techniques. Am J Ophthalmol 2014; 157:623–630
33. Søndergaard AP, Hjortdal J, Breitenbach T, Ivarsen A. Corneal distribution of riboflavin prior to collagen cross-linking. Curr Eye Res 2010; 35:116–121; erratum, 1044
34. Labate C, Lombardo M, Lombardo G, De Santo MP. Biomechanical strengthening of the human cornea induced by novel nanoplatform-based transepithelial riboflavin/UV-A corneal crosslinking. Invest Ophthalmol Vis Sci 2017; 58:179–184

Disclosures: *Drs. M. Lombardo and G. Lombardo are cofounders and shareholders of Vision Engineering Italy srl. They are coinventors on a pending patent application (WO2017130043A1) related to this work.*

First author:
Marco Lombardo, MD, PhD

Vision Engineering Italy srl, Rome, Italy