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Cornea & External Disease

High-Fluence Accelerated PACK-CXL for Bacterial Keratitis Using Riboflavin/UV-A or Rose Bengal/Green in the Ex Vivo Porcine Cornea

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Purpose: To investigate and compare the efficacy of high-fluence accelerated photoactivated chromophore for keratitis–corneal cross-linking (PACK-CXL) using either riboflavin/ultraviolet (UV)-A light or rose bengal/green light to treat *Staphylococcus aureus* or *Pseudomonas aeruginosa* infections in an ex vivo porcine cornea model.

Methods: One hundred and seventeen ex vivo porcine corneas were injected with clinical isolates of *S. aureus* or *P. aeruginosa*, divided into eight groups, and cultured for 24 hours. Then, either riboflavin with UV-A light irradiation (30 mW/cm^2; 8 minutes, 20 seconds; 15 J/cm^2) or rose bengal with green light irradiation (15 mW/cm^2, 16 minutes, 40 seconds; 15 J/cm^2) was applied; unirradiated infected groups served as controls. All corneas were incubated for another 24 hours. Next, corneal buttons were obtained and vortexed to release the bacterial cells. The irradiated and unirradiated solutions were then plated and incubated on agar plates. The amount of colony-forming units was quantified and the bacterial killing ratios (BKRs) resulting from different PACK-CXL protocols relative to non-treated controls were calculated.

Results: Riboflavin/UV-A light PACK-CXL resulted in median BKRs of 52.8% and 45.8% in *S. aureus* and *P. aeruginosa*, respectively, whereas rose bengal/green light PACK-CXL resulted in significantly greater BKRs of 76.7% and 81.0%, respectively (both $P < 0.01$).

Conclusions: Both accelerated PACK-CXL protocols significantly decreased *S. aureus* and *P. aeruginosa* bacterial loads. Comparing the riboflavin/UV-A light and rose bengal/green light PACK-CXL approaches in the same experimental setup may help develop strain-specific and depth-dependent PACK-CXL approaches that could be used alongside the current standard of care.

Translational Relevance: Our study used an animal model to gain insight into the efficacy of high-fluence accelerated PACK-CXL using either riboflavin/UV-A light or rose bengal/green light to treat *Staphylococcus aureus* or *Pseudomonas aeruginosa* infections.

Introduction

Infectious keratitis is an ophthalmic condition that can rapidly impair vision and requires immediate and effective treatment to avoid complications and minimize vision loss. Moreover, the incidence of infectious keratitis is increasing with the widespread use of contact lenses.[1] Although the principal causative organisms of infectious keratitis vary by geographical

region, a bacterial infection is the most common cause,[2] and the increasing global prevalence of antibiotic-resistant bacteria adds an important challenge to effectively treat bacterial keratitis with traditional topical antibiotic eyedrops,[3,4] highlighting the need for new treatments to overcome this challenge.

Corneal cross-linking (CXL) was initially developed to treat corneal ectasias such as keratoconus. The procedure starts with saturation of the stroma using riboflavin, followed by a 30-minute period of ultraviolet-A (UV-A) irradiation.[5] In the presence of oxygen, UV-A photons photoactivate riboflavin to create reactive oxygen species (ROS), which covalently cross-bind collagen and proteoglycans in the stroma and increase the biomechanical strength. In 2008, the antimicrobial effects of CXL were described.[6] Later, other wavelength/chromophore combinations such as green light/rose bengal, were proposed.[7] Accordingly, the term "photoactivated chromophore for keratitis–corneal cross-linking (PACK-CXL)" was introduced to describe this indication of CXL.[8]

The antimicrobial mechanism of PACK-CXL can be explained by two effects: (1) the direct killing effects of microorganisms through UV-A light and chromophores via generation of ROS, which impair the structural integrity of bacterial membranes and their replication; and (2) the development of collagenase resistance through steric hindrance.[9] In vitro experiments show that riboflavin/UV-A light (rf) PACK-CXL can effectively kill antibiotic-resistant bacteria.[10] In clinical studies, rf PACK-CXL with irradiation fluences between 5.4 and 7.2 J/cm^2 has already been used to treat bacterial keratitis of varying severity.[11–13] In a recent in vitro study, we showed that high-fluence rf PACK-CXL can be accelerated while maintaining the bacterial killing effect.[14] The purpose of this study was therefore to transfer the conclusions of the previously published in vitro study into an ex vivo model and to compare the bacterial killing effects of rf PACK-CXL with rose bengal/green light (rb) PACK-CXL.

Materials and Methods

Bacteria and Solution Preparation

Two species of clinically relevant bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, were chosen from independent clinical isolates of corneal infections for the following experiments. The bacterial strains were obtained from the Institute of Medical Microbiology, University of Zurich, Switzerland. Both bacterial species were cultured on Colombia agar + 5% sheep blood (COS; bioMérieux, Marcy l'Etoile, France) for 24 hours at 37°C. Colonies were suspended in sterile 0.9% NaCl and adjusted to McFarland 0.5, which corresponds to approximately 10^8 colony forming units (CFUs) per milliliter. The solutions were diluted 100-fold to 10^6 CFU/mL in 96-well microtiter plates (Costar Assay Plate; Corning, Inc., Corning, NY).

Ex Vivo Infectious Keratitis Model

Freshly enucleated porcine eyes were obtained from a local slaughterhouse and used within 4 hours. The corneas were excised with a 3-mm scleral rim individually immersed in povidone iodinate solution (Betadine; Mundipharma Medical, Basel, Switzerland) for 10 minutes, rinsed 10 times in distilled water, and individually placed in six-well plates (Costar Assay Plate; Corning) containing 4 mL of Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO) each. Plates were kept at 37°C with 5% CO_2 for 24 hours.

The culture medium was removed after incubation. Before that, the culture medium was plated on COS agar to check for potential bacterial contaminations. If bacteria were detected, the corresponding corneas were discarded. Bacteria free corneas were individually mounted on an artificial chamber (Moria, Antony, France) (Fig. 1a). A 33-gauge microsyringe (Hamilton Company, Reno, NV) with a fixed 2-mm needle containing 10 µL of the bacterial solution diluted to 10^6 CFU/mL was prepared, and the solution was injected into each cornea at a 45° angle (Fig. 1b). After the injection, corneas formed a localized bulge (Fig. 1c). Anterior segment optical coherence tomography (MS-39; CSO Italia, Firenze, Italy) was used to determine the deepest part of the formed bulge that was located at approximately 60% of stromal depth (Fig. 1d). All of the corneas were individually placed in new six-well plates containing 1 mL of DMEM medium and kept at 37°C with 5% CO_2 for another 24 hours. Corneal opacity, edema, and beginning ulceration were observed in all corneas at 24 hours after inoculation. This indicated successful establishment of the ex vivo infection keratitis model. The loose epithelium over the site of inoculation was removed using triangular sponges (EYETEC PVA Eye Spears; EYETEC, North Yorkshire, UK).

Irradiation Settings and PACK-CXL Protocols

Two different light sources were used: a commercially available UV-A light-emitting diode (LED) source (C-eye; EMAGine AG, Zug, Switzerland) and an experimental green light LED source (CSO Italia,

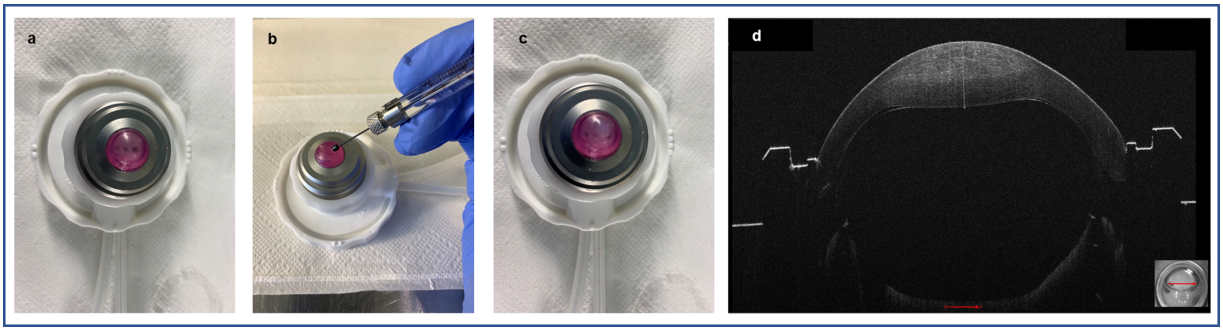

Figure 1. Corneas were mounted on an artificial chamber (**a**) and a microsyringe was injected into the cornea at a 45° angle (**b**). After the injection, all of the corneas formed a localized bulge (**c**). The bulge could be observed by the sectional scan of anterior segment optical coherence tomography (**d**).

Table. The Study Groups and PACK-CXL Technical Details

Group	Injected Bacteria	Light Source and Chromophore	Total Fluence (J/cm^2)	Irradiance Intensity (mW/cm^2)	Irradiance Time[a]
Group 1-control	*S. aureus*	—	—	—	—
Group 1-PACK-CXL	*S. aureus*	UV-A light + riboflavin	15	30	8′20″
Group 2-control	*S. aureus*	—	—	—	—
Group 2-PACK-CXL	*S. aureus*	Green light + rose bengal	15	15	16′40″
Group 3-control	*P. aeruginosa*	—	—	—	—
Group 3-PACK-CXL	*P. aeruginosa*	UV-A light + riboflavin	15	30	8′20″
Group 4-control	*P. aeruginosa*	—	—	—	—
Group 4-PACK-CXL	*P. aeruginosa*	Green light + rose bengal	15	15	16′40″

PACK-CXL, photoactivated chromophore for keratitis cross-linking; *S. aureus*, *Staphylococcus aureus*; *P. aeruginosa*, *Pseudomonas aeruginosa*; UVA, ultraviolet-A.

[a]Note that ′ indicates minutes and ″ indicates seconds.

Scandicci, Italy). Both light sources had a fixed 12-mm irradiation spot and performed two different types of PACK-CXL irradiation. The settings for rf PACK-CXL were UV-A light at 365 nm using 0.1% (w/v) riboflavin solution without carriers (Ribo-Ker, EMAGine) and irradiation settings of 30 mW/cm^2 for 8 minutes, 20 seconds, corresponding to a fluence of 15 J/cm^2. The settings for rb PACK-CXL were green light at 522 nm using 0.1% rose bengal solution (Bichsel Pharmacy, Interlaken, Switzerland) and irradiation settings of 15 mW/cm^2 for 16 minutes, 40 seconds, corresponding to a fluence of 15 J/cm^2.

Group Setting and PACK-CXL Procedures

The inoculated corneas were randomly assigned to the experimental (irradiated) or control groups. The Table provides an overview of the experimental and control groups; as indicated, we conducted rb PACK-CXL or rf PACK-CXL in corneas inoculated with one of two bacterial strains. Corneas in the experimental groups were mounted on an artificial chamber, and riboflavin or rose bengal was dropped every 20 seconds for 20 minutes, followed by irradiation with the aforementioned protocols (Figs. 2a, 2b). After irradiation, the corneas were placed into new six-well plates filled with 1 mL of fresh DMEM medium and incubated for 24 hours at 37°C with 5% CO_2. Corneas in the control groups were treated similarly to the experimental groups but were not irradiated.

Bacterial CFUs: Counting and Analysis

To address both biological and experimental variability and to obtain reliable and stable results, experiments were performed as follows: Each irradiation was repeated at least three times (technical replicate), and the whole experiment was repeated at least on three different days (biological replicate). For every irradiation, fresh bacterial solutions

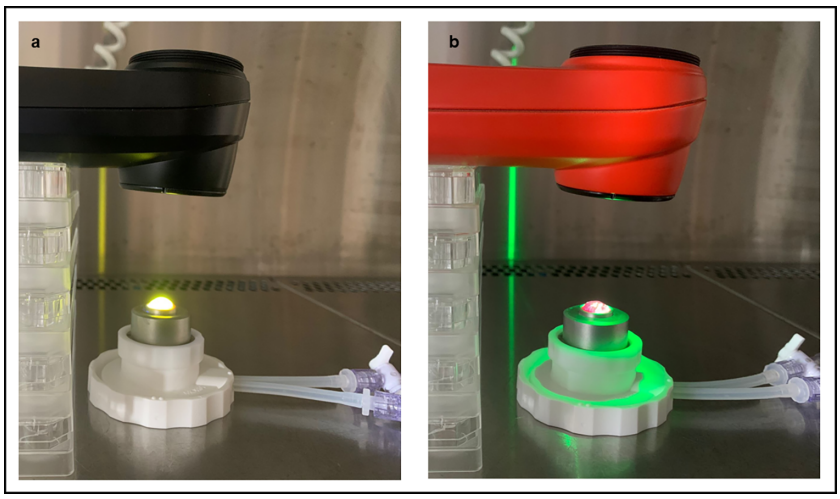

Figure 2. PACK-CXL treatment of a porcine cornea mounted in an artificial chamber using (**a**) UV-A light/riboflavin (rf) and (**b**) green light/rose bengal (rb).

were used. Corneal buttons were prepared using an 8-mm corneal punch (SMI AG, Brussels, Belgium). The buttons were placed in 2-mL tubes (Eppendorf AG, Hamburg, Germany) containing 1 mL 0.9% NaCl solution, vortexed, and then centrifuged for 10 minutes at 15,000 revolutions per minute (rpm) to release the bacteria from the cornea. Following centrifugation, the solutions were vortexed again, then aspirated and mixed with a 1-mL pipette (Eppendorf AG, Hamburg, Germany). The solutions then underwent three 10-fold dilutions, and 10 µL of the final dilution was plated on COS agar using its whole surface to obtain single bacterial colonies and incubated at 37°C for 24 hours.

After incubation, all agar plates were photographed, and the numbers of CFUs were counted as described previously.[15] Based on each CFU result, the bacterial killing ratios (BKRs) of three technical replicates were calculated by comparing the median CFU of each PACK-CXL irradiated plate ($CFU_{PACK\text{-}CXL}$) to its corresponding control plate ($CFU_{control}$), using the following formula:

$$BKR = \left(1 - \frac{CFU_{\ PACK-CXL}}{CFU_{\ Control}}\right) \times\ 100\ [\%]$$

For the two investigated bacteria, *S. aureus* and *P. aeruginosa*, the BKRs achieved by the two PACK-CXL protocols (rf PACK-CXL and rb PACK-CXL) were then compared.

Statistical Analysis

Statistical analyses and graph preparation were conducted using Prism 5 (GraphPad, San Diego, CA). In the case of determination of BKRs, descriptive statistics were calculated as medians with interquartile ranges. A non-parametric Mann–Whitney test was applied to analyze the equivalence among all groups. Means and standard deviations were used to calculate CFUs. $P < 0.05$ was considered as the threshold for statistical significance.

Results

Overall, 117 porcine corneas were used in our ex vivo model and included in the analyses. For *S. aureus*, the application of rf PACK-CXL yielded a mean CFU of 400 ± 106 for group 1-control ($n = 9$) and 181 ± 59 for group 1-PACK-CXL ($n = 9$), whereas rb PACK-CXL yielded a mean CFU of 507 ± 100 for group 2-control ($n = 20$) and 91 ± 55 for group 2-PACK-CXL ($n = 21$). For *P. aeruginosa*, rb PACK-CXL yielded a mean of CFU 257 ± 94 for group 3-control ($n = 13$) and 118 ± 61 group 3-PACK-CXL ($n = 13$), whereas rb PACK-CXL yielded a mean CFU of 260 ± 106 for group 4-control ($n = 15$) and 74 ± 53 for group 4-PACK-CXL ($n = 17$). The mean CFUs of all control groups were significantly higher (all $P < 0.001$) compared to the mean CFUs of all corresponding experimental groups (Fig. 3a).

The median BKRs of high-fluence rf PACK-CXL of *S. aureus* and *P. aeruginosa* were 52.8% and 45.8%, respectively, whereas the median BKRs of high-fluence rb PACK-CXL for *S. aureus* and *P. aeruginosa* were 76.7% and 81.0%, respectively (Fig. 3b). The higher BKRs were achieved by rb PACK-CXL for both bacteria (both $P < 0.01$).

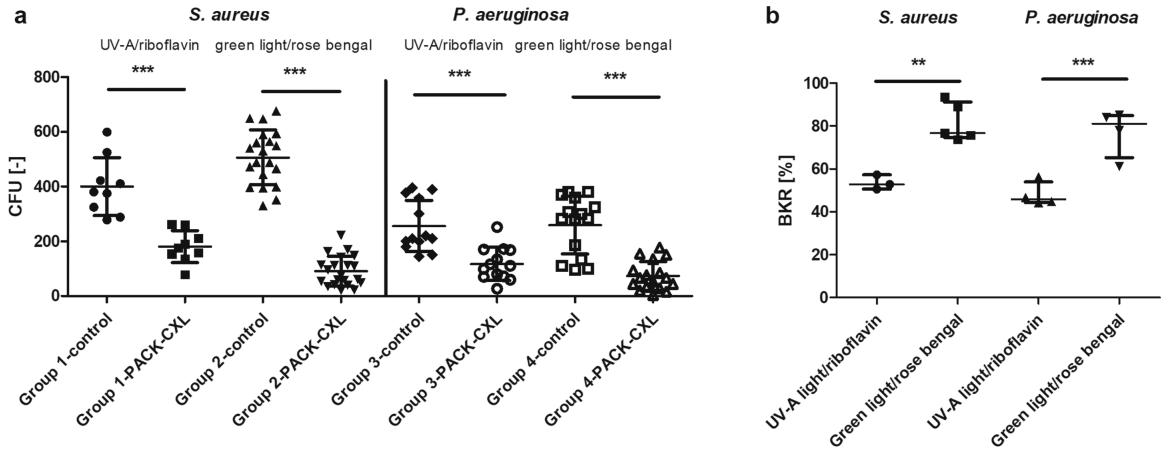

Figure 3. (**a**) The colony forming units (CFUs) of *S. aureus* and *P. aeruginosa* with or without two different protocols (UV-A light/riboflavin or green light/rose bengal) of PACK-CXL treatment were determined (***$P < 0.001$). The *scatters* represent each single experiments, and the *bars* show the means and standard deviations. (**b**) Bacterial killing rates (BKRs) were calculated from independent biological replicates. Higher BKRs were achieved when PACK-CXL was applied with green light/rose bengal (**$P < 0.01$, ***$P < 0.001$). The *scatters* represent each single biological replicates, which were calculated from at least three technical replicates, and the *bars* are medians with interquartile ranges.

Discussion

This ex vivo study demonstrates that accelerated high-fluence PACK-CXL can effectively reduce bacterial concentrations in a model for infectious keratitis using two clinically relevant bacterial species. These results suggest that a timely application of accelerated high-fluence PACK-CXL, potentially at the slit lamp, may effectively treat bacterial keratitis by decreasing the bacterial load at the lesion site and by increasing the resistance of the stroma to digestion.[16] Furthermore, this decrease in bacterial load may shorten the duration of infection and enhance the effects of concomitantly applied antimicrobial drugs, improving patients' outcomes.[16,17]

Two main ex vivo models of bacterial cornea infection are commonly used in the literature: (1) direct injection of bacterial solutions into the stroma with a needle, and (2) scratching the corneal surface and applying a bacterial solutions over the surface.[18,19] In this study, we chose the first method because it is highly reproducible, whereas the depth of a corneal scratch can be difficult to control. Additionally, applying bacterial solution to the cornea surface does not guarantee that the bacteria will fully penetrate the cornea. To investigate the bacterial killing effects of PACK-CXL to the maximum extent, a high initial bacterial count of 10^6 CFU/mL was used, which is consistent with a rat model for bacterial keratitis, in which a maximum bacterial count of 10^7 CFU/mL was found 48 hours after infection.[20]

Currently, the most widely used PACK-CXL protocol in UV-A light/riboflavin still is the classic "Dresden protocol" (3 mW/cm^2, 30 minutes, 5.4 J/cm^2).[21] This 20-year-old protocol may be appropriate for biomechanically strengthening ectatic corneas. However, the protocol is slow, assumes a transparent cornea, and has a limited depth of killing effect due to a low fluence of 5.4 J/cm^2. This constrains the efficacy of the procedure, as in infectious keratitis the infected tissue is opaque and the depth of infection may be far beyond the 330 µm that the Dresden protocol was designed for.[22]

Our group has previously shown in vitro that fluences of 10 J/cm^2 and 15 J/cm^2 (significantly greater than the Dresden protocol 5.4 J/cm^2) improve the antimicrobial efficacy of rf PACK-CXL.[15] Previous clinical studies using 15 J/cm^2 accelerated rf CXL did not decrease the endothelial cell density in transparent keratoconic and myopic corneas.[23,24] We therefore applied 15 J/cm^2 using high LED intensities for both UV-A light (30 mW/cm^2) and green light (15 mW/cm^2).

The current ex vivo study and our previous in vitro study used the same rf PACK-CXL irradiation protocols (UV-A light plus riboflavin; 30 mW/cm^2; 8 minutes, 20 seconds; 15 J/cm^2). Although we achieved lower BKRs than in the in vitro study (BKRs of *S. aureus* and *P. aeruginosa* were 97.50% and 82.27%, respectively), BKRs were still substantial. Potential reasons for this include the fact that, in the ex vivo model, the remaining bacteria continued to replicate in the corneas during the incubation after rf PACK-CXL, whereas in the in vitro experiments the remaining bacteria were directly plated on the agar plates.

Further, opaque corneas act to prevent the penetration of UV-A light, whereas in the in vitro experiments the solutions present in 96-well plates were almost transparent.

In this study, we evaluated two PACK-CXL protocols with a similar irradiation fluence delivered via different chromophores. For both bacterial species, the rb PACK-CXL BKRs were higher than the rf PACK-CXL BKRs. This is similar to the resistance to enzymatic digestion that rb PACK-CXL provides, which is greater resistance than rf PACK-CXL using the same fluence. In contrast, the biomechanical cross-linking effect of rb PACK-CXL was lower than the effect of rf PACK-CXL.[25]

The depth of the infection plays a critical role in the response of the cornea to PACK-CXL, and effective bacterial killing in deep keratitis is essential, as the continuing bacterial growth in the depth will impair corneal structure and may lead to corneal perforation. Therefore, although higher BKRs were achieved by rb PACK-CXL, the potential benefits of deep killing induced by rf PACK-CXL may be crucial. Accordingly, the application of both rf PACK-CXL and rb PACK-CXL protocols in the same session would appear to offer several advantages over either technique performed separately.

In the current study, we counted the remaining bacteria after PACK-CXL treatment by plating bacterial dilutions to agar, which is still the gold standard in microbiology for quantification of living bacteria. Thus, the location of the bacteria in the cornea that survived the treatment could not be determined. In a following study, we would like to address this question by infecting cornea with green fluorescent protein (GFP)-expressing bacteria and applying confocal laser scanning microscopy, which may help us to identify the location of remaining active bacteria in the cornea after PACK-CXL treatment.[26]

This study had several limitations. One is intrinsic to ex vivo experiments: the lack of an immune response from the host organism. Future in vivo experiments with corresponding histochemical studies would help us to further elucidate the role played by PACK-CXL in treating infectious keratitis. Additionally, our research examined only one strain from each bacterial species investigated. Investigating more strains that have been isolated clinically, especially antimicrobial resistant strains, will enhance the robustness of our findings and provide greater clinical relevance.

In conclusion, the results from our ex vivo model presented here confirm the findings of our previous in vitro study showing that high-fluence accelerated rf and rb PACK-CXL effectively reduce the concentration of *S. aureus* and *P. aeruginosa* in the cornea. In a clinical setting, the use of both chromophores simultaneously may have the potential to improve the outcomes of bacterial keratitis treatment significantly.

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