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Comparison between standard and transepithelial corneal crosslinking using a theranostic UV-A device

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Abstract

Purpose To assess corneal concentration of riboflavin in two different corneal crosslinking protocols performed by a novel image-guided therapeutic (or "theranostic") UV-A device.

Methods Ten human eye bank donor tissues were used in this work. The tissues underwent corneal cross-linking according to the conventional treatment protocol ($n = 5$; 30 min of stromal soaking followed by 30 min of 3 mW/cm^2 UV-A irradiance) and the iontophoresis-assisted transepithelial protocol ($n = 5$; soaking for 5 min at 1 mA/min and 9 min of 10 mW/cm^2 UV-A irradiance) using a theranostic UV-A device (Vision Engineering Italy srl, Italy). The device provided real time assessment of riboflavin concentration by hyperspectral image analysis of the cornea. A 0.1% riboflavin hypotonic solution (Ricrolin+, Sooft Italia Spa, Italy) was used in all cases.

Results Manual application of hypotonic riboflavin for 30 min into the stroma achieved greater corneal riboflavin concentration (425 ± 77 μg/cm^3) than transepithelial delivery of riboflavin by corneal iontophoresis (195 ± 35 μg/cm^3; $P = 0.001$). In both UV-A irradiation protocols, corneal riboflavin concentration decreased exponentially with a constant energy rate of 2.3 ± 0.5 J/cm^2 and 1.8 ± 0.3 J/cm^2 respectively. At the end of treatment, the average corneal concentration of riboflavin decreased by ≥ 85%, with values of 54 ± 29 μg/cm^3 and 31 ± 9 μg/cm^3 ($P = 0.11$), respectively.

Conclusion Manual application of riboflavin onto the stroma achieved almost 50% greater concentration of riboflavin than transepithelial delivery by corneal iontophoresis. The theranostic UV-A device provided a novel approach to estimate corneal concentration of riboflavin non-invasively during treatment.

Keywords Corneal crosslinking · Riboflavin · Theranostics · Keratoconus · Iontophoresis

Introduction

Novel objective optical methodologies for measuring the riboflavin concentration in the cornea are widening our understanding on the different stromal diffusion of ophthalmic riboflavin solutions in various treatment protocols [1–5]. Theranostics is an emerging therapeutic paradigm that enables image-guided therapy in clinic; theranostic devices make use of real-time non-invasive molecular analysis to achieve optimal treatment outcomes in the management of disease. In previous studies [4, 5], we have shown the reliability of a theranostic UV-A device for image-guided corneal crosslinking using the *Dresden protocol* in human donor tissues and have compared the conventional UV-A irradiation protocol (3 mW/cm^2 for 30 min) with accelerated UV-A irradiation of the stroma (10 mW/cm^2 for 9 min). The two UV-A irradiation protocols have been shown to be almost equivalent in terms of consumption of intrastromal riboflavin.

In this work, we used a theranostic UV-A device that, via the acquisition and analysis of the fluorescence emitted from the riboflavin when illuminated by UV-A light, measures in real time the concentration of the therapeutic molecule in the cornea. The scope of this work was to compare the corneal riboflavin concentration in two corneal crosslinking protocols, which were performed by the theranostic UV-A device, using the same dextran-free riboflavin hypotonic ophthalmic solution [6–15]. The protocols included (1) the manual stromal

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soaking for 30 min followed by 30 min of 3 mW/cm^2 UV-A irradiance of the cornea and (2) the iontophoresis-assisted transepithelial delivery of riboflavin (1 mA/min for 5 min) followed by 9 min of 10 mW/cm^2 UV-A irradiance of the cornea.

Material and methods

Human donor tissues

Ten eye bank human donor tissues from different donors were obtained from the Veneto Eye Bank Foundation (Venezia Zelarino, Italy); six were sclerocorneal tissues and four were whole eye globes. Inclusion criteria included an endothelial cell density > 1800 cells/mm^2; exclusion criteria included any corneal scar or previous ocular surgery. The study adhered to the tenets of Declaration of Helsinki for the use of human tissues.

UV-A device optical setup and operation

The optical setup of the prototype device has been described in detail in previous studies [4, 5]. The main components include a UV-A LED (365 ± 10 nm), a UV-A sensor and a RGB camera, which acquires the fluorescence images emitted by the cornea under treatment, and a device microcontroller. The controller manages the correct operation of the electro-optical components, processes the RGB images and provides real-time measurement of corneal riboflavin concentration during corneal crosslinking. The theranostic device sensitivity is 5 μg/cm^3.

In this study, the measurement of riboflavin is performed over a 6.0-mm central area of the cornea. At the beginning of each treatment, the device acquires, processes, and averages the green signals of the RGB camera of the cornea illuminated by UV-A light at very low density (< 0.6 mW/cm^2, which is lower than the photoactivation threshold of riboflavin) [4]; this pretreatment value corresponds to the native corneal fluorescence (B_G). This value is subtracted from the acquired averaged fluorescence image value (I_G) either after soaking or during UV-A irradiation period. At the end of soaking phase, i.e., immediately prior to UV-A irradiation, the riboflavin concentration is evaluated from the preset calibration curve of the device through the I_G value acquired by irradiating the cornea with < 0.6 mW/cm^2 UV-A power density [4, 5]. During UV-A irradiation, the theranostic device permits to measure the photodegradation of riboflavin in real time: the riboflavin concentration is determined from the acquired I_G through the preset calibration curve, which is proprietary. No riboflavin was applied over the corneal surface during irradiation in order to minimize bias between samples.

Corneal crosslinking treatments

Donors (70.9 ± 5.8 years) did not have history of corneal pathologies, traumas, or eye surgery. The mean cadaver time was 8.8 ± 6.7 h; the mean endothelial cell density (ECD) was 2100 ± 115 cells/mm^2 (Axiovert 25, Carl Zeiss Microscopy, Jena, Germany); the central corneal thickness (CCT) was 562 ± 30 μm (OCT-HS-100, Canon, Japan). The samples were shipped to the laboratory in 6% dextran-enriched corneal storage medium and were used for experiment within 24 h.

Each sclerocorneal tissue was placed in an artificial anterior chamber (AAC, Coronet, Network Medical Products Ltd., North Yorkshire, UK) pressurized with the AAC filled with 0.9% sodium chloride. The ACC was connected, through tubing, to a column manometer to maintain the pressure inside it at 18 mmHg during experiment. Each eye globe was mounted into a specially designed holder, facing upward. The eye was connected to a column manometer, filled with 0.9% sodium chloride solution, to maintain the pressure inside the eye at 18 mmHg during experiment. Each eye was pre-conditioned for 20 min before corneal crosslinking treatment, which allowed us to achieve a unique pre-stressing reference state of the ocular tissues before testing [16]. Corneal epithelial integrity was assessed by AS-OCT (OCT HS-100, Canon, Japan).

The tissues were randomized to receive either conventional corneal crosslinking ($n = 3$ sclerocorneal tissue and $n = 2$ eye globes) or iontophoresis-assisted transepithelial corneal crosslinking ($n = 3$ sclerocorneal tissue and $n = 2$ eye globes). A 0.1% riboflavin-5-phosphate hypotonic solution enriched with EDTA and trometamol as enhancers (Ricrolin+, Sooft Italia Spa, Italy) was used in both treatment protocols; the conventional corneal crosslinking consisted of soaking the stroma, after removing the corneal epithelium with an Amoils' brush (Innovative Excimer Solutions Inc., Toronto, Canada), for 30 min using a ring applicator placed onto the cornea (Fig. 1); thereafter, the stroma was irradiated by 3 mW/cm^2 for 30 min with the theranostic UV-A device. The transepithelial protocol consisted of using a commercially available iontophoresis device (Iontofor CXL, Sooft Italia Spa, Italy) with 1 mA/min current density for 5 min (Fig. 1), for delivering riboflavin into the corneal stroma; thereafter, the cornea was irradiated by 10 mW/cm^2 for 9 min with the theranostic UV-A device. The iontophoresis device included a generator, an applicator with an active electrode and a return, passive, electrode, which was applied to the optic nerve of the donor eyes or to the needle inserted in the sclera of the sclerocorneal tissues. The active electrode consisted in a plastic bath tube, which was applied to the corneal epithelial surface. After the tube was applied to the corneal epithelium using suction, it was filled with riboflavin solution.

The two UV-A irradiation protocols used in this study delivered the same total UV-A energy density of 5.4 J/cm^2 to the cornea; an irradiation area of 9.00-mm diameter was used in all cases.

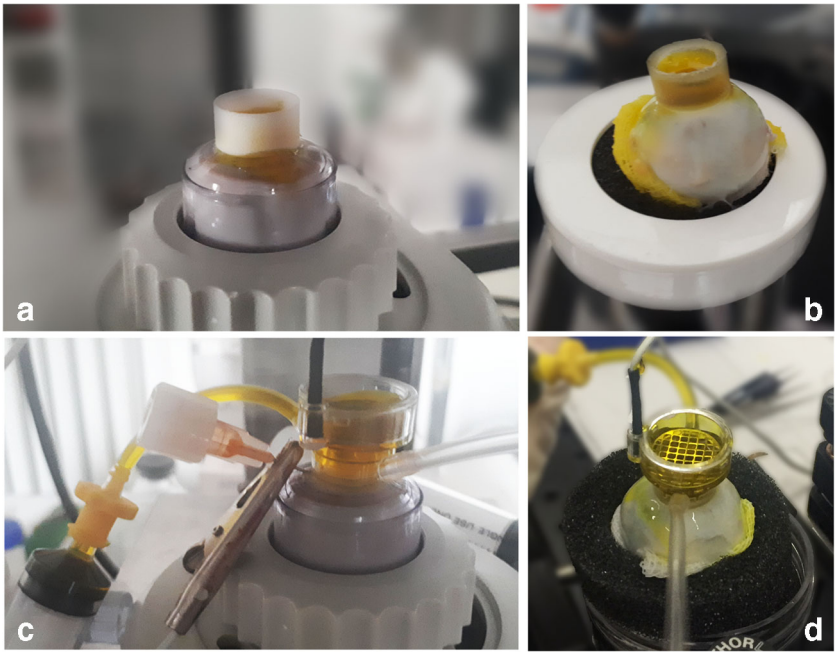

Fig. 1 Human donor sclerocorneal tissues and donor eye globes were used in this study in order to translate the laboratory results to clinic. The measurements by theranostic UV-A device were highly consistent between sclerocorneal tissues and eye globes for each treatment protocol. **a**, **b** The de-epithelialized samples undergoing stromal soaking for 30 min by filling a ring applicator with the 0.1% riboflavin hypotonic solution. **c**, **d** The samples with intact epithelium undergoing corneal iontophoresis with the 0.1% riboflavin hypotonic solution

Data analysis

In this study, data were given as mean ± standard deviation (SD), and the concentration of riboflavin was expressed as μg/cm^3 or as percentage (%), where 100 μg/cm^3 = 0.01% [1, 2, 4, 5]. The energy dependence of the riboflavin consumption for both crosslinking protocols was evaluated by fitting with an exponential function the evolution of corneal riboflavin concentration curve with respect to the riboflavin concentration measured at the end of soaking phase: $C(E) = C_0 e^{-E/Erate}$, where E is the UV-A energy density delivered to the cornea, E_{rate} is the energy constant, and C_0 is the concentration value obtained at the end of soaking phase. In order to assess any potential difference between the two UV-A irradiation protocols, the riboflavin concentration measured from all tissues was normalized to C_0 and plotted as a function of E. We also performed a linear fit on the normalized curve up to 0.5 J/cm^2 energy density delivered to the cornea and the slope of the fit was calculated for all tissues. The Student t test was used to compare data between the two corneal cross-linking protocols. The sample size (5:5) was calculated to achieve 89% power to detect average difference of 200 μg/cm^3 (SD: 100 μg/cm^3) between the two corneal crosslinking protocols.

Results

The application of riboflavin hypotonic solution for 30 min onto the stroma achieved high corneal riboflavin concentration values (425 ± 77 μg/cm^3); data were consistent between sclerocorneal tissues (400 ± 95 μg/cm^3) and eye globes (463 ± 32 μg/cm^3). During UV-A irradiation, the stromal riboflavin concentration decreased non linearly in all tissues; at the end of irradiation period, the mean intrastromal riboflavin concentration was 54 ± 29 μg/cm^3, i.e., on average 87% lower than the concentration value obtained at the end of soaking phase (Table 1). The CCT ranged from 562 ± 35 to 593 ± 43 μm (P = 0.29) before and after treatment respectively.

After corneal iontophoresis, the mean corneal concentration of riboflavin was 195 ± 35 μg/cm^3, with consistent values between sclerocorneal tissues (201 ± 41 μg/cm^3) and eye globes (176 ± 8 μg/cm^3). The riboflavin concentration values were significantly lower (P = 0.001) than conventional corneal soaking. Two tissues were de-epithelialized after iontophoresis in order to assess the true value of intrastromal riboflavin concentration, which was 171 ± 14 μg/cm^3 (almost 10% less riboflavin than with intact epithelium). The other three tissues underwent UV-A irradiation showing nonlinear decrease of corneal riboflavin concentration in all cases; at the end of

Table 1 Riboflavin concentration in the corneal stroma without epithelium immediately after soaking phase (C_0) and after UV-A irradiation at 3 mW/cm^2 for 30 min ($C_{UV\text{-}A3mW}$)

Tissue	C_0 (μg/cm^3)	$C_{UV\text{-}A3mW}$ (μg/cm^3)
cornea_st_1	500 μg/cm^3	–
cornea_st_2	310 μg/cm^3	97 μg/cm^3
cornea_st_3	390 μg/cm^3	39 μg/cm^3
eye_st_1	440 μg/cm^3	32 μg/cm^3
eye_st_1	485 μg/cm^3	45 μg/cm^3
M ± SD	425 ± 77 μg/cm^3	54 ± 29 μg/cm^3

Table 2 Riboflavin concentration in the cornea with epithelium immediately after corneal iontophoresis (C_0) and after UV-A irradiation at 10 mW/cm^2 for 9 min ($C_{UV\text{-}A10mW}$)

Tissue	C_0 (μg/cm^3)	$C_{UV\text{-}A10mW}$ (μg/cm^3)
cornea_ionto_1	256 μg/cm^3	40 μg/cm^3
cornea_ionto_2	180 μg/cm^3	–
cornea_ionto_3	190 μg/cm^3	–
eye_ionto_1	170 μg/cm^3	30 μg/cm^3
eye_ionto_1	180 μg/cm^3	25 μg/cm^3
M ± SD	195 ± 35 μg/cm^3	31 ± 9 μg/cm^3

irradiation period, the mean riboflavin concentration was 31 ± 9 μg/cm^3, i.e., on average 85% lower than the concentration value achieved at the end of soaking phase (Table 2). The CCT did not significantly change after transepithelial treatment protocol (from 561 ± 25 to 582 ± 88 μm; $P = 0.58$).

By delivering the same total energy to the corneal tissue, regardless of the radiated light power and exposure time, the two UV-A irradiation protocols achieved comparable consumption of riboflavin in the cornea ($P = 0.11$). The linear slope at the origin of the photodegradation curves was significant steeper ($P = 0.02$) in the rapid than conventional UV-A irradiation protocol; thereafter, the shape of the curves did not differ between protocols (Fig. 2). On average, the energy constant, E_{rate}, was 2.3 ± 0.5 J/cm^2 and 1.8 ± 0.3 J/cm^2 for standard 3 mW/cm^2 and transepithelial 10 mW/cm^2 irradiation protocols respectively.

Discussion

Application of riboflavin directly onto the corneal stroma for 30 min provided significantly greater drug concentration than transepithelial delivery by corneal iontophoresis at 1 mA/min for 5 min. In this study, corneal iontophoresis delivered 50% less amount of riboflavin into the stroma than conventional 30 min corneal soaking, which was in fair accordance with previous studies [17–19], though using a non-invasive measurement technology that can be used in humans. It is of note that 30 min of manual soaking with the hypotonic solution using the ring applicator achieved higher amount of riboflavin in the stroma than manual application of 20% dextran-enriched solution in the same period, as found in previous studies [4, 5, 12]. The use of hypotonic riboflavin solution did not significantly change the corneal thickness at the end of treatment, contrary to what found in several studies using dextran-enriched solutions, which induced stromal thinning [6, 8, 12].

The rapid transepithelial UV-A irradiation protocol achieved comparable consumption of stromal riboflavin with the conventional UV-A irradiation protocol of the corneal stroma. This means that, in the 3–10 mW/cm^2 range of power densities, riboflavin consumption in the corneal stroma is only energy dependent in accordance with the Bunsen-Roscoe law [10, 20, 21], and that the transepithelial irradiation with 10 mW/cm^2 for 9 min is effective in photodegrading intrastromal riboflavin as the conventional direct stromal irradiation by 3 mW/cm^2 for 30 min. As previously demonstrated [4, 5], for total energy density of 5.4 J/cm^2, the riboflavin in the cornea decreases exponentially during UV-A irradiation of

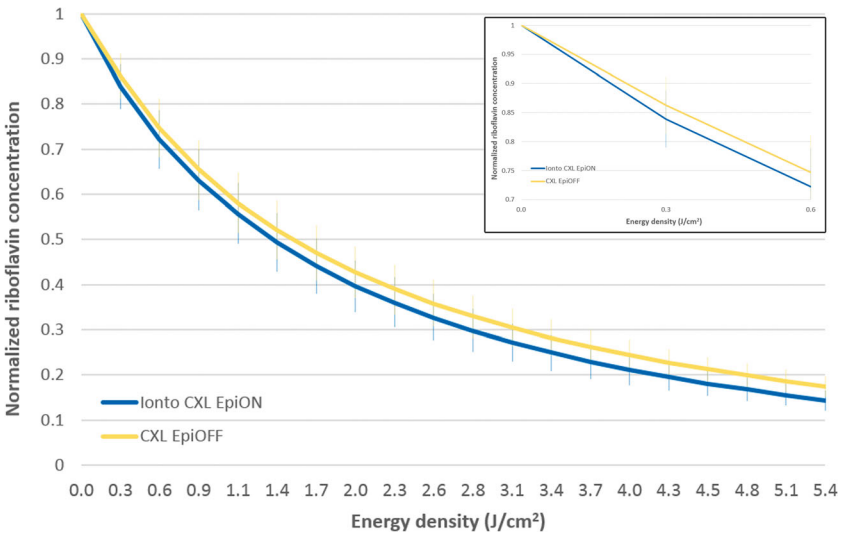

Fig. 2 Plot showing the normalized riboflavin consumption in the cornea during UV-A irradiation as a function of the UV-A energy density. The orange and blue curves show the mean values for 3 mW/cm^2 and 10 mW/cm^2 UV-A irradiance protocols respectively (bars indicate ± SD). The two UV-A irradiation protocols were almost equivalent in terms of riboflavin consumption in the cornea, with an average energy constant, E_{rate}, of 2.3 J/cm^2 and 1.8 J/cm^2 respectively. However, as shown in the inset, the rapid UV-A irradiation protocol (− 0.0005 ± 0.000) showed statistically significant ($P = 0.02$) steeper slope at the origin than conventional irradiation protocol (− 0.0003 ± 0.000)

the human cornea, with the 10 mW/cm^2 UV-A power density inducing faster riboflavin consumption than 3 mW/cm^2 at the origin of irradiation. The exponential photodegradation kinetic of riboflavin was found to be valid both for stromal and transepithelial and UV-A irradiation in the 3–10 mW/cm^2 power density range with $C_0 < 500$ μg/cm^3 [1, 5, 12, 22–27]. The epithelium did not influence the efficient photodegradation of stromal riboflavin by 10 mW/cm^2 UV-A irradiance.

The reliability and performance of the theranostic UV-A device, including the effect of very low light power density irradiation (< 0.6 mW/cm^2) on the cornea, have been assessed in previous studies by comparing the measurements in eye bank donor tissues with those obtained by spectrophotometry, which represents the state-of-the-art optical technology for measuring the UV and blue light absorption of riboflavin [1, 4, 5, 12, 22, 28, 29]. In this study, we assessed the performance of the device on eye globes showing highly consistent data with the sclerocorneal tissues.

Confocal imaging could theoretically improve the UV-A device's axial resolution and allow for analysis of corneal riboflavin at increasing tissue depth; on the other hand, the concentration of riboflavin has been shown to be almost constant up to 350-μm stromal depth[1,22] (keratoconus corneas have in general stromal thickness thinner than 450 μm). Therefore, increasing complexity in the system would not lead to a real advantage in the outcome measures.

In former study [4], we have shown that corneal riboflavin concentration ≥ 140 μg/cm^3 is needed for inducing significant mechanical stiffening of the stroma when delivering 5.4 J/cm^2 UV-A energy dose, with higher drug concentrations correlating with increasing stiffening effect. In controlled clinical studies [30–33], the *Dresden protocol* has shown to induce more K_{max} (i.e., maximum simulated keratometry value) flattening than iontophoresis-assisted transepithelial corneal cross-linking at 2 years postoperatively, though both protocols have been shown to be effective in stabilizing disease progression in the same period. The clinical differences between these two protocols may be strongly correlated with current evidence of greater concentration of riboflavin in the stroma, which may in turn generate, by proper UV-A irradiation, higher rates of additional crosslinking bonds between stromal proteins.

In conclusion, corneal riboflavin concentration is greatly influenced by the presence of epithelium and delivery method; the theranostic UV-A device provided an innovative image-guided therapeutic solution for real-time non-invasive quantification of the amount of riboflavin permeating the cornea during corneal cross-lining. Providing personalized patient care through the non-invasive measurement of corneal riboflavin during treatment can be advantageous to minimize the occurrence of crosslinking failure, regardless of treatment protocol.

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Compliance with ethical standards

Conflict of interest Marco Lombardo and Giuseppe Lombardo declare that they are co-inventors on an issued patent (IT102016000007349) and a pending patent application (WO2017130043A1) related to this work and that they are shareholders of Vision Engineering Italy srl. Sebastiano Serrao declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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