High Fluence Increases the Antibacterial Efficacy of PACK Cross-Linking

Sabine Kling, PhD, Fong Siang Hufschmid,† Emilio A. Torres-Netto, MD,*‡ J. Bradley Randleman, MD,§ Mark Willcox, PhD,¶ Reinhard Zbinden, MD,† and Farhad Hafezi, MD, PhD, FARVO*‖**††‡‡*

Purpose: Photoactivated chromophore for keratitis cross-linking (PACK-CXL) is used as an adjunct therapy to antibiotic medication in infectious keratitis. This experimental study aimed at quantifying the PACK-CXL efficacy as a function of UV fluence using several bacterial strains and irradiated volumes.

Methods: Six distinct bacterial strains, including standardized strains and clinically isolated strains from patients with keratitis, were analyzed. Bacterial concentrations between 10^2 and 10^8 cells/mL were used (simulating small corneal ulcers). Volumes of either 11 μL (≈285 μm stromal thickness) or 40 μL (≈1000 μm stromal thickness) were irradiated within a microtiter plate at different fluences (5.4–27 J/cm^2) and irradiances (3, 9 and 18 mW/cm^2). The ratio of bacterial killing (B†) was determined to evaluate the antimicrobial efficacy of PACK-CXL.

Results: B† was similar (51 ± 11%) in bacterial concentrations between 10^3 and 10^5 per ml. In 11 μL volume, *Staphylococcus aureus* (SA) 8325-4 ATCC 29213, *Bacillus subtilis* (BS) 212901, and *Pseudomonas aeruginosa* (PA) 2016-866624 were most sensitive to PACK-CXL at 5.4 J/cm^2 (on average B† = 49 ± 8%), whereas *Klebsiella oxytoca* (KO) 2016-86624 (B† = 25%) was least sensitive. When irradiating a larger volume, B† was on average lower in 40 μL (19 ± 18%), compared with 11 μL (45 ± 17%, $P < 0.001$). By contrast, applying a higher UV fluence increased B† of SA ATCC 29213, from 50% at 5.4 J/cm^2 to 92% at 10.8 J/cm^2, to 100% at 16.2 J/cm^2 and above.

Conclusions: Applying higher UV fluences substantially increases the bacterial killing rates. Safety limits for clinical application require further investigation.

Key Words: PACK-CXL, antibacterial efficacy, UV fluence, bacterial strain, infectious keratitis

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According to the World Health Organization (WHO), infectious keratitis constitutes one of the leading causes of blindness worldwide. It can result from minor ocular trauma because of occupational risk[1,2] combined with poor hygiene. Particularly, in the absence of immediate and adequate medical care—which is often the case in developing countries—it is a sight-threatening disease. Although the incidence rate in industrialized countries is significantly lower, infectious keratitis can arise from contact lens wear,[3] dry eyes, recent ocular surgery,[4] or systemic immunosuppression.[5,6]

The diverse nature of the underlying microorganisms—including bacteria, fungi, and protozoa—makes clinical diagnosis[7] and hence adequate antimicrobial treatment challenging, especially because laboratory analyses are not immediately available. In the absence of adequate antimicrobial treatment, infectious ulcers may progress rapidly. A further concern in the management of infectious keratitis is multiresistant bacterial strains.[8,9] If the first-line antibiotic medication is not effective, valuable time in halting the progression is lost.

Photoactivated chromophore for keratitis cross-linking (PACK-CXL),[10] reported first in 2008, is a novel and promising treatment for infectious keratitis.[11] It is based on a photochemical reaction initiated by the combination of a photosensitizer and UV-A light. Typical photosensitizers used for ophthalmic cross-linking are riboflavin[12,13] and rose bengal.[14] PACK-CXL has several benefits over pharmaceutical antimicrobial treatment. First, it is effective[15] against bacterial, fungal,[16] and mixed infections—although most effective against bacterial infections.[17] Second, theoretically, no resistance can develop because both cell membranes and DNA are destroyed during the treatment. Third, the formation of new cross-links increases the stiffness of the extracellular matrix and counteracts corneal melting, which is commonly observed in infections with matrix-metalloproteinase secreting bacterial strains.

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From the *University of Zurich, CABMM, Laboratory for Ocular Cell Biology Group, Zurich, Switzerland; †Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland; ‡Department of Ophthalmology, Paulista School of Medicine, Federal University of Sao Paulo, Sao Paulo, Brazil; §Cole Eye Institute, Clevelend Clinic, Cleveland, Ohio; ¶School of Optometry and Vision Science, University of New South Wales, Sydney, New South Wales, Australia; ‖kELZA Institute AG, Dietikon/Zurich, Switzerland; **Department of Ophthalmology, Wenzhou University, Wenzhou, China; ††Faculty of Medicine, University of Geneva, Geneva, Switzerland;and ‡‡Department of Ophthalmology, Keck School of Medicine, University of Southern California, Los Angeles, CA.

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Correspondence: Sabine Kling, PhD, University of Zurich, Ocular Cell Biology Lab, CABMM Y13-L86, Winterthurerstrasse 190, 8057 Zurich, Switzerland (e-mail: kling.sabine@gmail.com).

Several in vitro,[12,18,19] in vivo,[20,21] and clinical studies have confirmed the antimicrobial efficacy of PACK-CXL. Although in small[22,23] and moderate[24] ulcers, PACK-CXL significantly promoted healing and sped up the time until reepithelialization, in advanced ulcers, it was less effective.[25] Therefore, current clinical studies have investigated the potential of PACK-CXL as an adjunct treatment, which in an in vivo rabbit study was found to be superior[21] to PACK-CXL alone. The PACK-CXL protocol still needs further optimization,[13] regarding fluence (J/cm^2) levels. In vitro studies have reported different efficacies depending on the bacterial strain,[12] irradiation time, and riboflavin concentration.[18] Although a range of treatment parameters has been studied, 100% bacterial killing has not been achieved with any of the current PACK-CXL protocols. Potentially, the use of a higher UV energy (ie, fluence) in the PACK-CXL protocols could further increase the efficacy in infectious keratitis.

The purpose of the current study was to quantify the antimicrobial efficacy of PACK-CXL on different bacterial strains within a clinically realistic setting. In particular, we aimed at investigating the effect of the size of the irradiated volume (simulating the penetration into different stromal depths) and the minimal UV energy required to achieve complete bacterial eradication.

MATERIALS AND METHODS

Materials

Staphylococcus aureus (methicillin-resistant: *SA* 8325-4, SA 31 and methicillin-sensitive: SA ATCC 29213), *Bacillus subtilis* (BS 212901, Becton Dickinson [BD], Sparks, MD), *Pseudomonas aeruginosa* (PA 2016-866624, a clinical isolate from contact lens induced keratitis), and *Klebsiella oxytoca* (KO 2016-86624, a clinical isolate from contact lens induced keratitis).

Methods

Several distinct aspects of the antimicrobial effect of PACK-CXL were analyzed, including determining 1) the lethality dose at high- and low-bacterial concentrations, 2) the amount of bacterial killing (B†) for different bacterial strains with a standard PACK-CXL fluence of 5.4 J/cm^2, and 3) the B† for different irradiated volumes to evaluate stromal depth penetration. The corresponding protocols used for each purpose are described in the following section:

Determining the Lethality Dose at High and Low Concentrations

Preparation of Bacterial Solutions

Colonies from overnight subcultures of *S. aureus* SA ATCC 29213 on Columbia agar +5% sheep blood (COS, bioMérieux, Merci l'Etoile, France) were suspended in sterile 0.9% (wt/vol) NaCl and adjusted to a concentration of 0.5 McFarland (McF, 10^8 bacteria/mL) using a standard sample. Subsequently, dilutions containing 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 bacteria/mL and 0.1% riboflavin were prepared for the experimental analysis.

Experimental Protocol

First, to determine the minimal UV fluence to reach complete bacterial killing at the surface, a series of increasing UV fluences was tested with the highest bacterial concentration. A suspension of 10^8 bacteria/mL was spread out directly onto a Mueller Hinton agar plate using a cotton swab. Then, a central area was irradiated with a UV irradiance of either 3 or 18 mW/cm^2 for a duration ranging from 5 to 60 minutes. The surrounding area served as control. Directly after UV exposure, the plates were incubated at 35°C for 24 hours.

Next, a more realistic clinical scenario was simulated, that is, lower bacterial concentrations were investigated. For this purpose, volumes of 11 μL bacterial solution of *S. aureus* SA ATCC 29213 with a concentration of 10^4 bacteria/mL were pipetted into a 96-well microtiter plate. Each well had a diameter of 7 mm with a spherical base. Then, a series of increasing UV fluences was applied with an irradiance of 18 mW/cm^2 for 5, 10, 15, 20, and 25 minutes. For each treated sample, a control sample was prepared simultaneously and was not subjected to UV irradiation to determine the normal cell death during incubation and compensate for pipetting errors. Directly after UV exposure, the suspensions were transferred from the well onto a Mueller Hinton agar plate and incubated at 35°C for 24 hours.

Antimicrobial Efficacy in Different Bacterial Strains

Preparation of Bacterial Solutions

Six bacterial strains were used in this part of the study: *S. aureus*, both methicillin-sensitive (SA ATCC 29213) and multiresistant (SA 8325-4 and SA 31) strains; *P. aeruginosa* (PA 2016-866624); *K. oxytoca* (KO 2016-866624); and *B. subtilis* (BS 212901). Colonies from overnight bacterial subcultures on Colombia agar +5% sheep blood were suspended in sterile 0.9% NaCl, containing 0.1% (w/v) riboflavin at a concentration of 10^4 bacteria/mL.

Experimental Protocol

To investigate the antimicrobial efficacy in different irradiated volumes, volumes of 11 and 40 μL were pipetted into a 96-well microtiter plate for each strain in quadruplicate. The height of the 11 and 40 μL volumes correspond to a stromal depth of approximately 285 and 1000 μm, respectively. The volumes were chosen because 285 μm are within the effective penetration depth (300–350 μm) reported for CXL treatment, as quantified by the demarcation line. In contrast, 100 um thickness should stimulate a deep infection in a swollen cornea. Forty microliters were analyzed to represent deeper ulcers and corneal edema, which is a complication observed clinically in keratitis. For each volume and strain, a treatment (UV irradiation with 18 mW/cm^2 for 5 minutes, corresponding to 5.4 J/cm^2) and control (no irradiation) group was analyzed in quadruplicate. Directly after UV exposure, the suspension was transferred from the well onto a Mueller Hinton agar plate (212257, BD) and incubated at 35°C for 24 hours.

To investigate the antimicrobial efficacy with different clinically relevant UV fluences, *v*olumes of 11 μL were pipetted into a 96-well microtiter plate for each strain in triplicate. Two UV fluences were analyzed (5.4 vs. 7.2 J/cm^2, both at 18 mW/cm^2, ie, with an irradiation time of 5 and 6:40 minutes, respectively) and compared with nonirradiated controls that were prepared simultaneously. Directly after UV exposure, the suspension was transferred from the well onto a Mueller Hinton agar plate and incubated at 35°C for 24 hours. CXL with 5.4 J/cm^2 (3 mW/cm^2

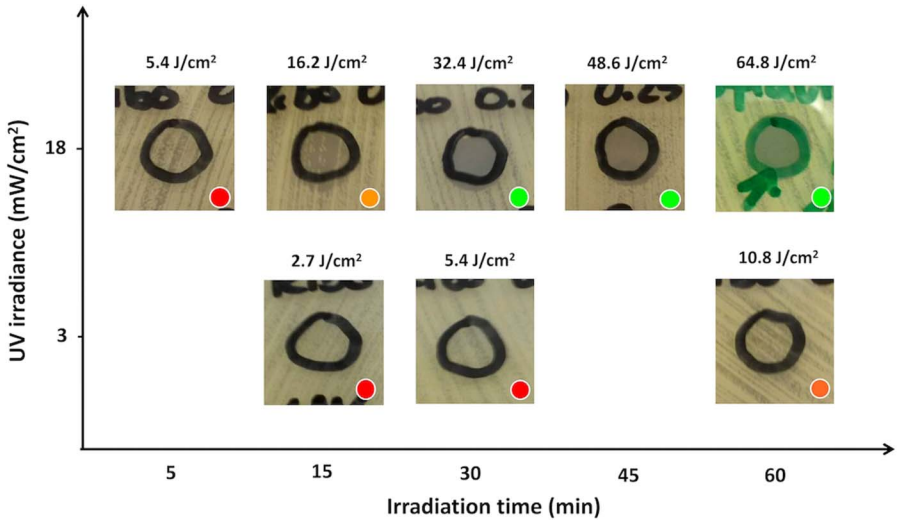

FIGURE 1. Determination of the amount of bacterial killing as a function of UV fluence and irradiation time in the *S. aureus* SA ATCC 29213 strain on agar plates and a concentration of 0.5 McF (10^8 bacteria/mL). The colored dot in the right bottom corner categorizes the treatment efficacy in 3 groups: Red = no visible effect; orange = noticeable effect; and green = clear antimicrobial effect.

for 30 minutes) is the standard protocol for the treatment of ectasia; nonetheless, newer protocols apply higher[26,27] fluences of 7.2 J/cm² (30 mW/cm² for 4 minutes) up to 10 J/cm² (9 mW/cm² for 18:30 minutes). According to the Bunsen Roscoe law, higher fluences are expected to have a stronger antimicrobial effect.

Analysis

After 24 hours of incubation, the agar plates were photographed and the mean number of bacterial colony forming units (CFUs) for a given strain and treatment setting were counted. Each treated plate was compared with its simultaneously prepared control to determine the bacterial killing ratio (B†) by using the following formula:

$$B_{\dagger} = 1 - \frac{CFU_{CXL}}{CFU_{control}}.$$

A 2-way analysis of variance (ANOVA) was applied (IBM SPSS Statistics, version 23) to statistically compare the observed number of CFUs between control and cross-linked groups for the different strains in a given experiment (11 µL with 5.4 J/cm² at 18 mW/cm² for 5 minutes, 11 µL with 7.2 J/cm² at18 mW/cm² for 6:40 minutes, and 40 µL with 5.4 J/cm² at 18 mW/cm² for 5 minutes). Subsequently, a two-way ANOVA was applied to analyze the effect of different fluences, irradiated volumes, and bacterial strains on B†. Upper and lower confidence intervals of the difference in B† were computed, as described in the literature.[28,29]

RESULTS

Determining the Lethality Dose

Figure 1 presents the amount of bacterial killing for high concentrations of bacteria (10^8 bacteria/mL). A UV fluence of at least 32.4 J/cm² (18 mW/cm² for 30 minutes) was necessary to kill all bacteria within the irradiated area. This demonstrates that UV fluence (J/cm²), but not irradiation time, determines the amount of bacterial killing. Figure 2 presents the relation

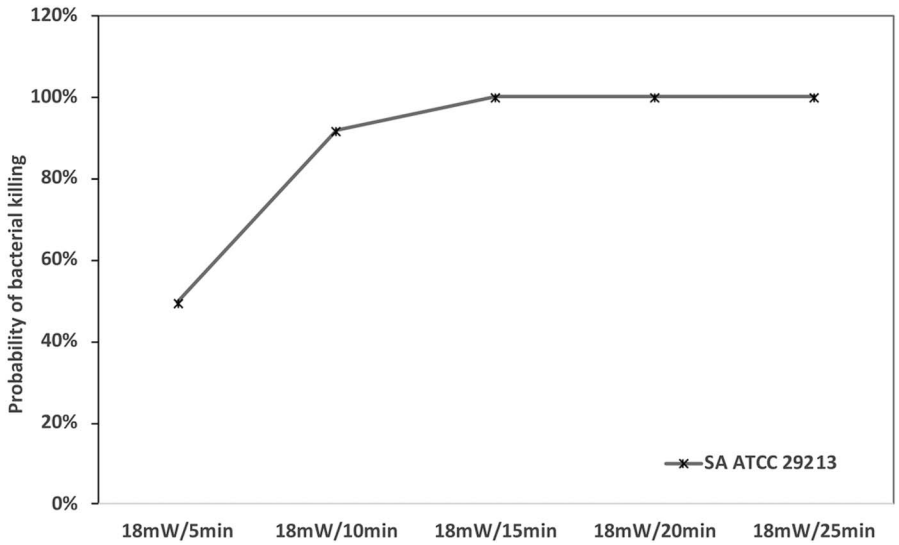

FIGURE 2. Determination of the lethality dose (LD) curve for the *S. aureus* SA ATCC 29213 strain in an irradiated volume of 10 µL.

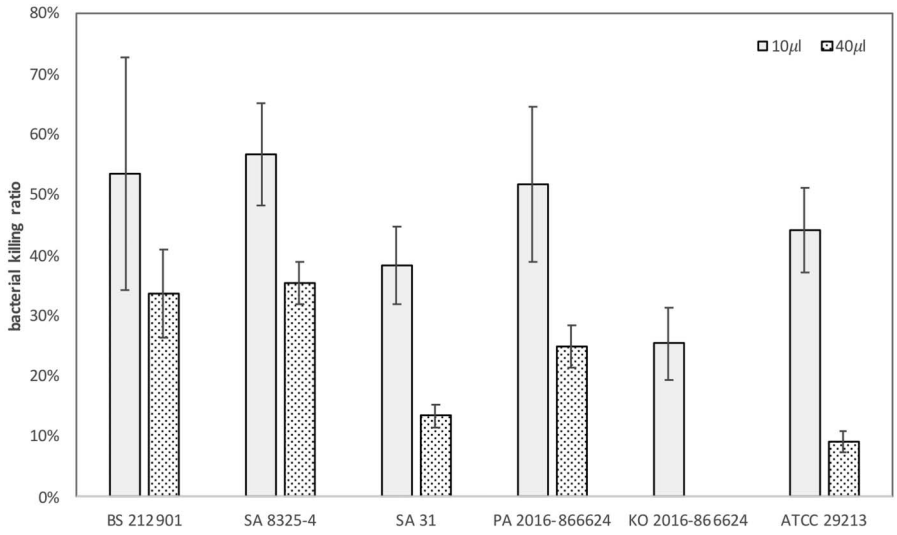

FIGURE 3. Determination of the bacterial killing ratio as a function of bacterial strain and irradiated volume at a UV fluence of 5.4 J/cm^2. Bars indicate standard error across N = 4 experimental repetitions.

between the probability of bacterial killing and UV dose for lower concentrations of bacteria. Increasing UV fluence to 16.2 J/cm^2 (corresponding to 18 mW/cm^2 for 15 minutes) or higher would likely kill approximately 100% of bacteria of *S. aureus* SA ATCC 29213 at this concentration (10^4 bacteria/mL).

Antimicrobial Efficacy in Different Bacterial Strains and in Different Irradiated Volumes

The ANOVA indicated a significant effect of UV irradiation on the CFU count in groups with 11 µL volume, both with 5.4 J/cm^2 (18 mW/cm^2 for 5 minutes) and 7.2 J/cm^2 (18 mW/cm^2 for 6:40 minutes) UV irradiation (each $P < 0.001$), but not in the group with 40 µL volume with 5.4 J/cm^2 (18 mW/cm^2 for 5 minutes) UV irradiation ($P = 0.176$). Figure 3 presents the ratio of bacterial killing for the different conditions. The bacterial strain had a significant effect on the killing ratio ($P = 0.001$). B† was highest in *S. aureus* SA 8352-4 and SA ATCC 29213, *B. subtilis* BS 212901, and *P. aeruginosa* PA 2016-866624. B† was significantly reduced for *K. oxytoca KO 2016-866624*, when compared with *B. subtilis* BS 212901 ($P = 0.007$), *S. aureus* SA 8352-4 ($P = 0.005$), and *P. aeruginosa* PA 2016-866624 ($P = 0.010$). In addition, B† was significantly reduced ($P = 0.045$) in *S. aureus* SA 31 compared with *S. aureus* SA 8352-4. Regarding the irradiated volume, B† was on average higher in 11 µL with 45 ± 17% than in 40 µL with an average of 19 ± 18%. A significant effect of the irradiated volume on the bacterial killing ratio was found ($P < 0.001$).

Antimicrobial Efficacy With Different Clinically Relevant UV Fluences

There was a significant effect of the overall UV fluence, both with 3 and 18 mW/cm^2, on the bacterial

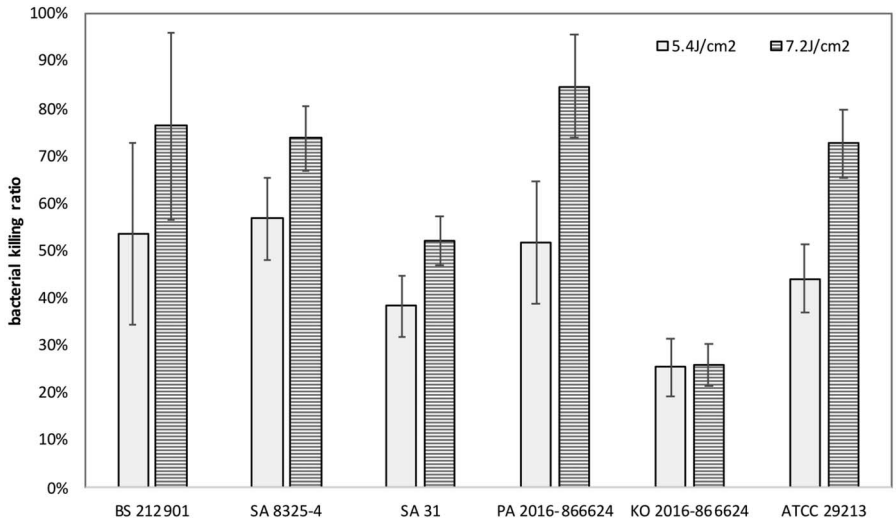

FIGURE 4. Determination of the bacterial killing ratio as a function of bacterial strain and clinically relevant UV fluences (5.4 and 7.2 J/cm^2) in an irradiated volume of 11 µL. Bars indicate standard error across N = 3 experimental repetitions.

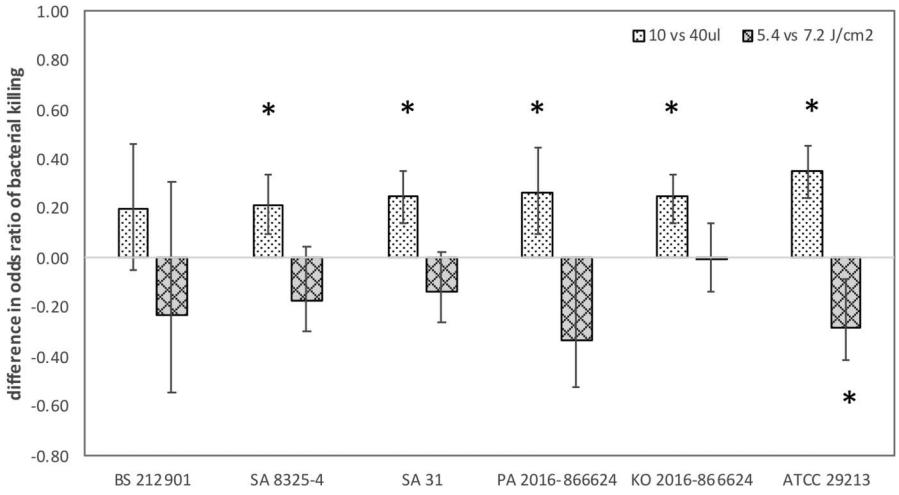

FIGURE 5. Differences in the odds ratio of bacterial killing with different sizes of irradiated volume (11 vs. 40 μL) and with different UV fluences (5.4 vs. 7.2 J/cm^2). Bars indicate 95% confidence intervals (CI). *Differences with CI excluding zero.

killing ratio ($P < 0.001$). Figure 4 presents the bacterial killing ratio for the different UV fluences. Compared with *K. oxytoca KO 2016-866624*, B† was significantly higher in *P. aeruginosa* PA 2016-866624 ($P < 0.001$), *B. subtilis* BS 212901 ($P < 0.001$), *S. aureus* SA 8352-4 ($P < 0.001$), and *P. aeruginosa* PA 2016-866624 ($P < 0.001$). Regarding the UV fluences, on average, B† was higher at 7.2 J/cm^2 (18 mW/cm^2 for 6:40 minutes), which killed 65 ± 24% of bacteria than at 5.4 J/cm^2 (18 mW/cm^2 for 5 minutes) with 45 ± 17% killing.

Figure 5 summarizes the differences of the killing ratio between 5.4 and 7.2 J/cm^2, and between 11 and 40 μL, along with the 95% confidence intervals. Table 1 lists the observed CFU counts for each group and condition.

DISCUSSION

We have determined the impact of UV fluence (ie, UV energy) and the size of the irradiated volume on the bacterial killing achieved with current clinical PACK-CXL protocols. In contrast to an earlier study, we found that the administered UV fluence—not the overall irradiation time[18]—determined the extent of bacterial killing.

The ratio of bacterial killing with PACK-CXL was significantly reduced in larger irradiated volumes (mimicking deeper corneal ulcers), which is in line with clinical observations[25] and likely occurs because of a lower UV intensity in deeper layers and a higher absolute number of surviving bacteria. Compared with the previous literature, the current study permitted for the first time to quantify the amount of killing in 2 different stromal depths (285 vs. 1000 μm). To

TABLE 1. Number of CFUs for Different Experimental Groups

	Control	Control	CXL	CXL	UV Fluence	Irradiance	Irradiation Time	Irradiated Volume	No. of Repetitions
	Mean	SD	Mean	SD	J/cm^2	mW/cm^2	min:s	μm	N
BS 212901	14.5	2.9	6.8	3.3	5.4	18	5:00	11	4.0
SA 8325-4	77.3	11.5	33.5	22.7	5.4	18	5:00	11	4.0
SA 31	91.0	13.7	56.3	7.0	5.4	18	5:00	11	4.0
PA 2016-866624	31.0	7.7	15.0	4.1	5.4	18	5:00	11	4.0
KO 2016-866624	70.3	9.7	52.5	12.0	5.4	18	5:00	11	4.0
ATCC 29213	88.0	9.1	49.3	4.9	5.4	18	5:00	11	4.0
BS 212901	19.7	4.9	4.7	4.0	7.2	18	6:40	11	3.0
SA 8325-4	157.0	18.1	41.3	31.1	7.2	18	6:40	11	3.0
SA 31	196.0	38.6	94.0	19.0	7.2	18	6:40	11	3.0
PA 2016-866624	71.3	10.7	11.0	8.9	7.2	18	6:40	11	3.0
KO 2016-866624	135.3	17.7	100.3	7.1	7.2	18	6:40	11	3.0
ATCC 29213	143.0	12.1	39.3	13.6	7.2	18	6:40	11	3.0
BS 212901	64.0	29.2	42.5	29.2	5.4	18	5:00	40	4.0
SA 8325-4	304.8	39.0	197.3	42.1	5.4	18	5:00	40	4.0
SA 31	367.5	10.9	318.3	27.5	5.4	18	5:00	40	4.0
PA 2016-866624	190.3	25.0	143.0	53.3	5.4	18	5:00	40	4.0
KO 2016-866624	289.3	15.1	291.0	49.3	5.4	18	5:00	40	4.0
ATCC 29213	308.3	57.1	280.5	57.1	5.4	18	5:00	40	4.0

achieve a higher treatment efficacy in larger volumes, a higher UV energy would be required. Almost all bacterial strains showed a trend to an increased ratio of bacterial killing at higher UV fluences, on average by +3.8% per J/cm.[2] Antibiotic resistant strains (*S. aureus* 8325-4 and 31) showed a similar susceptibility to bacterial killing as nonresistant strains. This finding is consistent with the clinical results in which PACK-CXL was successfully used for the treatment of ulcers resistant to conventional therapy.[2,30,31] On the other hand, a nonresistant strain (*K. oxytoca KO 2016-866624*) was *significantly less susceptible to PACK-CXL than most other strains—without any obvious reason.*

By further increasing the UV fluence for PACK-CXL, a ratio of bacterial killing of up to 100% can be achieved in low-bacterial concentrations. Given the experimental design of the current study, the precision at high bacterial killing does not extend to decimal places. Therefore, future studies are required to identify the exact UV threshold necessary to achieve killing amounts of several log units. An important concern with higher UV fluences is the safety of corneal endothelium, crystalline lens, and retina during and after the treatment. Initial safety estimations[32] that were performed for cross-linking in keratoconus concluded that corneas should be at least 400 μm thick for an irradiation with 5.4 J/cm^2, including a safety margin of approximately factor 2. Although the current in vitro study does not allow to evaluate the safety issues on endothelial cells, increased fluences, such as those used in the present study, are already being used clinically: more recent modifications of the CXL protocol for progressive keratoconus are based on higher UV fluences, reaching 7.2 J/cm^2 in pulsed CXL[27] and even 18 J/cm^2 in customized CXL.[26,33] None of these studies have reported endothelial failure.[26,33] Recently, it has been shown that actual riboflavin concentration at the endothelium is by a factor of 1.7 lower than the theoretically estimate,[34] suggesting that the previously defined UV damage threshold for corneal endothelium is too conservative. Such further evidence may potentially enable higher UV-fluence application in clinical practice in future.[34] Regarding PACK-CXL, corneal ulcers are less transparent than normal corneal tissue and often present local edema and increased corneal thickness. Both decreased transparency and increased corneal thickness would theoretically justify the use of a higher UV fluence, if the irradiation were restricted to the ulcer.

In the current study, bacterial concentrations from 10^4 to 10^8 per ml were used, which likely represent a small ulcer, in which PACK-CXL is most effective. If we consider a corneal ulcer of 2-mm diameter and 150-μm depth (ie, a volume of 0.47 μL), this concentration represents the amount of bacteria present after 10 to 27 cycles of bacterial cell doubling. With further assuming a bacterial doubling time of 120 minutes, this would correspond to an incubation time of approximately 20 to 54 hours. Although time from infection to clinical manifestation is highly variable and depends on the strain, its virulence, and the in vivo growth rate (ie, with suboptimal conditions), this range of incubation time can be considered a representative for a small corneal ulcer.

Owing to the particular design of the experimental setting, the results cannot provide a measure of log reductions in the bacterial killing ratio. A further limitation is that different bacterial concentrations were studied for only one strain (SA ATCC 29213). However, as Figure 5 shows, the extent of an increased killing ratio with lower irradiated volumes or higher UV fluences was similar in all strains. Hence, we also may expect a similar trend with different bacterial concentrations.

With these given limitations and the fact that so far all clinical studies have used a (suboptimal) standard fluence of 5.4 J/cm^2 to treat infectious ulcers, it is impressive that clinical benefits could already be observed.[2,30,35–37] In a clinical study with 40 eyes of all stages of infectious keratitis (0–12 mm), Price et al[37] demonstrated that only 15% of keratitis required an additional treatment and small ulcers did not require any. Even in moderate-sized therapy-resistant infectious keratitis, Knyazer et al[2] recently showed that PACK-CXL plays a decisive role. Similarly, PACK-CXL has been successfully used as an option for limiting the spread of infection in the cap-stromal bed interface after a small incision lenticule extraction (SMILE) procedure.[35] Therefore, even if we are unable to completely eradicate the microorganisms clinically, an increase in the amount of microorganism killing could potentially improve the current treatment protocols.

In conclusion, the efficacy of PACK-CXL can be further improved by using a higher UV fluence. Considering that PACK-CXL follows the Bunsen Roscoe law,[19] an increased fluence (J/cm^2) does not necessarily imply increased treatment duration but could also be achieved with a higher UV irradiance (mW/cm^2). Increasing the currently used UV fluence by a factor 3 leads to an increase in the amount of bacterial killing from 50% to 100% in low-bacterial concentrations. In view of recent findings by Seiler et al[34] showing that the effective irradiation damage threshold for the endothelium might be substantially higher than previously assumed, such an increase by a factor 3 might be feasible clinically. Further studies are required to determine the bacterial killing in higher bacterial concentrations and the maximal UV fluence that can be safely applied in different sizes of corneal ulcers.

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