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Cornea & External Disease

Combining Riboflavin/UV-A Light and Rose Bengal/Green Light Corneal Cross-Linking Increases the Resistance of Corneal Enzymatic Digestion

M. Enes Aydemir[1], Nikki L. Hafezi[1,2], Nan-Ji Lu[1,2], Emilio A. Torres-Netto[1,3], Mark Hillen[1], Carina Koppen[2], and Farhad Hafezi[1,3–5]

[1] ELZA Institute, Dietikon, Switzerland
[2] Department of Ophthalmology, Antwerp University Hospital, Edegem, Antwerp, Belgium
[3] Ocular Cell Biology Laboratory, University of Zurich, Zurich, Switzerland
[4] Faculty of Medicine, University of Geneva, Geneva, Switzerland
[5] Department of Ophthalmology at New York University Grossman School of Medicine, New York University, New York, NY, USA

Correspondence: Farhad Hafezi. University of Zurich, Center for Applied Biotechnology and Molecular Medicine, Winterthurerstrasse 190, Zurich, Switzerland. e-mail: farhad@hafezi.ch

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Purpose: The purpose of this study was to determine if concurrent riboflavin/UV-A light (RF/UV-A) and rose Bengal/green light (RB/green) epi-off PACK-CXL enhances corneal resistance to enzymatic digestion compared to separate chromophore/light treatments.

Methods: Ex vivo porcine corneas were allocated as follows. Group A corneas were soaked with riboflavin (RF) and were either not irradiated (A1, controls) or were irradiated with 10 (A2) or 15 J/cm^2 (A3) UV-A light at 365 nm, respectively. Group B corneas were soaked with RB and either not irradiated (B1, controls) or were illuminated with 10 (B2) or 15 J/cm^2 (B3) green light at 525 nm, respectively. Corneas in group C were soaked with both RF and RB and were either not irradiated (C1, controls) or were subjected to the same session consecutive 10 J/cm^2 (C2) or 15 J/cm^2 (C3) UV-A and green light exposure. Following treatment, all corneas were exposed to 0.3% collagenase A to assess digestion time until corneal button dissolution.

Results: A1 to A3 digestion times were 21.38, 30.5, and 32.25 hours, respectively, with A2 and A3 showing increased resistance to A1. B1-3 had digestion times of 31.2, 33.81, and 34.38 hours, with B3 resisting more than B1. C1 to C3 times were 33.47, 39.81, and 51.94 hours; C3 exhibited superior resistance to C1 and C2 (both $P < 0.05$).

Conclusions: Same-session combined RF/UV-A and RB/green PACK-cross-linking significantly increases corneal enzymatic digestion resistance over standalone treatments.

Translational Relevance: Combining RF-based and RB-based PACK-CXL considerably increases corneal collagenase digestion resistance, potentially minimizing ulcer size in clinical contexts.

Introduction

Infectious keratitis (IK) is a major cause of global blindness[1,2] and can be caused by several causative organisms, including bacteria, fungi, amoebae, and viruses. Prompt treatment, ideally before stromal involvement and ulcer onset, significantly improves prognosis.[3] Identifying pathogens and selecting the proper therapy for IK can be challenging. Inappropriate treatment choices waste time and risk poorer outcomes due to the rapid progression of the condition.

Corneal cross-linking (CXL),[4] initially used to treat corneal ectasias, has developed into an IK therapy. CXL involves applying a chromophore to the corneal stroma, and irradiation of stroma with a specific wavelength of light to activate the chromophore. When riboflavin (RF) and UV light (365-370 nm) are used, this process yields reactive oxygen species (ROS)

that induce the cross-linking of stromal molecules—primarily collagen fibers and proteoglycans in the extracellular matrix—thereby stiffening the corneal tissue.[5] Two primary antiseptic mechanisms underpin the effectiveness of CXL in reducing pathogen load. The first is the direct killing of pathogens through ROS-mediated damage to cellular membranes and nucleic acids.[6,7] The second is steric hindrance: ROS-mediated cross-linking obstructs collagenase binding sites and increases the cornea's resistance to enzymatic digestion, which should help limit ulcer development and constrain the eventual corneal scar size.[8] These mechanisms are pathogen-agnostic, and in an era of increasing pathogen antimicrobial resistance, has made CXL a promising approach for IK treatment.[9] Initially explored as an adjuvant therapy for advanced ulcerative IK by Iseli et al., the technique was later termed "photoactivated chromophore for infectious keratitis corneal cross-linking" (PACK-CXL).[9,10] PACK-CXL currently serves as an adjuvant treatment for advanced ulcerative IK, but a recently published randomized prospective phase III trial has shown that a single standalone PACK-CXL application is as effective as standard-of-care antimicrobial treatment for early to moderate bacterial or fungal keratitis.[11]

Besides riboflavin and UV-A light at 365 nm (RF/UV-A), PACK-CXL can also be performed using rose Bengal (RB) and 532 nm green light (RB/green). Laboratory studies demonstrated varying pathogen susceptibility for PACK-CXL employing RF/UV-A and RB/green.[12,13] The stromal penetration depth of both chromophores also varies: RF/UV-A cross-links 400 to 600 µm into the corneal stroma, depending on its formulation and application, whereas RB/green only cross-links to a depth of 100 µm.[12,14–17] Notably, the absorption spectra of both chromophores show only minimal overlap meaning they mostly do not compete for the same wavelengths of light energy. Here, we investigated whether corneal resistance to digestion could be further augmented by a same-session application of RF/UV-A and RB/green in PACK-CXL when compared to the use of a single chromophore/light combination.

Materials and Methods

Specimen Acquisition and Preparation

Freshly enucleated porcine eyes (less than 6 hours postmortem) were obtained from a local abattoir. First, the epithelium was removed using a hockey knife (FEATHER, PFM Medical AG, Cologne, Germany). Corneas were circumferentially excised using Westcott scissors leaving a 3 mm corneoscleral rim. Corneal buttons were immersed in a 400 mOsmol/L phosphate-buffered saline (PBS) solution for 10 minutes before being trephined centrally with an 8 mm biopsy punch (SMI AG, St. Vith, Belgium) to obtain corneal buttons. The corneal buttons were then soaked in either 0.1% RF solution (Ribo-Ker, EMAGine AG, Zug, Switzerland) or 0.1% RB solution (Bengalrosa, Bichsel AG, Interlaken, Switzerland) for 10 minutes. After soaking, the buttons were rinsed with a 400 mOsmol/L PBS solution and placed in a 24-well plate (Merck AG, Darmstadt, Germany). Last, the RF-treated corneas underwent CXL using a 365 nm UV-A light commercially available light source (C-eye device, EMAGine, Zug, Switzerland), whereas RB-treated corneas underwent CXL using a 522 nm green light source (custom-built experimental device).

Study Group Allocation and CXL Protocols

We randomly assigned 144 porcine eyes to one of 3 study groups (A, B, and C), each containing 48 corneas (see the Table).

Group A (RF/UV-A): All corneas underwent 10 minutes of RF soaking. Non-irradiated A1 corneas ($n = 16$) served as controls whereas A2 and A3 corneas ($n = 16$ each) were irradiated with 365 nm UV-A light at either 10 J/cm^2 (18 mW/cm^2, for 9 minutes and 15 seconds) or 15 J/cm^2 (30 mW/cm^2 for 8 minutes and 20 seconds), respectively.

Group B (RB/green): All corneas underwent 10 minutes of RB soaking. Non-illuminated B1 corneas served as controls whereas B2 and B3 corneas were illuminated with 522 nm green light at either 10 J/cm^2 (15 mW/cm^2, for 11 minutes and 7 seconds) or 15 J/cm^2 (15 mW/cm^2, for 16 minutes and 40 seconds), respectively.

Group C (same-session RF/UV-A and RB/green): All corneas underwent 10 minutes of RF soaking, followed by 10 minutes of RB soaking. Non-illuminated/irradiated C1 corneas served as controls. C2 corneas were irradiated with UV-A (18 mW/cm^2, for 9 minutes and 15 seconds), immediately followed by illumination with green light (15 mW/cm^2, for 11 minutes and 7 seconds). C3 corneas were irradiated with UV-A (30 mW/cm^2, for 8 minutes and 20 seconds), immediately followed by illumination with green light (15 mW/cm^2, for 16 minutes and 40 seconds).

Table. Experimental Setup

Experimental Groups	Chromophore Riboflavin/Rose Bengal	Description	Intensity (mW/cm^2), Irradiation Time (Min, S)	Fluence (Irradiance) J/cm^2
A1	√/x	Control nonirradiated	None	None
A2	√/x	10.0 J/cm^2	18 mW/cm^2, 9 min 15 s	10
A3	√/x	15.0 J/cm^2	30 mW/cm^2, 8 min 20 s	15
B1	x/√	Control nonirradiated	None	None
B2	x/√	10.0 J/cm^2	15 mW/cm^2, 11 min 7 s	10
B3	x/√	15.0 J/cm^2	15 mW/cm^2, 16 min 40 s	15
C1	√/√	Control nonirradiated	None	None
C2	√/√	10.0 J/cm^2 + 10.0 J/cm^2	18 mW/cm^2, 9 min 15 s + 15 mW/cm^2, 11 min 7 s	20
C3	√/√	15.0 J/cm^2 + 15.0 J/cm^2	30 mW/cm^2, 8 min 20 s + 15 mW/cm^2, 16 min 40 s	30

Riboflavin groups (control [A1], 10 J/cm^2 [A2], and 15 J/cm^2 [A3]), rose Bengal irradiation groups (control [B1], 10 J/cm^2 [B2], and 15 J/cm^2 [B3]), combined irradiation groups (control [C1], 20 J/cm^2 [C2], and 30 J/cm^2 [C3]) min = minutes; s = seconds; mW/cm^2 = milliwatts per square centimeter.

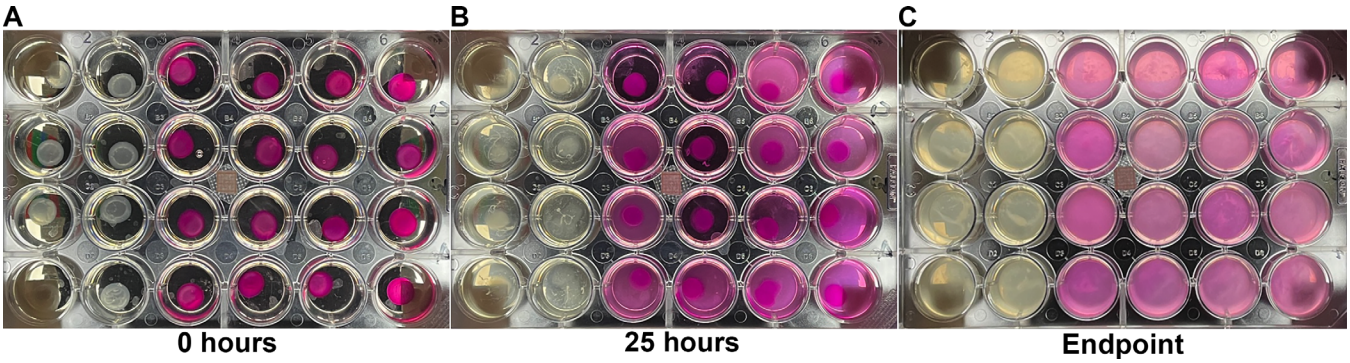

Figure 1. Representative image of a 24-well plate containing corneas immediately upon immersion in 0.3% collagenase solution (**A**) and after 25 hours (**B**) of digestion in 0.3% collagenase A solution, and at the end point (**C**).

Enzymatic Digestion of Corneal Buttons and Assessment

Post-PACK-CXL, all corneas were transferred to a fresh 24-well plate (Merck AG, Darmstadt, Germany) filled with 2.0 mL of a 0.3% collagenase A solution (Roche, Basel, Switzerland) per well. The plate was placed on a thermoshaker at 37°C with 150 revolutions per minute. The corneal buttons were visually inspected and photographed hourly (Fig. 1). The duration of enzymatic digestion for each cornea was recorded. Complete digestion was achieved when corneas fragmented completely, forming a dust-like layer.

Statistical Analysis

Statistical analysis was performed by using R Studio (version 2022.12.0), Prism 9 (version 9.5.1), and Microsoft Excel (version 16.70). The descriptive statistics were presented as mean ± standard deviation. Analysis of variance (ANOVA) was used to analyze the differences between the groups, with a value of $P < 0.05$ indicating statistical significance.

Results

This study included a total of 144 corneas. Digestion results are displayed in Figure 2.

For RF groups A1 to A3, the mean digestion times were 21.38 ± 1.15, 30.5 ± 1.63, and 32.25 ± 2.27 hours, respectively. Corneas in groups A3 and A2 displayed significantly greater digestion resistance than group A1 corneas ($P < 0.05$), but no differences in digestion resistance between corneas in groups A3 and A2 were observed ($P = 1$).

Among the RB groups (B1 to B3), the mean digestion times were 31.2 ± 2.78, 33.81 ± 1.52, and 34.38 ± 1.2 hours, respectively. Group B3 corneas were more digestion-resistant than those in group B1 ($P < 0.05$). Group B2 corneas displayed no significant difference in resistance compared to group B1 or B3 corneas ($P = 0.087$ and $P = 1$, respectively).

For the combination groups C1 to C3, the mean digestion times were 33.47 ± 2.58, 39.81 ± 2.23, and 51.94 ± 4.2 hours, respectively. Group C3 corneas were more digestion-resistant than group C1 and C2-corneas (both $P < 0.05$). In addition, group C2 corneas displayed a significantly greater mean digestion time than those in group C1 ($P < 0.05$).

Discussion

The use of RF/UV-A and RB/green light PACK-CXL, applied consecutively during the same session, distinctly enhances corneal resistance to enzymatic digestion when compared to a single chromophore/light PACK-CXL protocol. This enhancement could be due to a number of reasons, including total fluence, the different chromophores' absorption spectra, and penetration depths, causing cross-linking effects at various corneal layers. However, we focused solely on overall digestion times, not depth-specific effects.

The highest resistance to digestion was exhibited in corneas that underwent combined RF and RB PACK-CXL, receiving a fluence of 15 J/cm^2 in each procedure. In our recent studies, we found that corneas treated with RB/green light PACK-CXL show an enzymatic resistance that is higher than corneas treated with RF/UV-A PACK-CXL, in both unirradiated controls and following irradiation. A possible explanation for the increased resistance in the control groups might be that the saturation of the corneal stroma with the chromophores alters the accessibility of the cleavage sites for the collagenase. Why RB shows an even higher inhibitory effect than RF cannot easily be explained.

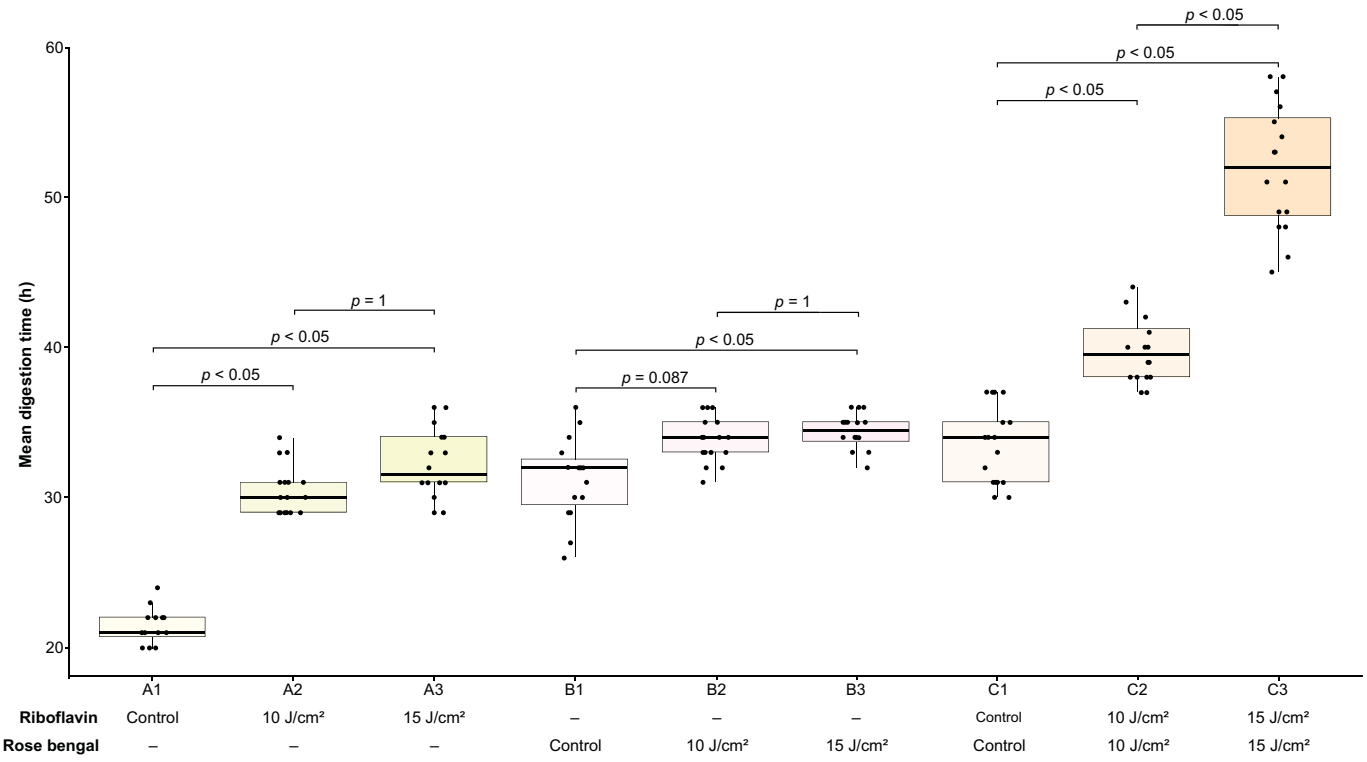

Figure 2. Comparative digestion times across experimental groups.

Enzymatic digestion with subsequent tissue defects is a hallmark of IK and is caused by the production of excessive amounts of proteolytic enzymes released by the pathogens. In this study, we used collagenase A to digest the corneal buttons. Collagenase A is a bacterial collagenase and is widely used as a standard enzyme in evaluating biomaterials' enzymatic resistance[18] and its high potency and broad specificity enables it to digest all collagen types and to target multiple sites on the collagen helix.[19,20] Spoerl et al.[18] compared the effectiveness of collagenase A to other collagen-digesting enzymes, pepsin and trypsin. They found that collagenase A was more potent than pepsin at digesting the cornea.

Our findings align with previous studies that investigated the effect of single chromophore/light combinations, using either RF/UV-A or RB/green light. These studies demonstrated significantly increased resistance with both RF/UV-A[21,23] and RB/green Light CXL.[21–23] Morgan et al.[23] observed increased resistance in porcine corneal buttons treated with 0.3% collagenase A and accelerated cross-linking protocols. Our study represents the first same-session use of combined RF and RB-mediated PACK-CXL. The total fluence used in these same-session combination treatments was 20 and 30 J/cm^2, respectively. This is distinctly higher than the maximum of 15 J/cm^2 used so far in RF-mediated PACK-CXL.[24] However, this combined fluence was achieved with 2 × 10 and 2 × 15 J/cm^2 per chromophore using different wavelengths with, potentially, different endothelial threshold levels.

Besides resistance to digestion, another crucial aspect of PACK-CXL's effectiveness is its direct microbial killing via oxidative stress and the inhibition of replication through DNA/RNA intercalation with the photoactivated chromophore, which together constitute the foundational pillars of PACK-CXL. Whether a combined fluence of 30 J/cm^2 also represents an optimum for the microbial killing rate should be assessed in future studies.

The limitations of this study include its ex vivo nature. Although selecting irradiation protocols delivering 10 and 15 J/cm2 that represent novel CXL protocols that are currently in preclinical and clinical use for progressive keratoconus and infectious keratitis,[24–27] we want to emphasize that the present study represents a first cautious step to set the framework for such later use. There are no clinical data published in the combination of RF and RB in the same session yet.

Additionally, we examined transparent corneas, whereas IK typically renders corneas opaque, which has the potential to diminish the penetration of light into the cornea. Further investigation is required to better understand the impact PACK-CXL has on infected cornea digestion rates, particularly in a clinical setting.

In conclusion, combining RF/UV-A and RB/green PACK-CXL in a single procedure may present a novel approach for treating IK. This approach may further strengthen the impact that PACK-CXL may have in times of increasing antimicrobial resistance.

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