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# Decreased Riboflavin Impregnation Time Does Not Increase the Risk for Endothelial Phototoxicity During Corneal Cross-Linking

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**Purpose:** To evaluate the riboflavin (RF) concentration and distribution in the corneal stroma and the risk for endothelial photodamage during corneal crosslinking (CXL) following 10- and 30-minute impregnation.

**Methods:** De-epithelialized rabbit corneas were subjected to impregnation for 10 and 30 minutes with different RF formulations. Human corneal endothelial cells (HCECs) were subjected to different RF concentrations and ultraviolet A (UVA) dosages. Assays included fluorescence imaging, absorption spectroscopy of corneal buttons and anterior chamber humor, and cell viability staining.

**Results:** After 10 and 30 minutes of impregnation, respectively, anterior chamber fluid showed an RF concentration of $(1.6 \pm 0.21)\cdot10^{-4}$% and $(5.4 \pm 0.21)\cdot10^{-4}$%, and transcorneal absorption reported an average corneal RF concentration of 0.0266% and 0.0345%. This results in a decrease in endothelial RF concentration from 0.019% to 0.0056%, whereas endothelial UVA irradiance increases by 1.3-fold when changing from 30 to 10 minutes of impregnation. HCEC viability in cultures exposed to UVA illumination and RF concentrations as concluded for the endothelium after 10- and 30-minute impregnation was nonstatistically different at 51.0% ± 3.9 and 41.3 ± 5.0%, respectively.

**Conclusions:** The risk for endothelial damage in CXL by RF/UVA treatment does not increase by shortened impregnation because the 30% increase in light intensity is accompanied by a 3.4-fold decrease of the RF concentration in the posterior stroma. This is substantiated by similar endothelial cell toxicity seen in vitro, which in fact appears to favor 10-minute impregnation.

**Translational Relevance:** This study offers compelling arguments for (safely) shortening RF impregnation duration, reducing patients' burden and costly operation room time.

## Introduction

Keratoconus is a common degenerative corneal disorder that has been reported in 0.05% to 2.3% of the population depending on diagnosis criteria and country studied.[1,2] Symptoms include irregular astigmatism, myopia, corneal scarring and structural changes of the cornea including thinning, protrusion, and bulging forward to a more conical shape. The

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disorder is proposed to be affected by family history, eye rubbing, sex, race, atopy, allergy, and other conditions, such as connective tissue disease.[2–4] Up to 1998, treatment was symptomatic using contact lenses, or in severe cases, invasive by penetrating keratoplasty. In 1998, corneal collagen crosslinking (CXL) with riboflavin (RF) and ultraviolet A (UVA) light was introduced, allowing to stabilize progressing keratoconus by stiffening the collagenous corneal tissue. To overcome practical hurdles, increase safety, and reduce patient's discomfort, multiple protocols of RF/UVA CXL have been studied, varying in RF formulation and impregnation times, UVA intensity and duration, and epithelial debridement (Supplementary Table S1).

In the first reported clinical treatment with epithelial removal, dextran enriched RF (RF-D500) was applied for 5 minutes before consecutive UVA irradiation was applied.[5] Later, the RF-D500 impregnation was prolonged to 20 to 30 minutes.[6–10] Currently, a prolonged 30-minute presoaking of the cornea with RF-D500 is regularly applied (Dresden protocol).[11] This impregnation time was chosen to assure sufficient corneal and RF UVA absorption, thereby preventing toxic photodynamic activity at the endothelial level. Based on in vitro studies Wollensak et al.[12,13] determined an endothelial phototoxic level of 0.35 $mW/cm^2$, and determined a minimal corneal thickness of 400 µm are needed to assure safe RF/UVA CXL. Despite these precautions, postoperative corneal edema suggestive of endothelial damage has been reported also in thicker corneas.[14] Although shorter impregnation times are emerging to reduce the discomforting procedure duration and operating theater time, established protocols along with safety justifications are still lacking.

Here we address the correlations between RF impregnation time and RF concentration, distribution, and subsequent UVA/RF phototoxicity to the endothelium based on an in vitro study using human corneal endothelial cells (HCECs). We follow 10 and 30 minutes of impregnation durations because these two periods were found effective in CXL treatment of de-epithelialized cornea with RF and RF-D500, and the 30-minute impregnation has been assumed safer as providing better UVA attenuation.[11,15–19] We hypothesize that, although the attenuated UVA is lower after a 30-minute impregnation period, the overall risk for photodynamic damage at the endothelium is actually higher because of the increased endothelial RF concentration. To test this hypothesis, we applied RF/UVA combinations using the experimentally based calculated RF concentrations and light intensities for the two CXL protocols to in vitro grown HCECs.

# Methods

## Sample Preparation

### RF Formulations

Two formulations of RF were used: (1) hypo-osmolar riboflavin-5'-phosphate (F6750; Sigma-Aldrich, St. Louis, MO) in saline solution, adjusted to pH 7.3 (RF); and (2) hyper-osmolar commercial riboflavin-5'-phosphate in 20% dextran 500 kDa (RF-D500), (MedioCross, Kiel, Germany; pH 6.8). For the in vivo and ex vivo experiments a concentration of 0.1% was applied, whereas for the in vitro studies a range of RF concentrations was used.

### Animal Models

Both ex vivo (n = 17) and in vivo (n = 14) experiments were performed with New Zealand White (NZW) rabbits of 12 to 25 weeks old (2.5–3.7 kg weight), respectively, either obtained from a local abattoir, or housed with ad libitum access to food and water at the animal facility of the Weizmann Institute of Science (Rehovot, Israel). All experimental procedures were approved by the Institutional Animal Care and Use Committee, and in adherence to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Visual Research.

For the ex vivo experiments, eyes were obtained within several hours after enucleation, de-epithelialized, and left unimpregnated (n = 3), or impregnated with RF for 10 (n = 5) or 30 (n = 9) minutes. For the in vivo experiments the rabbits were anesthetized by intramuscular injection of 35 mg/kg ketamine (Rhone Merieux, Lyon, France) and 5 mg/kg xylazine (Vitamed, Binyamina, Israel). After treatment, rabbits were euthanized by intravenous injection of pentobarbital sodium (CTS Chemical Industries Ltd, Kiryat Malachi, Israel). As negative controls for stromal RF distribution measurements by fluorescent microscopy, this experiment included two corneas in the in vivo group, in which the epithelium was left intact prior to RF impregnation. All other eyes were de-epithelialized, after which the cornea was incubated with either RF or RF-D500 using an eye cap (12 mm in diameter) for 10 (n = 2 and n = 2, respectively) or 30 (n = 3 and n = 4, respectively) minutes, or with saline solution for 30 minutes (n = 1), as previously described.[9] The corneas were then briefly rinsed with saline solution, and a central 8-mm corneal button was trephined for further processing.

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## Stromal RF Distribution

Immediately after euthanization and corneal button trephination, buttons were frozen on dry ice until further processing. Twenty-micrometer thick sagittal slices were cut using a cryotome from both RF and RF-D500 in vivo treated corneas, and mounted on a microscope glass slide, stored frozen and in the dark until consecutive fluorescence readings. Fluorescence intensity from three serial slices per cornea was recorded using a fluorescence microscope (BX61 Olympus, Tokyo, Japan) equipped with a CCD camera (Cascade 512B, Roper Scientific, Inc., Tucson, AZ). An excitation wavelength of 488 nm was used, and fluorescence intensity above 530 nm was recorded using a filter. Intensity of 50 lines within each image of a cross section were averaged using ImageJ software (National Institutes of Health, Bethesda, MD). To avoid bias, the analysis was done blinded with respect to sample treatment.

## Absorption Measurements

### RF Calibration Curve

A calibration curve was prepared for deriving the RF concentration from the 446 nm optical absorption. The stock solution used included the same RF used for the impregnation, that is, RF-D500 (0.1% RF, 20% dextran, MedioCross), in a mixture of 2.4:1 methanol and water to provide optimal RF solubility, which was adjusted to pH 7.3 and used to prepare 5, 10, 15, and 20 µg/mL solutions for calibration. Optical absorption of the four solutions was measured through a 1-cm path quartz cuvette using a spectrophotometer (V750; Jasco, Oklahoma City, OK).

### Anterior Chamber Humor Measurements

After corneal impregnation in vivo, as described earlier, corneal paracentesis was performed, and anterior chamber humor was drawn and frozen on dry ice until further analysis. The humor (100 µL) was diluted with methanol (240 µL) and centrifuged (21,000*g* x 5 minutes) to remove large particles. Optical absorption of a filled 1-cm path cuvette was measured (V750; Jasco), the diluting mixture's baseline spectrum was removed, and zero absorption was set at 600 nm.

### Trans-Corneal Absorption

Ex vivo treated corneal buttons were placed on a specially designed 1-mm light pass cuvette (Fig. 1). Recorded trans-corneal absorption spectra (Evolution 220 spectrophotometer; Thermo Scientific, Waltham, MA) were normalized to the deviation from the average thickness after de-epithelialization. The trans-corneal spectrum of untreated de-epithelialized eyes (average of three eyes) was considered baseline and subtracted from each measured spectrum. In addition, the optical absorption was set to zero at 600 nm. The obtained corneal absorption at 446 nm ($A_{446}$) was used to calculate the average RF concentration in the stroma according to the Beer Lambert law:

(1) $A_{446} = \varepsilon_{RF}^{446} \bullet \bar{C} \bullet \mathfrak{l}$, where $\varepsilon_{RF}^{446} = 12{,}550\ \mathrm{M}^{-1}\mathrm{cm}^{-1}$, $\mathfrak{l} = 0.045$ cm, and $\bar{C}$ = is the average concentration of the RF-5'-phosphate (molecular weight: 456 [g/mol]) in the corneal stroma.

To estimate the RF concentration in the posterior stroma (i.e., endothelial layer) after 30 minutes of impregnation, we follow the near-linear cross-corneal fluorescence decay and assume that $\bar{C}$ is roughly given by:

(2) $\bar{C} = \frac{(\bar{C}_{ant} + \bar{C}_{post})}{2}$, where $\bar{C}_{ant}$ and $\bar{C}_{post}$ are the RF concentrations in the anterior (0–200 µm) and posterior (200–400 µm) corneal stroma. $\bar{C}_{ant}$ is known from the literature to range between 0.05 and 0.09%, the lowest value was chosen to not underestimate the posterior RF concentration[20–23]

As stromal diffusion after 10 minutes of RF impregnation is known not to be linear, posterior RF concentration after 10 minutes impregnation was estimated by dividing the obtained value after 30 minutes by the ratio between aqueous humor concentration after 10 and 30 minutes of RF impregnation.

## In Vitro Endothelial Toxicity Measurements

Immortalized HCECs (HCEC-12; DSMZ, Braunschweig, Germany) were grown in culture medium consisting of 95% Dulbecco's Modified Eagle Medium, supplemented with 5% fetal bovine serum, 1/100 penicillin-streptomycin, and 1/125 amphotericin B in an incubator at 37°C, according previously published protocol.[24,25] Cells were plated at $2.5{\bullet}10^4$ cells per well in a flat-bottomed transparent 96-well plate. After 24 hours, the culture medium in three wells per plate was replaced with 200-µL culture medium containing 0.04%, 0.02%, 0.01%, 0.005%, 0.0025%, or no RF (F6750; Sigma-Aldrich) and kept in the dark for 10 minutes. Consecutively, irradiation was performed from below, accounting for the plate's UVA absorption (measured at 18.6% by spectrophotometry), with environmental temperature and oxygen concentration at 25°C and 20%, respectively. UVA intensity (365

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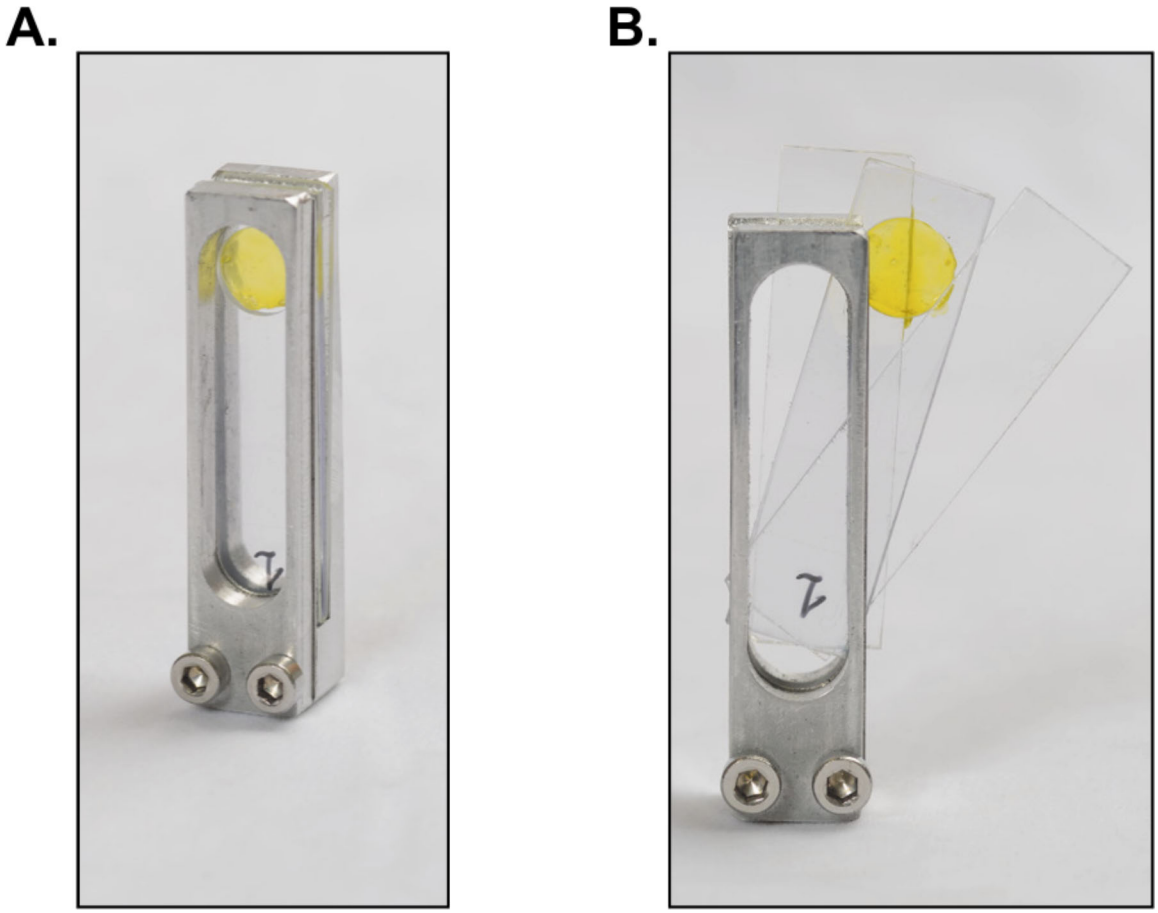


**Figure 1.** Assembled (A) and disassembled (B) views of the specially designed cuvette for measuring trans-corneal absorption spectra of a corneal button with a corneal button impregnated with RF in the cuvette spacer between two glass holders.

nm) was set to deliver either no UVA or ranging between 0.3 and 1.3 mW/cm$^2$ at 0.2 mW/cm$^2$ intervals. After irradiation, the solution in all wells was replaced by fresh culture medium. Twenty-four hours later, the medium was replaced by a 9:1 mixture of culture medium and WST1 viability staining (Sigma-Aldrich) and kept for 1 hour, per manufacturer's protocol. Per plate, three empty wells were filled with the same culture medium and WST1 mixture for baseline determination. Luminescence of each well was measured (Infinite m200pro; Tecan, Männedorf, Switzerland) and baseline corrected. A total of nine plates (three plates per irradiation group each) were seeded and treated as described earlier, resulting in nine readings per combination of RF concentration and UVA irradiation (three plates x three wells). Cell survival was calculated by dividing each well's baseline corrected luminescence reading by the average luminescence reading of the wells that did not contain any RF and did not receive UVA irradiation. Significance was determined using a linear regression model (IBM SPSS Statistics for Windows, Version 23, IBM Corp., Armonk, NY).

## Results

### Baseline Characteristics

Corneal thickness measured before epithelium debridement was 497 ± 48 µm. On average 50 µm were removed on epithelium debridement.

### Stromal RF Distribution

Negligible fluorescence was detected from de-epithelialized corneas incubated in vivo for 30 minutes with saline solution (one eye), or with RF without de-epithelialization (two eyes) (Figs. 2A, 2B). In contrast, after 10 minutes of impregnation to de-epithelialized eyes (two eyes), RF penetrated the corneal stroma entirely. Stromal fluorescence peaked



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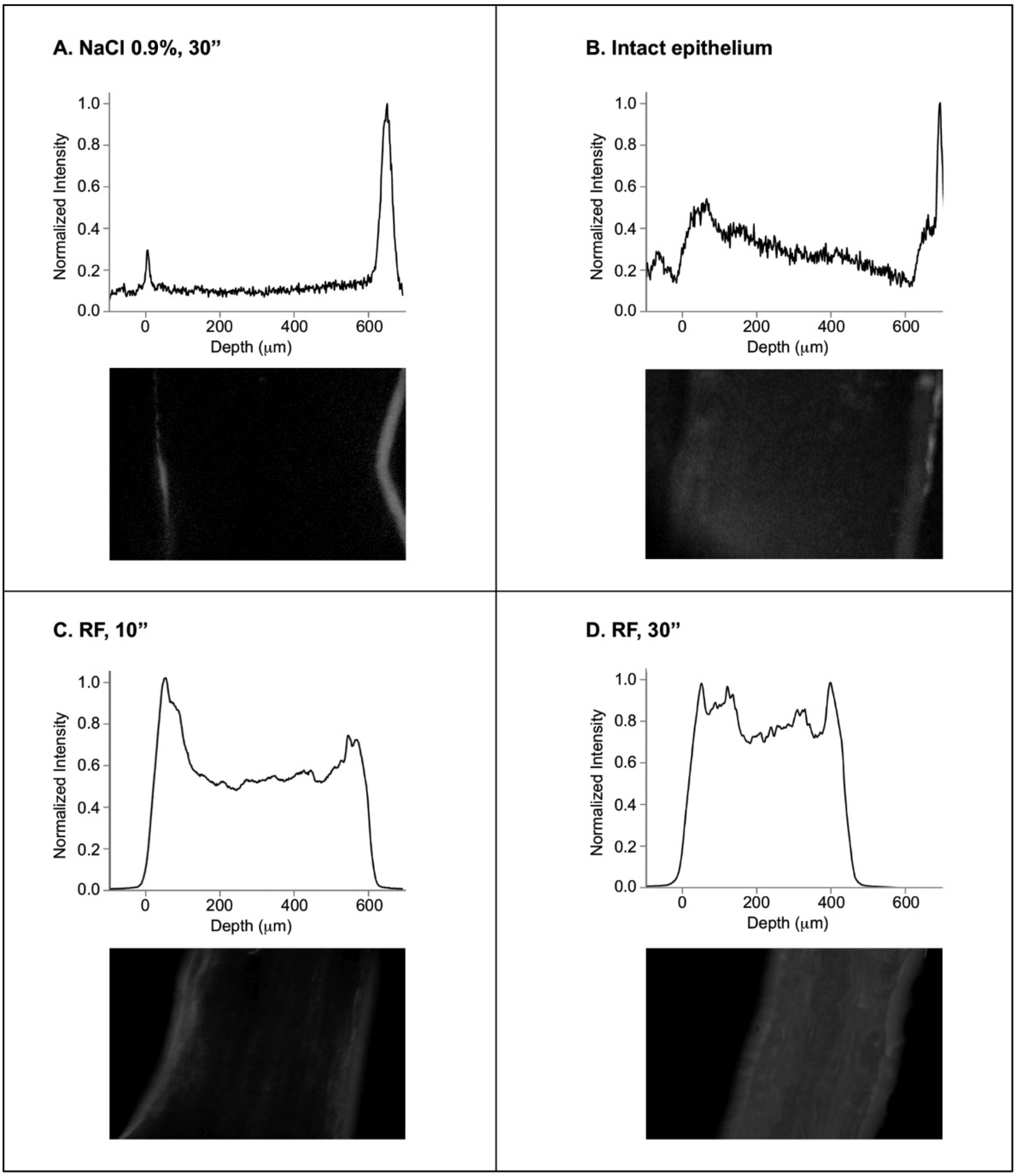


**Figure 2.** In vivo cross-corneal penetration of RF in saline solution across rabbit corneas. No signal was detected from a de-epithelialized cornea incubated with saline solution for 30 minutes (A). Likewise, no signal was detected from the intact cornea with the epithelium on (B). Rabbit corneas were exposed to RF in saline solution for (C) 10 minutes and (D) 30 minutes. The graphs represent the trans-corneal fluorescence signal as averaged over 50 lines of the fluorescence image.

at the anterior cornea and decayed by approximately 50% at a depth of ~200 μm in which it leveled off and remained so until reaching the posterior stroma. After 30 minutes of RF incubation (three eyes), the fluorescence intensity at the anterior cornea decayed only by 20% at 200 μm and remained so until reaching the Descemet membrane (Figs. 2C, 2D). Application of the clinically applied RF-D500 resulted in significant changes in the photosensitizer distribution within the cornea, compared with the dextran-free solution (Figs. 3A, 3B). The cornea impregnated for 10 minutes (two eyes) presented a gradient decline in RF

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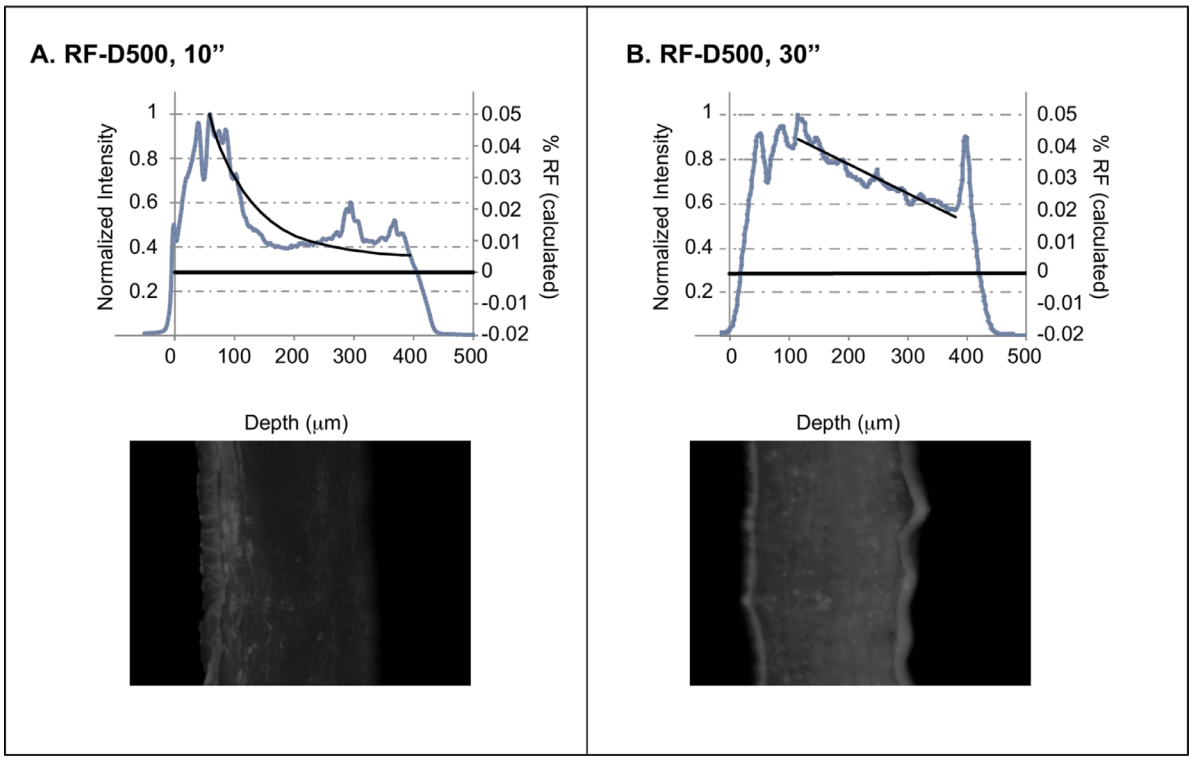


**Figure 3.** In vivo corneal penetration of RF-D500 after impregnation for 10 (A) and 30 (B) minutes. The normalized intensity graphs depict the distribution of RF-D500 concentration across the corneal sagittal slice. The graph was normalized to give the same thickness (400 μm) in the two panels. The exponential trend line for panel (A) is y = 0.045*e-0.11*X555 + 0.005.

concentration reaching a minimum at a depth of 209 μm. In contrast, the 30-minute impregnation (four eyes) showed a mild gradient across the entire cornea with a measured minimum at 366 μm.

## Absorption Measurements

### RF Calibration Curve

All spectra displayed a flat absorption from approximately 520 to 600 nm. The background noise level was negligible with $R^2 = 0.994$, and a low error for the lowest measured RF concentration (0.09 ± 0.0007 μg RF/mL; Figs. 4A, 4B). Using Equation 1, this calibration curve provides value of $\varepsilon_{RF}^{446} = 12{,}550\ M^{-1}cm^{-1}$, similar to literature value of $\varepsilon_{RF}^{446} = 12{,}500\ M^{-1}cm^{-1}$ for RF.[26]

### Anterior Chamber Humor Measurements

Using Equation (1) with $l = 1$ cm and $\varepsilon_{RF}^{446} = 12{,}550\ M^{-1}cm^{-1}$, the obtained concentrations of RF in the anterior chamber were found to be $(1.6 \pm 0.21) \cdot 10^{-4}$ and $(5.4 \pm 0.59) \cdot 10^{-4}$ % ($P < 0.01$) for 10- and 30-minute impregnation, respectively (Fig. 4D).

### Trans-Corneal Absorption

Trans-corneal spectra clustered tightly according to the impregnation duration, except for two outliers in the 30-minute group (n = 9) that were excluded. The trans-corneal optical absorption at 446 nm ($A_{446}$) measured 0.398 ± 0.021 and 0.496 ± 0.033 for the 10 (five eyes) and 30 (seven eyes) minutes of RF impregnation, respectively (Fig. 5A). For nonimpregnated corneas, $A_{446}$ was 0.069 ± 0.005 (three eyes). After removing this contribution to the light absorption by the corneal stroma, the RF absorption was 0.329 and 0.427 after 10- and 30-minute impregnation, respectively (Fig. 5B).

### Stromal RF Concentrations

Applied to Equation 1 and multiplied by the molecular weight (456 g/mol), the measured $A_{446}$ values as mentioned earlier, result in an average stromal concentration of 0.0266% and 0.0345% after 10- and 30-minute RF impregnation, respectively. Thus already after 10 minutes of RF impregnation, there is 77% of the RF concentration across the stroma relative to 30-minute impregnation. The average RF concentration in the posterior stroma for the 30-minute impregnation ($\bar{C}_{post\ 30min}$) is then 0.019%. After 10-minute RF

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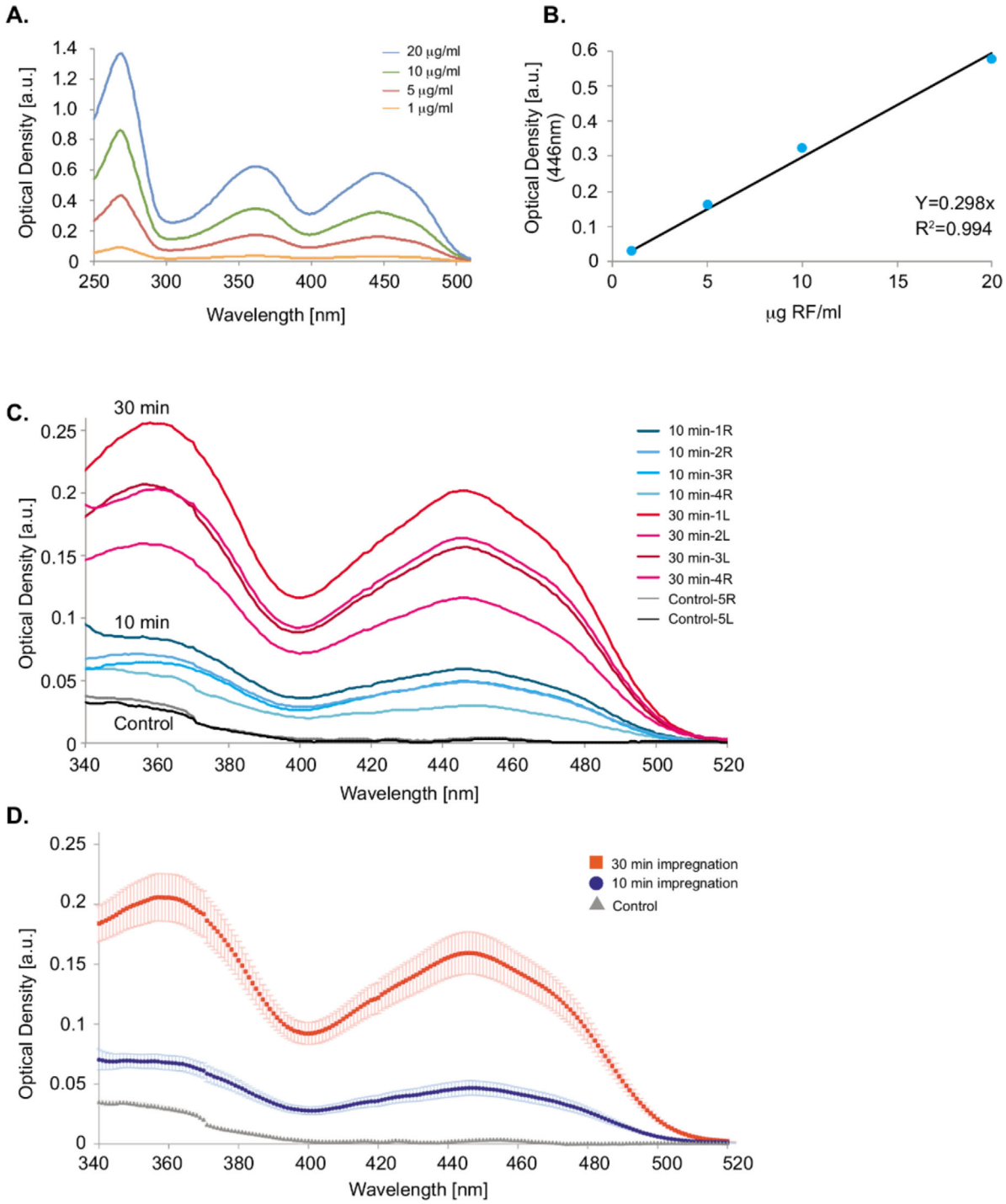


**Figure 4.** Anterior chamber concentrations of RF following 10 and 30 minutes of RF-D500 impregnation. (A) Different concentrations of RF in solution were used to construct a calibration curve (B) using the spectrum peak at 446 nm. The equation was set to pass at (0,0). (C) Spectra of the anterior chamber humor drawn from rabbits after the de-epithelialized cornea was incubated, under anesthesia, for 10 (right eye, denoted "R") and 30 minutes (left eye, denoted "L") of impregnation with RF-D500. The numbers in the *inset* legend refer to the rabbit number to enable comparison of the spectra between the two eyes. Eyes of an untreated rabbit served as a control. (D) Averages for the data presented in panel (C). *Error bars* represent the standard error.

impregnation, the average posterior stromal concentration ($\bar{C}_{post\ 10min}$) equals 0.0056% (0.019% divided by the ratio found in anterior chamber RF concentrations: 3.4).

## Expected Light Fluency at the Endothelium

Considering both the optical density due to RF absorption and the absorption and scattering of

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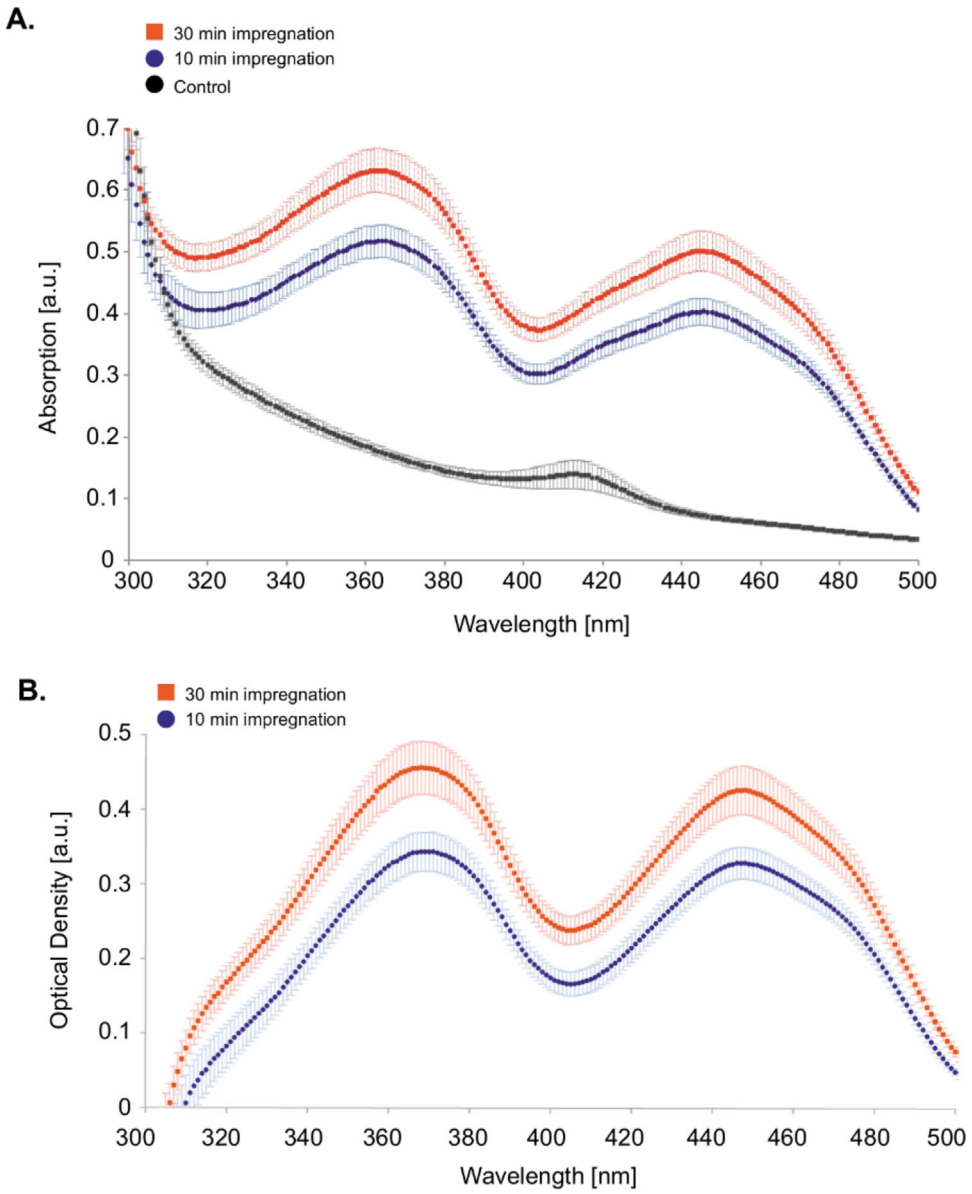


**Figure 5.** Trans-corneal absorption spectra (A). Ex vivo de-epithelialized trans-corneal absorption spectrum following 10 (*blue*) and 30 (*red*) minutes of RF impregnation and the absorption of the cornea without impregnation (*gray*). (B) The absorption spectra of the two impregnation durations after deducting the contribution of the native cornea. Averaging was conducted for five and seven eyes for the two spectra, respectively. *Error bars* represent the standard error.

the untreated cornea (control, Fig. 5A), the absorption at 370 nm is 0.5116 and 0.6255, for the 10- and 30-minute impregnated corneas, respectively. Calculated transmitted light intensity at 370 nm, by substituting the relevant values in $T = 10^{-A}$, was then 30.08% and 23.7%, respectively. Thus with a fluency of 3 mW/cm$^2$ at the corneal anterior surface, the endothelium is subjected to 0.9 and 0.7 mW/cm$^2$, after 10 and 30 minutes of RF impregnation, respectively.

## In Vitro Corneal Endothelial Toxicity Measurements

Cell survival of HCECs for a range of RF concentrations and UVA intensities, including the parameters we found after 10 minutes (0.005% RF and 0.9 mW/cm$^2$ UVA) and 30 minutes (0.02% RF and 0.7 mW/cm$^2$ UVA) of RF impregnation, are shown in Figure 6 and Supplementary Table S2. We found no significant difference in cell survival between the

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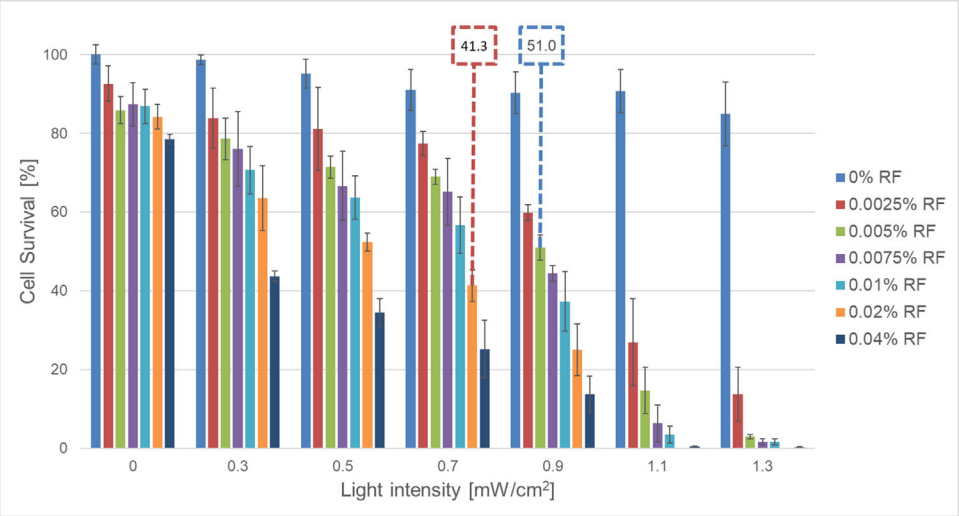


**Figure 6.** Cell survival of in vitro RF/UVA treated HCEC-12. Bars were normalized to the average luminescence reading of nine wells containing no RF and receiving no UVA irradiation. *Error bars* represent the standard deviation. Mean endothelial cell survival corresponding to the 10 minutes (*blue dashed box*) and 30 minutes (*red dashed box*) are highlighted.

10- and 30-minute impregnation protocols ($P = 0.056$). In fact, there appears to be a slightly lower photodynamic toxicity for the shorter impregnation, with a mean cell survival of 51.0% ± 3.9% and 41.3% ± 5.0%, for the 10- and 30-minute impregnation protocol, respectively. UVA alone, RF alone, and the combination of RF and consecutive UVA irradiation show a highly significant trend toward reduced cell viability (all $P < 0.001$). $\beta$-Coefficients for UVA irradiation, RF concentration, and their interaction term are −29.3% per mW/cm$^2$ UVA, −605.2% per % RF, and −1206.9% per mW/cm$^2$ UVA * % RF, respectively.

## Discussion

The main goal of CXL is to arrest progression of keratoconus and corneal ectasia with minimal side effects and risk.[3–6,8,27–30] An established benchmark protocol (Dresden[11]) includes RF impregnation of the corneal stroma for 30 minutes and subsequent irradiation with UVA light. The RF impregnation time is guided by the minimal RF concentration to allow for sufficient CXL, and the minimal RF concentration across the cornea needed to sufficiently attenuate the UVA light, minimizing the photodamage to the corneal endothelium.[11,31–33] Following the apparent conflict of the risk/benefit of shorter impregnation times, we explored the time-dependent distribution of RF across the corneal stroma and the corresponding endothelial cell survival. Although RF distribution has been shown before by several authors, to the best of our knowledge, little attention has been paid to the safety implication of reduced RF impregnation times.

On the ground of theoretical and experimental considerations, 30 minutes of impregnation followed by 30 minutes of illumination at 3 mW/cm$^2$ have been recommended for safe and effective treatment.[11,13,32] Yet, to reduce patient burden and adverse effects, numerous reports demonstrated efficient stiffening without compromising clinical safety following shorter times of RF impregnation (Supplementary Table S1). Not less important, Supplementary Table S1 shows that there is currently no established protocol as to RF impregnation duration. Previous studies show deep stromal RF penetration already occurs after 8 to 10 minutes of impregnation, with a further moderate rise to a plateau after 30 minutes.[31,34,35] Although Baiocchi et al.[36] measured a ratio of 75% for the average total RF concentration, Spoerl et al.[31] showed that in the range of 0 to 180 µm there is no difference between the RF concentration after 10 and 30 minutes of impregnation. However, when looking at the endothelium (360–540 µm), after 10 minutes of impregnation the RF concentration is 30% to 40% of that achieved after 30 minutes of impregnation. The overall distribution demonstrated therein is in agreement with the change of RF fluorescence across the cornea as presented in this study (Figs. 3A, 3B). The data from our study, as displayed in Figure 5, shows the 30-minute incubation decreases the transmitted light intensity by merely a factor of ~1.3 compared with the transmitted intensity after 10 minutes (23.7% and 30.8% for the long



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and short impregnation durations, respectively). Thus although RF concentration at the endothelial level increases over three-fold after 30 minutes compared with 10 minutes of impregnation, the attenuation of UVA light only decreases by a factor of ~1.3 in the longer 30-minute incubation protocol.

Photodynamic toxicity is usually achieved when the product of a photosensitizing agent concentration by the light energy overcomes a threshold value given by Equation 3:

(3) $PDT_{PS} = [PS] \bullet E_I$ where $PDT_{PS}$, $[PS]$ and $E_I$ denote photodynamic therapy, photosensitizer concentration (in percent, defined as g/100 mL) and light intensity energy (in Joules), respectively, which show initiation of photodynamic damage.

Wollensak et al.[12] found out that for endothelial cells in culture a cytotoxic threshold was set at $PDT_{RF} = 0.024 \bullet 0.63 = 0.0151$ where 0.024% and 0.63 J/cm$^2$ are the RF concentration and UVA light energy, respectively. Our data demonstrates that the UVA light intensity of 3 mW/cm$^2$ at the anterior stroma will be attenuated to 0.9 and 0.7 mW/cm$^2$ at the posterior stroma after 10 and 30 minutes of RF impregnation, respectively. The total delivered energy at the endothelium will thus be 1.62 and 1.26 J/cm$^2$ for the 10- and 30-minute impregnation protocols, respectively, neglecting the additional attenuation by the RF film, after the standard 30 minutes of UVA irradiation. Applying these numbers and the corresponding RF concentrations at the posterior stroma as found in this study in Equation 3, results in a respective $PDT_{RF}$ value of 0.00903 and 0.0239. Thus the probability for photodamage actually increases by over a two-fold and above the 0.0151 threshold determined by Wollensak et al.,[12] for the prolonged incubation time, whereas that of the short time stays below.

Current methods to assess corneal stromal RF concentration lack the ability to directly measure concentrations and are subjected to some degree of variability due to factors, such as light attenuation, physiological state of the measured tissue (e.g., hydration, de-epithelialization, and others), or resolution of confocal sectioning. The lack of a gold standard is clear from the variety of methods applied in the literature.[21,37] Our study suffers from similar limitations. First, although corneal hydration was monitored and in vivo pachymetry was performed in physiological state, our cohort of NZW rabbits had thicker pachymetry measurements than what was to be expected.[38] Second, our calculations rely on previously published data on anterior stromal RF concentrations.[20–23] Although these values correspond well, this does allow for variation. Nevertheless, our data are in line with recent reported literature.[39] Notably, our data also agrees with reports in which 30 minutes of RF impregnation cannot bring the corneal absorption to sufficiently attenuate the UVA below the putative threshold of 0.63 J/cm$^2$ suggested by Wollensak et al.[13] for 0.024% RF. Only on considering the precorneal RF film, the attained UVA light intensity is lower than this threshold.[32] Thus when considering the RF concentration in the posterior stroma for 10- and 30-minute impregnation, the shorter time of incubation appears to be the safer regimen, as shown earlier. The first series of successful CXL treatments applied 5 minutes of impregnation without expressing endothelial damage.[5] Recent practices of reduced impregnation times (Supplementary Table S1) further substantiate the notion that a long impregnation duration is not required. When applying RF during the CXL, the resulting 70-µm RF film can further attenuate the irradiance in a significant manner.[32] As the film breaks up only after 20 minutes,[32] it will remain during CXL, let alone during accelerated CXL even without more frequent RF drops as commonly applied.

Several studies have investigated the effect of RF/UVA CXL on the corneal endothelium, applying different models and cell lines (Supplementary Table S3). Wollensak et al.[13] provided data on CXL endothelial toxicity that led to a threshold corneal thickness of 400 µm to allow for safe CXL. In vitro studies with corneal endothelium derived from different animal species showed toxicity at 100 µM (Cho et al.[40]) or 50 µM (Wollensak et al.[12]). A study by Mooren et al.[41] using human donor corneas has, however, demonstrated that HCECs may be more resistant than what is suggested by the earlier mentioned in vitro studies using animal cell lines. Notably, in this ex vivo setup, it is not clear what the final RF concentration is at the endothelium at the time of irradiation. It is unlikely that a difference between the biology of human and animal endothelial cells accounts for the observed difference in toxicity. Most probably, this difference is related to differences in the experimental conditions used.

To our knowledge, our study is the first to use HCECs grown in vitro to assess toxicity. Our results indicate endothelial toxicity is related to RF concentration and applied UVA intensity, both parameters influenced by altering RF impregnation time. Furthermore, we show toxicity of UVA irradiation alone for both intensities applied, which was seen in vitro using porcine corneal endothelial cells only at much higher intensities.[12] Although the in vitro setup and immortalization of the HCECs may not exactly mimic the in vivo situation, it does allow for comparison between

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treatment protocols with accurate control over applied RF concentrations. Greater oxygen availability in vitro will most likely overestimate the corresponding in vivo endothelial toxicity. However, when comparing RF concentration and UVA intensity as applicable during CXL after 10- or 30-minute RF impregnation, we find a similar toxicity of 50% to 60%, in favor of the 10-minute impregnation protocol. This indicates that lowering RF impregnation time from 30 to 10 minutes does not increase corneal endothelial toxicity and may in fact even be safer.

## Conclusions

Our study suggests that 30-minute RF impregnation does not increase the CXL safety compared with 10 minutes of impregnation, as confirmed by our in vitro endothelial toxicity study. Rather, theoretically the increased posterior stromal RF concentration may enhance the risk for photodynamic damage of the endothelium. When combined with other preclinical and clinical data, this supports the current trends of shortening the impregnation time (Supplementary Table S1), thus decreasing associated corneal thinning and drying, and overall treatment duration along with patient discomfort.

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