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Customized Corneal Cross-Linking—A Mathematical Model

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Purpose: To improve the safety, reproducibility, and depth of effect of corneal cross-linking with the ultraviolet A (UV-A) exposure time and fluence customized according to the corneal thickness.

Methods: Twelve human corneas were used for the experimental protocol. They were soaked using a transepithelial (EPI-ON) technique using riboflavin with the permeation enhancer vitamin E–tocopheryl polyethylene glycol succinate. The corneas were then placed on microscope slides and irradiated at 3 mW/cm^2 for 30 minutes. The UV-A output parameters were measured to build a new equation describing the time-dependent loss of endothelial protection induced by riboflavin during cross-linking, as well as a pachymetry-dependent and exposure time-dependent prescription for input UV-A fluence. The proposed equation was used to establish graphs prescribing the maximum UV-A fluence input versus exposure time that always maintains corneal endothelium exposure below toxicity limits.

Results: Analysis modifying the Lambert–Beer law for riboflavin oxidation leads to graphs of the maximum safe level of UV-A radiation fluence versus the time applied and thickness of the treated cornea. These graphs prescribe UV-A fluence levels below 1.8 mW/cm^2 for corneas of thickness 540 μm down to 1.2 mW/cm^2 for corneas of thickness 350 μm. Irradiation times are typically below 15 minutes.

Conclusions: The experimental and mathematical analyses establish the basis for graphs that prescribe maximum safe fluence and UV-A exposure time for corneas of different thicknesses. Because this clinically tested protocol specifies a corneal surface clear of shielding riboflavin on the corneal surface during UV-A irradiation, it allows for shorter UV-A irradiation time and lower fluence than in the Dresden protocol.

Key Words: corneal cross-linking, transepithelial cross-linking, low fluence cross-linking

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Corneal collagen cross-linking (CXL), originally described by Spoerl et al,[1] is now an important corneal strengthening procedure in treatment of keratoconus[2,3] and postoperative laser in situ keratomileusis corneal ectasia.[4] Cross-linking can significantly increase biomechanical stiffness of the cornea.[2,3,5] In the standard Dresden cross-linking protocol for keratoconus treatment, the cornea is deepithelialized and then soaked in riboflavin for 30 minutes. The treated cornea is then irradiated with ultraviolet A (UV-A; 370 nm) at a power of 3 mW/cm^2 for 30 minutes for a total input energy from the UV-A source of 5.4 J/cm^2 while continually replenishing the corneal surface with riboflavin phosphate 0.1%.[1,6] Cross-linking with the Dresden protocol produces cross-linking to a depth of 250 to 350 μm; thus, a minimum corneal thickness of 400 μm is recommended[7] or application of hypotonic solution to the cornea to create a corneal thickness of 400 μm.[8]

Two basic assumptions for determining proper UV-A fluence and total energy for cross-linking are flawed assumptions. Reexamining these assumptions seems to lead to a possibly safer, more widely usable, and more reproducible cross-linking procedure.

One assumption, called the Bunsen–Roscoe law, has been used to justify more rapid cross-linking than the 1-hour Dresden protocol. The Bunsen–Roscoe law implies that the same corneal strengthening effect could be achieved by applying higher UV-A fluence over a shorter time but with the same total energy of 5.4 J/cm^2 as in the Dresden protocol. But endothelial toxicity and existing published data on effectiveness limit the use of the Bunsen–Roscoe law. Endothelial toxicity determines the safety limit of UV-A power. A study by Wollensak et al[9] demonstrated that UV-A rays elicit cytotoxic activity at 0.65 J/cm^2 (0.35 mW/cm^2). Furthermore, higher-fluence shorter-duration cross-linking seems to produce only a relatively shallow cross-linking effect. Kymionis et al[10] demonstrated clinically that the 30-minute 3-mW/cm^2 UV-A

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intensity Dresden protocol for CXL yielded a deeper corneal effect than did a shorter more intense treatment of a 10-minute 9-mw/cm^2 CXL protocol. Recent experimental work by Wernli et al[11] also disproved the Bunsen–Roscoe law for UV-A fluence much higher than in the Dresden protocol. In fact, clinical results by Caruso et al[12] indicated that effective cross-linking could be accomplished using half the UV-A fluence of the Dresden protocol and also a shorter exposure time than described in the Dresden protocol.

The second flawed assumption is the Lambert–Beer law used to describe an exponential decline in UV-A power versus depth in the irradiated cornea during cross-linking.[13] In addition, experimental data provided the basis for the proposed 400-μm lower limit of minimal pachymetry in the original Dresden protocol for safe cross-linking. It is also the reason for continually adding riboflavin during UV-A irradiation.[14] But the Lambert–Beer law grossly oversimplifies the actual UV-A absorption kinetics during cross-linking. When a cornea is fully soaked in riboflavin phosphate for 30 minutes, most of the UV-A absorption and endothelial protection result from the unoxidized riboflavin within the corneal tissue, not the corneal tissue itself. But endothelial protection from riboflavin in epithelium-off (EPI-OFF) cross-linking has been shown to be time-varying during treatment as the riboflavin disappears from oxidation.[15]

The Dresden protocol is well tested, safe, and effective, and its safety depends on adding more riboflavin during the UV-A irradiation process. The surface riboflavin absorbs much of the UV-A energy input, thus protecting the endothelium. But adding more riboflavin onto the corneal surface produces a time-varying thickness of riboflavin on the corneal surface that blocks UV-A absorption in an erratic and highly time-variable way by acting as a "sunscreen" for UV-A transmission during treatment. The thickness of the riboflavin film varies constantly, thus inducing large variations in UV-A intensity and transmission to the corneal stroma where corneal strengthening is needed.[14]

UV-A blockage can vary by the patient and by the technique used by the person applying the riboflavin. This variation could cause a differential stiffening effect at different depths within the treated corneas and among the treated eyes.

This study explores the mathematics of cross-linking and leads to a prescription of a customized cross-linking treatment based on corneal pachymetry that includes corneas thinner than 400 μm. We previously investigated the pharmacological, chemical, physical, and biological basis underlying CXL treatments with laboratory experiments, and we demonstrated the clinical effectiveness and safety of TE-CXL treatments with a newly proposed permeation enhancer (vitamin E–tocopheryl polyethylene glycol succinate) (VIT-E-TPGS) and lower-fluence shorter-duration UV-A exposure with respect to the Dresden protocol as discussed in previous studies.[12,15,16]

MATERIALS AND METHODS

This research adhered to the tenets of the Declaration of Helsinki. Approval from the Institutional Review Board/Ethics Committee and Animal Care and Use Committee was obtained (authorization no. 1269).

Cross-Linking Solution

The solution contained riboflavin 0.125%, D-alpha-tocopheryl polyethylene glycol 1000 succinate (VE-TPGS) (IROMED, Rome, Italy).

Corneal Samples

Twelve human corneas were used for the experimental protocol. Human corneas, not suitable for transplantation because of biological contamination of the carrier solution (Optisol GS; Bausch & Lomb, Rochester, NY), were provided by the spin-off consortium Fast-linking S.r.l. and by the Pellegrini Hospital Regional Eye Bank, Naples, Italy.

All samples showed intact epithelium, normal density of endothelial cells, and absence of epithelial or stromal opacification. Before experimentation, each cornea was excised circularly 2 mm from the limbus, and central thickness (d) of the sample was measured with a 5-μm resolution ultrasound pocket pachymeter (Quantel Medical, Clermont, France).

Spectrophotometric Measurements

The UV-A source used in this study (CF–X Linker Modulated; IROMED) contains light-emitting diodes with a 370-nm wavelength and an intensity variable (I) from 1 to 20 mW/cm^2. The distance between this device and the corneal surface was set at 5 cm. The UV detector used was a LaserMate Q-2000 (LaserMate Group, Wessling, Germany) set orthogonally to the radiation wavefront. The detector was previously calibrated and had an SD of ±0.01 mW/cm^2. The UV-A intensity (I) values were measured placing the microscope glass directly in contact with this device. Beam intensity of the UV-A emerging from the posterior surface of every microscope slide (I) when exposed to UV-A (3.0 mW/cm^2) was previously measured. Only slides presenting clear and unscratched glass, with I values of 2.527 ± 0.01 mW/cm^2, were used in this study. The specific absorbance value ε of the riboflavin solution was then measured using a spectrophotometer (PerkinElmer Lambda 25 UV/Vis; PerkinElmer, Waltham, MA). The test was repeated with 10 different samples. I values emerging from the posterior surface of each corneal sample were initially measured as described above. These values at time 0 (t_0) were used as a comparative control for the following readings. Each cornea, with an approximately 2-mm ring of sclera, was then mounted on a modified Franz-type diffusion cell (0.9 mm, 5 mL receptor volume; SES GmbH–Analysesysteme, Bechenheim, Germany), with the endothelium facing the receptor compartment. This compartment was constituted by isotonic phosphate buffer at pH 7.4 and was maintained at 37 ± 1°C. Before each experiment, corneas were equilibrated using 0.5 mL of balanced salt solution for 10 minutes. Then, 1.0 mL of riboflavin solution was applied. After 30 minutes, the diffusion cells were dismantled, excess donor solution was washed away with balanced salt solution, and the samples were set on the microscope slide to be exposed to UV-A radiation. The samples were then irradiated for 30 minutes at 3 mW/cm^2. The intensity of UV-A light emerging from the posterior surface of each cornea (i_0) was measured every 5 minutes, for a total of 30 minutes.

Statistical Analysis

Mathematical and statistical analyses were conducted using Mathematica 7.0 (Wolfram Research Software Inc, Champaign, IL), and GraphPad Prism (GraphPad Software Inc, San Diego, CA). The significance of differences between groups was determined by 1-way analyses of variance followed by Bonferroni post hoc tests for multiple comparisons. Differences with $P < 0.05$ were considered statistically significant in this study.

RESULTS

The I values measured for human corneas are reported as Supplemental Digital Content 1 (see Table, measured UV-A output intensities through 12 cornea samples, http://links.lww.com/ICO/A494); they are expressed in milliwatts per square centimeter and were measured before and after topical riboflavin application, and every 5 minutes up to 30 minutes during UV-A exposure. The thickness values (d) of every sample, expressed in centimeters, are reported in Supplemental Digital Content 2 (see Table, corneal central thickness and UV-A absorbance values A and β, http://links.lww.com/ICO/A495). In these tables are also reported the corneal absorbance values (calculated with the Lambert–Beer law) A (dimensionless) and β (specific, cm^{-1}). These values were measured before the topical riboflavin application (A_{t0}, β_{t0}), after 30 minutes of continuous riboflavin soaking (A_{t30}, β_{t30}), and after 5 minutes of UV-A irradiation (ACXL, βCXL). The corneas showed a significant decrease in I values after 30 minutes of riboflavin soaking (0.27 ± 0.03 mW/cm^2) compared with those at t_0 (1.13 ± 0.08 mW/cm^2, $P < 0.01$, 2-tail, heteroscedastic); the decrease was significant also after the first 5 minutes of UV-A exposure (0.85 ± 0.04 mW/cm^2). At 10 minutes of UV-A exposure, I values increased and no statistical differences were evidenced with t_0, before riboflavin soaking. Significant differences were also present in the same group for the absorbance values ($A_{t30} = 2.43 \pm 0.124$ and $\beta_{t30} = 52.913 \pm 2.576$ cm^{-1}) with respect t_0 (t_0) ($A_{t0} = 0.981 \pm 0.070$ and $\beta_{t0} = 19.248 \pm 1.531$ cm^{-1}; $P < 0.01$).

DISCUSSION

The EPI-ON model used for this experimentation allows riboflavin corneal penetration similar to the classical protocols, in which the epithelium is removed (EPI-OFF) before soaking with the riboflavin solution.[7] Spectroscopic analysis of UV-A rays emerging from the endothelial side of the cornea shows that the intensity is time-varying, not constant as needed for the application of the Lambert–Beer law.

After 30 minutes of riboflavin soaking, the intensity of radiation emerging from the rear corneal surface (I) is only 0.27 ± 0.033 mW/cm^2, considered a safe level for the endothelium. But, soon, that I value increases to 0.85 mW/cm^2, which is beyond the endothelial safety threshold. This increase is due to intrastromal oxidation (photolysis or "bleaching") of the riboflavin and its disappearance as a UV-A absorber.

Other authors including Wollensak et al[14] have described the increased endothelial irradiation by a factor of 2 or 3 that occurs during treatment and have prescribed a superficial riboflavin film during the cross-linking procedure. But the use of the precorneal film limits cross-linking to the superficial stroma, thus producing the so-called "demarcation line" described by Spörl et al.[17]

By avoiding the use of the precorneal riboflavin film, endothelial safety can be achieved instead by lowering UV-A intensities and shortening the treatment time, and, at the same time, producing a suitable cross-linking effect. Without the extremely variable riboflavin film causing a great variation in energy absorbed into the cornea, and using UV-A treatment with lower energy, it should be possible to customize and optimize treatment for corneas of different thicknesses.

Mathematical Model

The experimental data were used to calculate the main parameters describing the rate of photolysis of riboflavin by unit of volume (rate equation). The legend of the mathematical model is described in Table 1. In the cornea soaked with riboflavin, UV-A radiation, I, is absorbed by both corneal tissues and riboflavin molecules. The absorbance of riboflavin in the corneal tissue at time 0 (A_{t0}) can be described using the following formula, according to the Lambert–Beer law:

$$A_{t0} = -\ln\frac{I}{I_0} = \frac{\varepsilon W_R}{S} + \beta d \qquad (1)$$

where ε is the specific absorbance value of the riboflavin solution, already measured; d is corneal thickness; S is the corneal surface exposed to riboflavin soaking (0.64 cm^2); βd is the corneal absorbance, as previously measured; $\varepsilon W_R/S$ is the absorbance of the corneal tissue due to riboflavin; and W_R, is the amount of riboflavin in corneal stroma, calculated from the ratio I/I_0.

At t_0, the absorbance, due to riboflavin (W_R) accumulated in stroma, can be calculated as follows:

$$\frac{A_{t30}}{d-0.005} - \frac{A_{t0}}{d} = \varepsilon\frac{W_R}{S\times(d-0.005)} \qquad (2)$$

TABLE 1. Legend of the Mathematical Model

$R_A(z,t)$ = concentration (mg/cm^3) of riboflavin available for cross-linking at depth z in the corneal thickness at time t
$I(z,t)$ = intensity of the UV-A radiation in the corneal thickness at depth z and time t
$\partial I_R(z,t)$ = intensity of the UV-A radiation absorbed by the available riboflavin contained in an infinitesimally thick corneal layer dz at depth z and at time t
S = exposed surface area
W_{mol} = molecular weight
ε = specific riboflavin absorbance
α = specific exhaust solution absorbance
β_{t0} = specific corneal UV-A absorbance (cm^{-1}) before topical application of the riboflavin solution
P = photolysis probability for a riboflavin molecule during the cross-linking process
γ = proportionality factor [approximately 935,500 ($m^2 \cdot s^{-2}$) for riboflavin and 639,727 ($m^2 \cdot s^{-2}$) for riboflavin phosphate]

Having measured the corneal thickness d in centimeters, a correction factor (0.005) has been used for human corneal thickness, considering epithelium removal (50 μm of supposed thickness as reported in the literature).[18]

The key issue in this model is that CXL consumes the riboflavin molecules within the cornea, thus, contribution to UV-A absorption from riboflavin decreases during the procedure. Therefore, the Lambert–Beer law is not sufficient in and of itself to describe the phenomenon.

For the sake of ease, in the ensuing mathematical description, the cornea will be assumed to be substantially planar. With this simplification, the intensity I of UV-A in the cornea may be considered a function only of the time t and distance (depth) z from the epithelial surface.

Hence, the intensity of UV-A in a cornea soaked with riboflavin can be described by the following differential Equation 3:

$$\frac{\partial I(z,t)}{\partial z} = -I(z,t)\times[(\varepsilon-\alpha)\times R_A(z,t)+\alpha\times R_A(z,0)+\beta] \tag{3}$$

where α is the specific absorbance value of the exhaust riboflavin solution; t is the time elapsed from the beginning of UV-A irradiation (at the instant $t = 0$); z is the distance between a considered point in the stroma and the epithelial surface; $I(z,t)$ is the intensity of the UV-A radiation in the corneal thickness at depth z and time t; $\partial I_R(z,t)$ is the intensity of UV-A radiation absorbed by the available riboflavin contained in an infinitesimally thick corneal layer dz at depth z and at time t; $R_A(z,t)$ is the concentration (milligram per cubic centimeter) of riboflavin available for cross-linking at depth z in the corneal thickness at time t; and $\alpha \times [R_A(z,0) - R_A(z,t)]$ is the contribution of the products of photolysis of riboflavin (herein referred also as "exhaust solution") to the UV-A absorbance.

The time rate of riboflavin consumption during CXL is not described by the Lambert–Beer law. The following rate Equation 4 is adapted to describe the photolysis of riboflavin:

$$\frac{\partial R_A(z,t)}{\partial t} = -\frac{\varepsilon\times P}{\gamma}\times I(z,t)\times R_A(z,t) \tag{4}$$

where P is the photolysis probability for a riboflavin molecule during the cross-linking process and $\frac{\varepsilon}{\gamma}$ is a proportionality factor.

The probability of riboflavin photolysis is considered constant in this model at any point within the cornea and at any time during cross-linking treatment.

γ is calculated as follows: U being the number of UV-A photons absorbed by riboflavin in an infinitesimally thick layer of the cornea in time dt, the corresponding energy $-S\times\partial I_R(z,t)\times dt$ absorbed by this layer is as follows:

$$-S\times\partial I_R(z,t)\times dt = U\times\frac{h\times c}{\lambda} \tag{5}$$

where c is the speed of light in a vacuum, S is the exposed corneal area, h is the Planck constant, and λ is the UV-A wavelength.

The number of molecules of riboflavin M split by photolysis in an infinitesimally thick layer of the cornea by UV-A in time dt is assumed proportional to the number of photons absorbed by riboflavin:

$$M = P\times U \tag{6}$$

where P is the probability that a photon succeeds in splitting a riboflavin molecule. The corresponding riboflavin consumption in the infinitesimally thick layer dz of the cornea during time dt is as follows:

$$S\times\partial R_A(z,t)\times dz = -M\times\frac{W_{mol}}{N} \tag{7}$$

where W_{mol} is the molecular weight of riboflavin and N is the Avogadro number. By combining the above equations,

$$\partial I_R(z,t)\times dt = \frac{h\times c}{P\lambda}\times\frac{N}{W_{mol}}\times\partial R_A(z,t)\times dz \tag{8}$$

According to the Lambert–Beer law, the amount of radiation $\partial I_R(z,t)$ absorbed into the infinitesimally thick layer by the available riboflavin is proportional to the incident radiation $I(z,t)$ and to the product of the concentration of available riboflavin $R_A(z,t)$ by thickness dz of the crossed layer, whereby,

$$\partial I_R(z,t) = -\varepsilon\times I(z,t)\times R_A(z,t)\times dz \tag{9}$$

Being:

$$\gamma = \frac{h\cdot c}{\lambda}\times\frac{N}{W_{mol}} \tag{10}$$

So, rate Equation 4 is obtained.

The parameter γ is inversely proportional to the UV wavelength and also to the molecular weight of the target substance (riboflavin), according to the proportionality constant:

$$hcN = 0.11314208\ \mathrm{J\cdot m\cdot mole^{-1}} \tag{11}$$

For a wavelength of 370 nm,

$$\frac{h\cdot c\cdot N}{\lambda} = 305789.41\ \mathrm{J\cdot mole^{-1}} \tag{12}$$

This value represents the energy per mole of UV-A photons (sometimes referred to as "1 Einstein").

The parameter γ is equal to 935,500 ($m^2\cdot s^{-2}$) for riboflavin and 639,727 ($m^2\cdot s^{-2}$) for riboflavin-5-phosphate. It represents the ratio between the energy of 1 mole of 370-nm UV-A photons and the mass of a mole of riboflavin. Then, $\frac{P}{\gamma}$ indicates the number of consumed riboflavin moles for 1 J of UV-A photons absorbed by this substance. Using Mathematica 7.0 software, the following value was calculated from Equations 3 and 4 and from the experimental values reported in Supplemental Digital Content 1 (see Table 1, http://links.lww.com/ICO/A494):

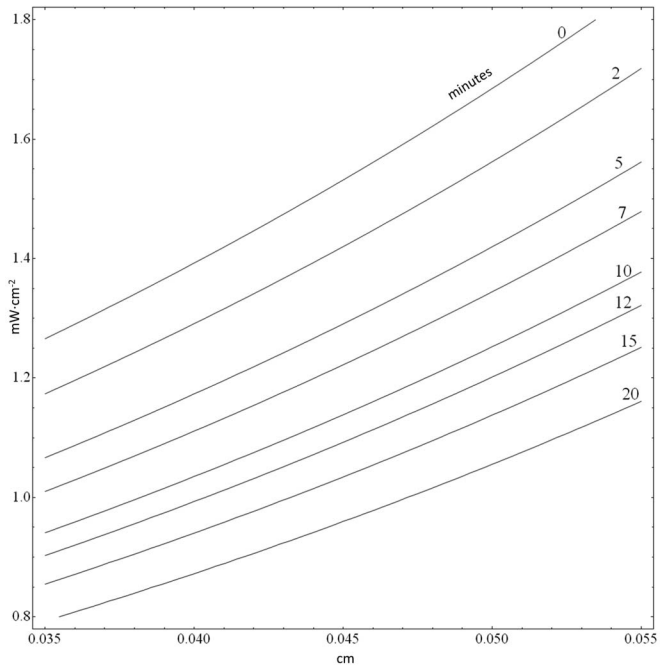

FIGURE 1. Maximum UV fluence in mW/cm^2 (*y* axis) versus corneal minimal thickness in centimeters (*x* axis) to assure less than 0.3 mW/cm^2 on the corneal endothelium. The number attached to each line on the chart is the number of minutes for application of UV light needed to accomplish effective cross-linking. The 0 plot represents the safety limit conditions for treatment, showing that an UV-A intensity of 1.8 mW/cm^2 should not be used on the human cornea with a thickness less than 0.054 cm.

$$\frac{P}{\gamma} = 0.14\ \mathrm{mg \cdot J^{-1}} = 1.4 \cdot 10^{-7} \mathrm{m^{-2} \cdot s^2} \qquad (13)$$

where P is the probability of splitting a riboflavin-5-phosphate molecule with a UV-A photon (wavelength 370 nm) and is calculated from Equation 13 to approximately 9%.

Theoretical Results of the Model

A "customized CXL plot" can be obtained from differential Equations 3 and 10 previously described. In this diagram (Fig. 1), corneal thicknesses (*x* axis, expressed in centimeters) are plotted against the UV-A source intensity (*y* axis, expressed in Joule per square centimeter) at different treatment times (expressed in minutes). The diagram has been built using average specific absorbance and riboflavin corneal concentrations after soaking (see Supplemental Digital Content 1 and 2, http://links.lww.com/ICO/A494, http://links.lww.com/ICO/A495) and assuming the hypothesis that products of photolysis of riboflavin do not contribute to shield against UV-A ($\alpha = 0$). Once corneal thickness and UV-A source intensity have been assessed, the treatment time can be derived. The longer the treatment time is, the deeper is the effect produced, but from the safety point of view, the limit of 0.35 mW/cm^2 is never exceeded and the longest treatment time used is 15 minutes. Moreover, the 0 plot represents the safety limit conditions for treatment, showing that an UV-A intensity of 1.8 mW/cm^2 should not be used on the human cornea with a thickness of less than 0.054 cm, thus presenting a slightly more conservative prescription for UV-A fluence than may be necessary.

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