# Riboflavin Concentrations at the Endothelium During Corneal Cross-Linking in Humans

Theo G. Seiler,[1-3] Ana Batista,[4,5] Beatrice E. Frueh,[1] and Karsten Koenig[4,5]

[1]Universitätsklinik für Augenheilkunde, Inselspital, Universität Bern, Bern, Switzerland
[2]Institut für Refraktive und Ophthalmo-Chirurgie (IROC), Zürich, Switzerland
[3]Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, United States
[4]JenLab GmbH, Berlin, Germany
[5]Lehrstuhl für Biophotonik und Lasertechnologie, Universität des Saarlandes, Saarbrücken, Germany

Correspondence: Theo G. Seiler, Universitätsklinik für Augenheilkunde, Inselspital, Universität Bern, Bern 3010, Switzerland; theo@seiler.tv.

TGS and AB contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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**Purpose.** To determine the riboflavin concentration in the posterior corneal stroma, Descemet's membrane, and endothelium prior to UV irradiation in corneal cross-linking (CXL) in humans.

**Methods.** Five human deepithelialized cadaver corneas were mounted into artificial anterior chambers. After the establishment of stable physiological hydration, two-photon imaging with a certified multiphoton tomograph was used to determine fluorescence intensity and second harmonic generation signals from collagen throughout each cornea by optical sectioning, with a step size of 2.5 µm. Afterward, 0.1% riboflavin solution was applied to the anterior corneal surface, similar to the standard CXL protocol. To determine the absolute riboflavin concentration immediately before UV irradiation, corneas were measured by two-photon imaging just at the end of the riboflavin imbibition and after riboflavin saturation.

**Results.** The topical application of 0.1% riboflavin results in a riboflavin concentration that decreases to 0.035% in the posterior stroma. Inside Descemet's membrane and endothelium, the concentration drops further to only approximately 0.015% at the endothelial level. Local riboflavin distribution indicates a predominantly paracellular passive diffusion of riboflavin into the anterior chamber.

**Conclusion.** The experimentally determined riboflavin concentration of 0.015% at the endothelium shows a substantial discrepancy of a factor of 1.7 to the previously theoretically calculated 0.025%. A lower riboflavin concentration at the endothelium may enable higher radiant exposures and further improve the efficacy of CXL.

Keywords: cornea, endothelium, safety, riboflavin, corneal cross-linking, CXL, two-photon

Corneal cross-linking (CXL) is considered to be a safe and effective procedure to halt the progression of keratectasia such as keratoconus[1,2] or post-LASIK ectasia.[3] By means of UV-A irradiation and riboflavin as the photosensitizer, reactive oxygen species (ROS) are created, inducing new bonds in the extracellular matrix of the cornea.[4] Beside the beneficial tissue-stiffening effect, these radicals cause damage in cellular membranes, leading to keratocyte apoptosis.[5,6] This can be observed one month after CXL by using the optical imaging methods confocal microscopy or optical coherence tomography visualizing the corneal demarcation line.[7,8]

When ROS reach the posterior surface of the cornea, vulnerable structures such as the endothelium may be affected and potentially become necrotic or apoptotic. Therefore, prior to the clinical introduction of CXL by the Dresden group, this risk was identified, and safety thresholds were determined in order to protect the corneal endothelium.[9,10] However, these studies used a rough estimation of the riboflavin concentration based on theoretical calculations because no technique existed at that time for a precise determination of the local concentration of riboflavin.[11] These considerations lead directly to the "400-µm rule", which demands a minimal corneal thickness during UV irradiation.[11]

In the 15 years since the clinical introduction of CXL, only few reports of endothelial damage have been published.[12–17] In addition, Mooren et al.[18] could not find endothelial cell damage even with a suprathreshold UV treatment of the human endothelium.

The purpose of this laboratory study is to measure the riboflavin gradient in the posterior human cornea by sectioning based on two-photon excited riboflavin fluorescence, thus verifying or disproving the theoretical values of the riboflavin concentration at the endothelial level. Two-photon imaging has the advantage of using (1) a high light-penetration depth in the near infrared, as well as (2) the absence of out-of-focus one-photon absorption, photobleaching, and photochemical reactions.

## Materials and Methods

### Cornea Preparation for Two-Photon Fluorescence Microscopy

Five human corneoscleral disks unsuitable for transplantation were excised from intact globes not later than 6 hours post mortem and evaluated for endothelial cell count (ECC) and morphology by inverted phase-contrast microscopy (TS100

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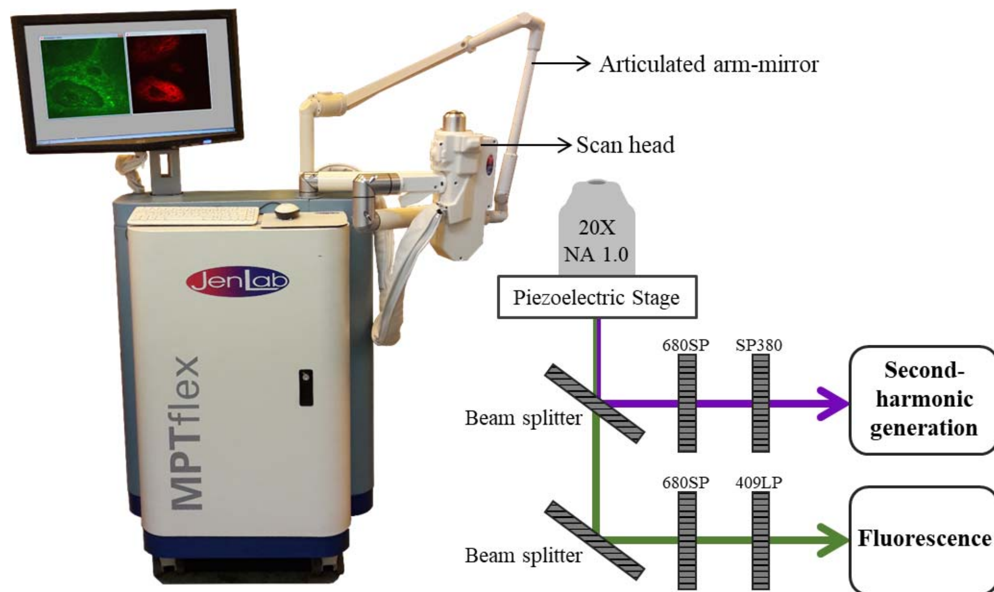


FIGURE 1. Two-photon imaging with a CE-certified multiphoton tomograph provides high-resolution optical sections based on riboflavin and NAD(P)H fluorescence signals and second harmonic generation (SHG) signals from collagen. The instrumental setup inside the system's scan head is presented schematically.

Eclipse; Nikon, Tokyo, Japan). Subsequently, the eyes were stored in a corneal culture medium consisting of 2% fetal bovine serum and minimal essential medium (Sigma-Aldrich, St. Louis, MO, USA), following the standard protocol of the eye bank of the University Hospital of Bern. Twelve hours before the experiments, dextran T-500 (Sigma-Aldrich Corp.) was added at a concentration of 6% (wt/wt) to de-swell the cornea to physiological hydration. For the experiments, corneas were mounted into an artificial anterior chamber (Barron Precision Instruments, Grand Blanc, MI, USA) with an IOP equivalent to 18 mm Hg and an aqueous 15% dextran T-500 solution as aqueous humor. Afterward, remnants of epithelium were removed using a blunt hockey knife. As previously described,[19] a reservoir containing 15% dextran T-500 solution was placed on the anterior side of the cornea to achieve stable physiological hydration via both surfaces of the cornea. After a stable hydration state had been reached (three identical consecutive pachymetries, 10 minutes apart) the first two-photon microscopy scan was performed. Afterward, the reservoir on the anterior surface was filled with 0.1% riboflavin-5-monophosphat in 15% dextran T-500 solution for 10 minutes (stirred each two minutes) to imitate the clinical riboflavin imbibition. The reservoir was then removed, the corneal surface was wiped clean without liquid, and the second two-photon microscopy scan of the cornea was acquired. For individual calibration, each cornea was saturated with riboflavin by applying 0.1% riboflavin in 15% dextran solution in the reservoir on the anterior surface of the cornea and in the anterior chamber for 2 hours. Subsequently, a third two-photon measurement was performed of the saturated corneas. At each step of the experiment, the content of the reservoirs was replaced by freshly prepared solutions. During the entire procedure, a pressure equivalent to 18 mm Hg was applied. Ultrasound pachymetry (SP-100; Tomey, Nagoya, Japan) was performed after every step of the process. Dextran T-500 in a concentration of 15% (wt/wt) was chosen as the osmotic agent instead of the clinically used 1.1% hydroxypropyl methylcellulose (HPMC) because it yielded better cellular imaging quality while keeping the same physiological hydration.[20,21] All experiments were performed at a temperature of 19°C.

## Two-Photon Fluorescence Microscopy and Intensity Analysis

Two-photon images were acquired using a CE-certified multiphoton tomograph (MPTflex; JenLab GmbH, Berlin, Germany) (Fig. 1). Sample excitation was accomplished using a 80-MHz near-infrared Ti:Sapphire tunable laser with pulsed widths of 100 femtoseconds. The laser beam is guided to the system's scan head via an articulated mirror arm (Fig. 1). Once in the scan head, the laser beam is focused on the sample using a 20× numerical aperture (NA) 1.0 water immersion objective with a working distance of 1.7 mm to cover the entire corneal thickness. Galvanometric scanners and a piezo-driven z-scanner to change the scanning position in $x$, $y$, and $z$ directions are included in the system's scan head. Second harmonic generation (SHG) and two-photon fluorescence–generated photons are collected by the objective, separated based on their emission wavelength using beam splitters, and detected simultaneously by single-photon counting using two photomultiplier tubes (Fig. 1). Using this system, the human cornea can be imaged with high repeatability. The mean absolute percentage of deviation of photon counts in consecutive measurements is 0.69% (maximum of 1.54%).

In this study, image acquisition was performed using an excitation wavelength of 760 nm. Acquisition times were 3.2 seconds for a scanning area of $512 \times 512$ pixels, corresponding to pixel dwell times of 0.012 milliseconds/pixel. Volumes with $400 \times 400 \times 200$ $\mu m^3$ at the interface between the posterior cornea and anterior chamber were acquired with a 2.5-µm step between the images. Prior to riboflavin application, laser powers of 50 mW were used to image the tissue autofluorescence. Following the application of the photosensitizer, the laser power was reduced to 27.5 mW to avoid saturation of the signal. Laser power was kept constant throughout the entire volume. Images were acquired with lateral and axial resolutions of about 0.5 µm and 2 to 3 µm, respectively.

A first measurement was performed prior to the administration of riboflavin to gain images by two-photon excited autofluorescence based on endogenous fluorophore NAD(P)H and the SHG signal from collagen. The beginning of Descemet's membrane was determined by loss of SHG-signals in the

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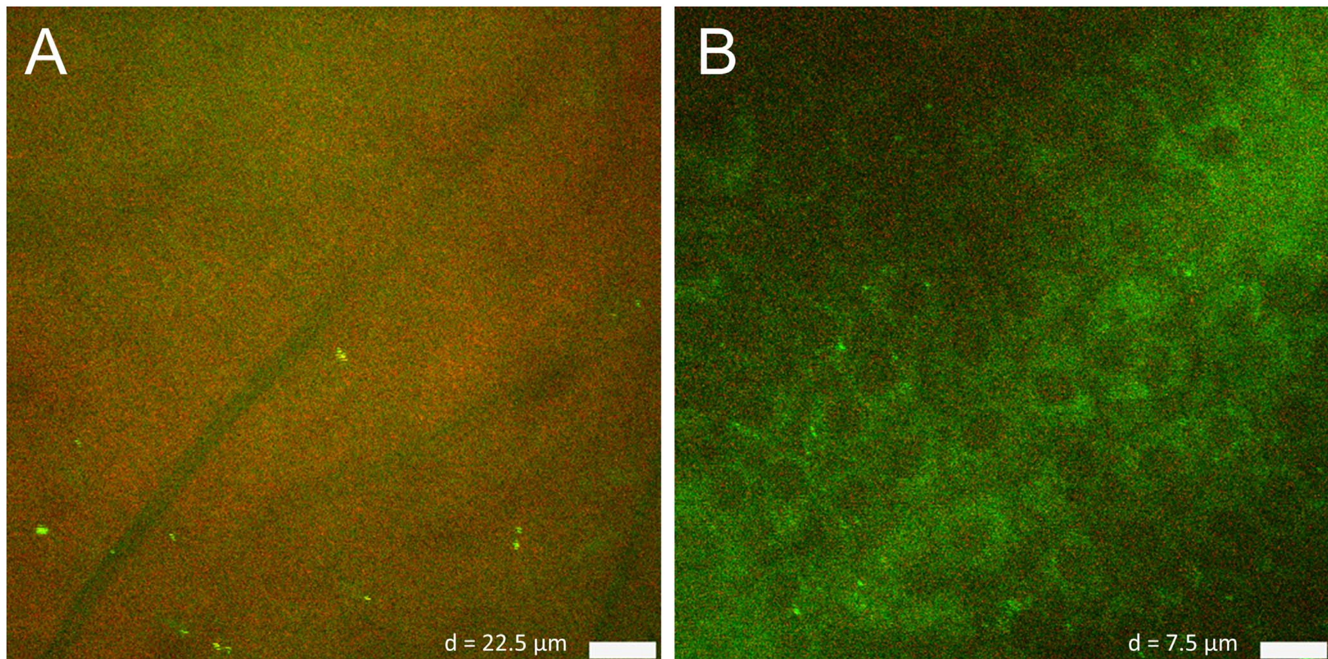


FIGURE 2. A typical SHG image (**A**) and autofluorescence image (**B**) of an untreated cornea after the establishment of stable hydration.

posterior stroma.[22,23] The first image in the absence of endothelial cells indicated the beginning of the aqueous humor, allowing the thickness calculation of Descemet's membrane and corneal endothelium. As corneal thickness was maintained constant throughout the entire course of the experiment, this landmark was used for the determination of absolute riboflavin concentration, which was performed as previously described.[19] In brief, obtained mean gray values of each corneal depth for a predetermined region of interest (ROI) were compared to those of the same corneal depth in corresponding corneal stacks (after 10 minutes of riboflavin imbibition and after riboflavin saturation) calibrating the signal attenuation due to absorption and scattering effects within the cornea.

## Numerical Analysis

To compare absolute riboflavin concentrations between different depths of the cornea, Mann-Whitney $U$ tests were performed using statistical software (Winstat for Excel; R. Fitch, Bad Krozingen, Germany). Significance was accepted if $P < 0.05$.

# RESULTS

## Autofluorescence and SHG Imaging Prior to the Administration of Riboflavin

Figure 2A shows a typical SHG image of the corneal stroma after the establishment of stable hydration, whereas Figure 2B depicts an autofluorescence image at the endothelial level. The fluorescence intensity is low due to the fact that the endogenous fluorophore NAD(P)H has a low fluorescence quantum efficiency.

## Riboflavin Concentration at the Endothelial Level

The application of riboflavin resulted in a strong two-photon fluorescence signal. The photomultiplier detecting the fluorescence became saturated when using the same mean power as in the case of a riboflavin-free cornea. Therefore, the power was reduced to 27.5 mW.

Supplementary Figure S1 illustrates averaged riboflavin fluorescence signals after the riboflavin imbibition and after saturation. The ratio of gray values of the fluorescence signals in the predetermined ROI yields the absolute riboflavin concentration in the corresponding depth.

The average of the absolute riboflavin concentrations of all five corneas is illustrated in Figure 3. Riboflavin concentration decreases toward posterior, experiencing a substantial decay within Descemet's membrane, and reaching 0.019% ± 0.007% at the Descemet's membrane side of the corneal endothelium and 0.013% ± 0.005% at the aqueous side of the endothelium, resulting in an average concentration of 0.015% ± 0.005% within the endothelium. Within the aqueous humor, only minor changes of riboflavin concentrations are detected, with an average concentration of 0.012% ± 0.004%.

When comparing the average riboflavin concentrations at different depths, significant differences were observed (average Descemet's membrane versus endothelium, $P = 0.047$; Descemet's membrane versus aqueous humor, $P = 0.009$).

Figure 4 depicts two-photon riboflavin fluorescence of two representative corneas at the endothelial level after riboflavin saturation. When analyzing the riboflavin distribution in the endothelium, it becomes obvious that the riboflavin diffusion pathway is predominantly paracellular. Immediately in front and behind the endothelium the fluorescence has a homogenous pattern.

TABLE. Average Corneal Pachymetry Measured by Ultrasound Throughout the Course of the Experiment

| | Steady State | After Riboflavin Application | After Riboflavin Saturation |
|---|---|---|---|
| Central corneal pachymetry, μm | 485 ± 10 | 494 ± 13 | 532 ± 15 |

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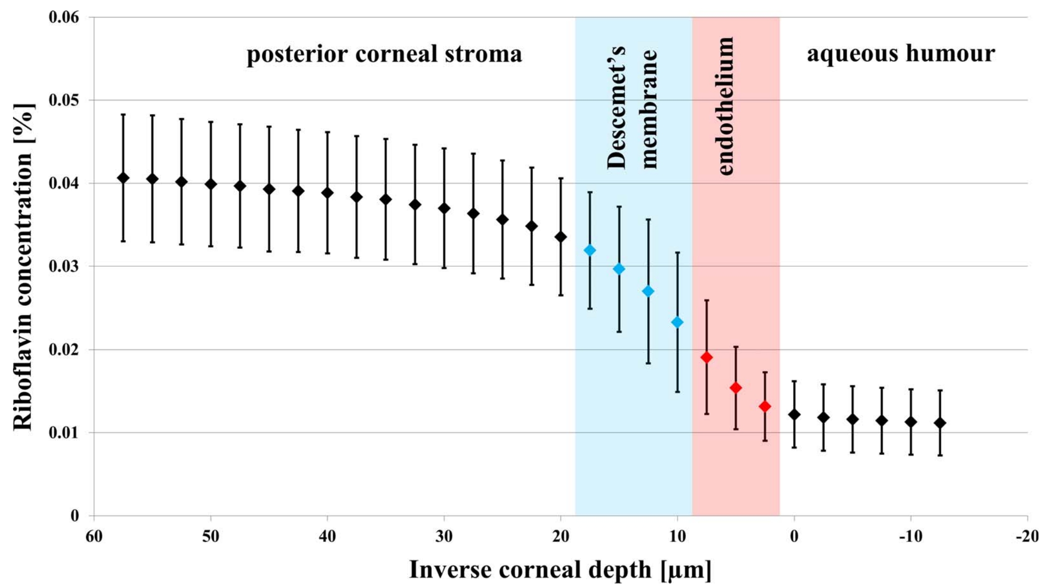



FIGURE 3. Absolute riboflavin concentrations in the posterior cornea and aqueous humor prior to CXL. A substantial drop within Descemet's membrane is observed. *Blue area* indicates Descemet's membrane, *red area* the endothelium.

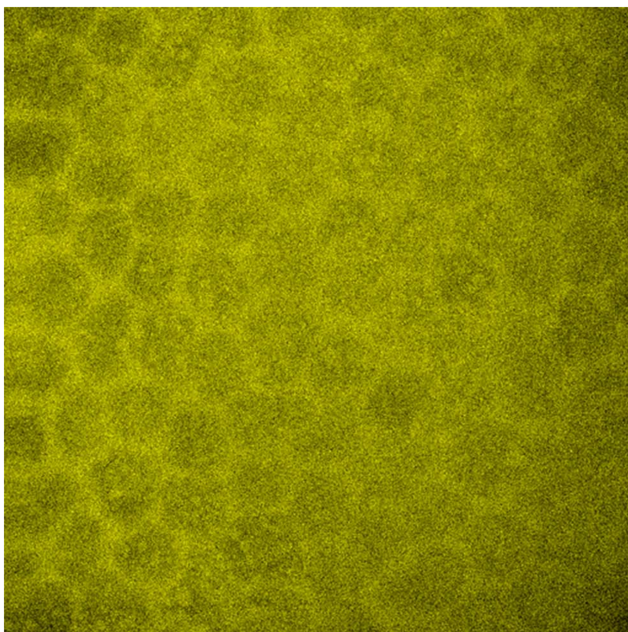
### Endothelial Cell Density and Corneal Hydration States During the Experiments

Average ECC was 2810 ± 317 cells/mm$^2$. Average central corneal pachymetry at different times is listed in the Table.

## DISCUSSION

The main findings of this study are that (1) the measured riboflavin concentration of 0.015% at the endothelial level during CXL in humans is approximately half of the theoretically calculated concentration of 0.025% and (2) riboflavin diffusion into the anterior chamber happens predominantly through a passive paracellular pathway.

Before the clinical application of CXL, the Dresden group investigated the potential impact of CXL on endothelial cells. At that time, no precise method to measure riboflavin concentration at the endothelial level was available. With the assumption that riboflavin has a diffusion constant similar to that of fluorescein, a riboflavin concentration of 0.025% was theoretically calculated for the human endothelium prior to CXL.[9,11] In the past, the diffusion of fluorescein was extensively studied by Araie and Maurice[24] using fluorophotometry. A riboflavin concentration of 0.04% was calculated for a depth of 400 µm, and 0.025% was determined for the endothelium level by Spoerl and Seiler.[11] Apoptosis UV-A



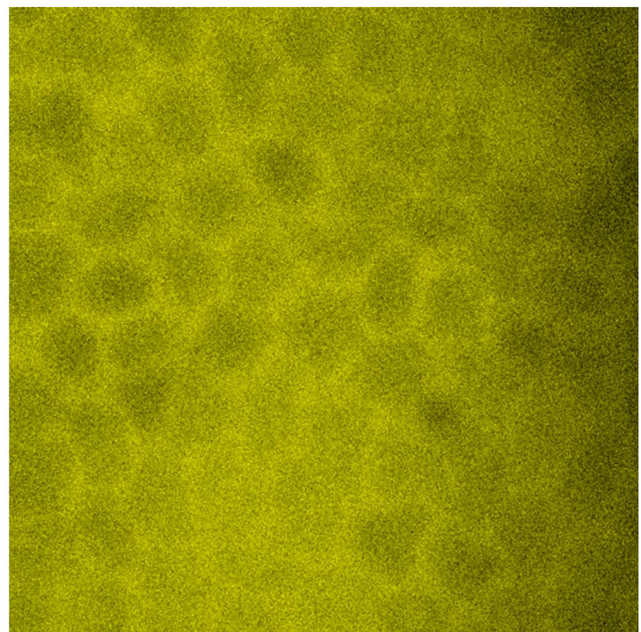


FIGURE 4. Two-photon excited riboflavin fluorescence within the endothelium in two typical samples. Inside the endothelium, the intracellular riboflavin concentration is substantially lower compared to the extracellular concentration, indicating a paracellular diffusion pathway.

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thresholds with 0.025% riboflavin were evaluated in porcine endothelial cells in vitro,[9] leading to a UV-A threshold of 0.35 $mW/cm^2$ for a 30-minute exposure (radiant exposure 0.63 $J/cm^2$). For UV irradiation without riboflavin, a threshold of 4 $mW/cm^2$ for 30-minute exposure (radiant exposure 7.2 $J/cm^2$) was found. This underlined the need of riboflavin not only for the creation of ROS but also as a shielding agent to protect the endothelium, reducing the total corneal UV-A transmission to only 13% in humans.[25] The experiments were performed in vitro (no physiological cell arrangement) using a 100-μm-thick cell culture layer under unlimited access to atmospheric oxygen.

In contrast to the roughly estimated riboflavin concentration of 0.025%, we measured experimentally an average riboflavin concentration at the endothelium of only 0.015%. The substantially smaller riboflavin concentration (0.015% vs. 0.025%), taken together with a lower available oxygen amount in vivo, indicate that the thresholds currently used might be too high.

A second study from the Dresden group examined in vivo the threshold of endothelial apoptosis in rabbits and found threshold UV doses[10] similar to in vitro experiments of porcine eyes.[9] However, the translation to human eyes may not be correct for the following reasons: (1) deepithelialized New Zealand White rabbit corneas (weight, 2.5 kg) have a central thickness of less than 350 μm[26]; (2) during a 35-minute application of 0.1% riboflavin in 20% dextran, the cornea shrinks to a thickness of 300 μm or less[27]; and (3) the assumption of only a 12.5% UV transmission appears to be unrealistic because other groups found a UV transmission ranging from 9% to 13% in 600- to 800-μm-thick riboflavin-soaked human and porcine corneas.[25,28] The fact that rabbit endothelial cells can regenerate[29] may explain a possibly lower damage threshold than that in humans. Therefore, the model rabbit should be critically scrutinized to perform endothelial safety experiments.

The theory of photochemical cross-linking shows that the number of produced ROS is related to the product $I \times c$ of UV-A light intensity $I$ and riboflavin concentration $c$.[30] This means a concentration threshold smaller by a factor of 2 may be compensated by two-fold higher light intensity without changing the number of induced ROS and, therefore, the potential damage. In clinical CXL with fixed combinations of $I$ and $c$, the reduction of the threshold may be equivalent, with a smaller minimal thickness, which means that the 400-μm rule is falsely high. Theoretical predictions of new threshold thickness based on the results presented here would be too speculative because we do not have access to reliable damage thresholds for human endothelium with riboflavin and UV light. Therefore, such thresholds should be determined experimentally in human cornea.

In 2016, Mooren et al.[18] tried to reproduce the results of the Dresden group using human cadaver corneoscleral disks. Using a riboflavin concentration of 0.025% and a UV irradiance of 18 $mW/cm^2$ for 5 minutes (radiant exposure 5.4 $J/cm^2$) the endothelial side was directly exposed under unlimited atmospheric oxygen access. Endothelial microscopy 5 days later revealed no endothelial cell damage (neither necrosis nor apoptosis), indicating at least a 10-fold higher UV/riboflavin oxidative damage threshold in human eyes.

In research of PubMed (search terms: CXL, complication), six reports with 19 eyes can be found describing endothelial damage and/or inflammation after CXL. In contrast to the tolerated UV values found by Mooren et al.[18], Hafezi et al.[12] described in 2007 a case of a localized transient corneal edema at the thinnest pachymetry after CXL in post-LASIK ectasia using the standard Dresden protocol.The preoperative corneal thickness including the epithelium was 400 μm. Subtracting the epithelium by 50 μm and considering an up to 20% shrinkage of the cornea due to the 30-minute imbibition with riboflavin/20% dextran,[31] a preirradiation thickness of approximately 300 μm has to be assumed. However, 6 weeks after CXL, the cornea cleared up again.

In 2012, Kymionis et al.[13] prospectively evaluated corneal cross-linking in thin corneas, reporting ECCs of 14 eyes with a follow-up of 12 months. Corneal thickness after epithelial removal was on average 373 μm (range, 340 to 399 μm). Including a 20% shrinkage due to the riboflavin/20% dextran imbibition process,[31] a preirradiation thickness of about 300 μm is suspected. A significant reduction in ECC by −290 cells/$mm^2$, on average, is reported, but no significant correlation was found with preoperative corneal thickness. Only one eye substantially lost endothelial cells (preoperative, 2708 cell/$mm^2$; postoperative, 1448 cells/$mm^2$), which had an estimated preirradiation corneal thickness of less than 300 μm.

Sharma et al.[14] published a case series with persistent corneal edema after CXL. However, no preoperative ECC was measured, and all eyes had a preoperative (including the epithelium) thinnest pachymetry of between 449 and 496 μm. Subtracting the roughly 50 μm of epithelium and considering a further shrinkage due to imbibition, a corneal thickness of 350 μm can be assumed prior to UV-A irradiation. In contrast to the reports of Hafezi et al.[12] and Kymionis et al.[13], the endothelial damage went along with severe anterior segment inflammation accompanied by iris atrophy, pigment dispersion, or corneal infection, making an isolated endothelial damage caused by CXL rather questionable. Bagga et al.[15] and Gokhale[16] each reported a case of persistent corneal edema after CXL. The preoperative situation was similar to the cases of Sharma et al.[14], with the lack of preoperative ECC and pachymetry. Gumus[17] reported a case of endotheliitis after CXL, which did not lead to a significant endothelial cell loss compared to the fellow eye 6 months after CXL. These clinical reports emphasize the importance of measuring the preirradiation corneal thickness and indicate a critical preirradiation thickness of 300 to 350 μm.

Another interesting issue arises from our study when interpreting the riboflavin distribution at the endothelial level. Obviously, riboflavin does not penetrate the endothelial cell itself (Fig. 4) and is found mainly in the intercellular space. As the mechanism of endothelial fluid transport has not been fully understood in detail until today,[32] this observation might help to analyze the paracellular water flow across the endothelium.

A limitation of the study is the use of 15% dextran as the osmotic active dilution, although in modern CXL, dextran has been frequently replaced by 1.1% HPMC. Another limitation occurs due to the areal averaging of the riboflavin concertation at the endothelial level. Locally (between the endothelial cells), a higher concentration may exist. However, due to the flat cylindrical shape and the tight-surface parallel arrangement of endothelial cells, this effect might be negligible. Human cadaver eyes might have a reduced endothelial cell metabolism, which can affect cellular riboflavin uptake or transport. In the near future, clinical in vivo two-photon imaging of the cornea will be available as an instrument to optimize the treatment parameters.[33,34]

In conclusion, the results presented here reveal a riboflavin concentration at the endothelium substantially lower than initially anticipated. Based on our findings, the 400-μm rule is too high. New human endothelial UV safety thresholds need to be evaluated in order to determine a more solid guideline for thin corneas with a pachymetry of less than 400 μm.[35] In particular with respect to customized[36] and refractive CXL,[37] using substantially higher energy parameters, which are currently entering the clinical routine.

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