Impact of Fluorescein on the Antimicrobial Efficacy of Photoactivated Riboflavin in Corneal Collagen Cross-linking

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ABSTRACT

PURPOSE: To investigate the effect of fluorescein on the antimicrobial efficacy of photoactivated riboflavin in corneal collagen cross-linking (CXL) in vitro.

METHODS: The ultraviolet light-A (UVA) absorption by fluorescein and riboflavin in different concentrations was analyzed with a spectrophotometer. The killing rate of *Staphylococcus aureus* strains after CXL with UV-A irradiation using different solutions containing riboflavin and/or fluorescein, was evaluated in vitro.

RESULTS: Fluorescein absorbed UV-A to a significant extent, which augmented with increasing concentration. Moreover, addition of fluorescein to the riboflavin solution contributed to a significant reduction of the killing rate of *S. aureus* strains after 30 minutes of UV-A irradiation in vitro with a killing rate of 85%, whereas it was 47% in the presence of 2% fluorescein ($P = .0247$).

CONCLUSIONS: The results indicate that fluorescein competes with riboflavin for the absorption of UV-A during CXL and reduces the antimicrobial effect of the procedure. The authors recommend that physicians treating infectious ulcerative keratitis by CXL should not stain the cornea with fluorescein for visualization of the corneal ulceration prior to CXL.

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Bacterial keratitis is a potentially sight-threatening condition, which may cause severe visual loss if not treated adequately at an early stage. If the appropriate antimicrobial treatment is delayed, only 50% of patients' eyes show satisfactory visual recovery.[1] On the other hand, early antimicrobial treatment does not always ensure rapid and complete cure, because resistance to antimicrobial agents and misdiagnosis of fungal keratitis increase the rate of devastating complications such as deep corneal ulceration and perforation.[2]

Collagen cross-linking (CXL) of the cornea by ultraviolet-A (UVA) irradiation and riboflavin is a therapeutic procedure introduced initially for the treatment of keratoconus and corneal ectatic diseases.[3-5] More recently, Martins et al. demonstrated the antimicrobial potential of CXL against common pathogens in vitro,[6] and photoactivated riboflavin/CXL has been successfully applied for treatment of bacterial and fungal keratitis with corneal melting.[7-12]

Many surgeons stain a corneal ulcer with high concentrations of fluorescein to better assess the size of the lesion prior to a CXL procedure. Fluorescein is a low toxic fluorophore with excitation peaks at 325 and 494 nm and an emission peak at 521 nm. During an eye examination the amount of fluorescein used (fluorescein sodium 2%; Bausch & Lomb, Rochester, NY) is 20 times the concentration of riboflavin (0.1%) used during CXL.

We have investigated whether fluorescein might influence CXL by competing with riboflavin for UV-A absorption during the irradiation.

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Drs. Richoz and Hafezi hold a patent on a UV light source (PCT/CH 2012/000090). The remaining authors have no financial or proprietary interest in the materials presented herein.

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METHODS

UV-A Absorption Analysis for Riboflavin and Fluorescein

We analyzed the UV-A absorption of riboflavin and fluorescein in NaCl 0.9% at pH 7 with a spectrophotometer (Lightwave S2000; Biochrom WPA, Cambridge, United Kingdom) using a 1-mm cuvettes manufactured from Suprasil quartz glass (Hellma Analytics, Müllheim, Germany). The following concentrations were tested: 0.1%, 0.01%, and 0.001% for riboflavin and 10%, 1%, 0.1%, and 0.01% for fluorescein.

Experimental Set-up

NRS1 strains, reference *Staphylococcus aureus* strains obtained from the 'Network on Antimicrobial Resistance in Staphylococcus aureus' (NARSA) collection, were used in all experiments. The isolate is a methicillin-resistant *S. aureus* (MRSA)-isolate showing resistance against macrolide, quinolones, and aminoglycosides. The suspension was prepared from fresh subcultures grown on Mueller Hinton Agar at a titer of 0.5 McFarland, corresponding to a cell density of 1×10^8 bacterial cells/mL. A 1:10 dilution in NaCl 0.9% was pre-incubated during 30 minutes with 5 µL of fluorescein 10% or 5% and riboflavin solution for a final concentration of 0.1% riboflavin.

The mixture (10 µL) was introduced in a 0.4-mm thick quartz cuvette and exposed to UV-A at an intensity of 9 mW/cm^2 for 10 minutes (novel protocol). Following UV-A exposure, the sample was collected and diluted to a final volume of 500 µL, which was plated on Mueller Hinton Agar (Becton Dickinson, Franklin Lakes, NJ). The number of colony-forming units was evaluated after 24-hour incubation at 37°C.

For each experiment, we estimated the difference between the MRSA-killing rate with UV-A irradiation (UVA+) and the MRSA-killing rate without UV-A irradiation (UVA-), which we defined as UV-mediated killing rate [= (killing rate UVA+) – (killing rate UVA-) = Δ killing rate].

Statistical Analysis

Statistical analysis was performed with the SPSS Statistics program (Version 19; IBM, Armonk, New York). The Student's *t* test was applied for statistical comparisons. *P* values less than .05 were considered statistically significant. All experiments were performed in duplicate.

RESULTS

Absorption analysis at 365 nm (UV-A) showed 100% absorption for 0.1% riboflavin solution, whereas absorption decreased to 40% for riboflavin 0.01% solution (**Figure 1**). At a concentration of 0.001%, the absorption of riboflavin was close to 0% and could not be measured in a reliable manner (data not shown). Fluorescein demonstrated an absorption of 100% for 10% (data not shown) and for 1% solution and an absorption of 10% for 0.01% fluorescein solution (**Figure 1**). **Figure 2** shows the UV-A–mediated killing rate of MRSA after 30 minutes of UV-A irradiation. The killing rate was 85% for the suspension containing riboflavin only, whereas it was 47% in the presence of 2% fluorescein and 52% in the presence of 1% fluorescein. Solutions containing NaCl only and NaCl with fluorescein served as controls.

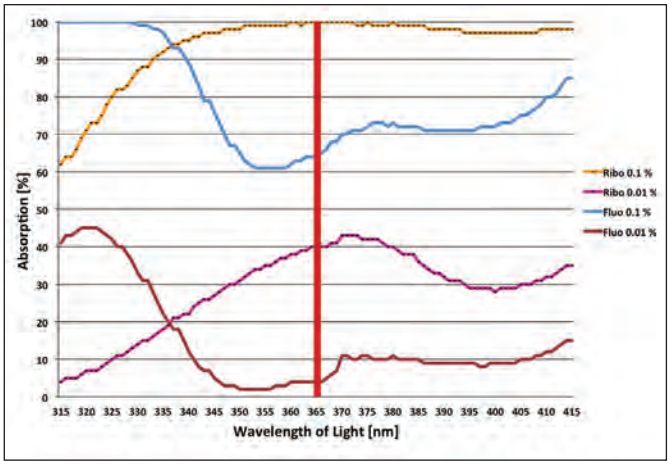

Figure 1. Absorption spectrum for 0.1% and 0.01% riboflavin and fluorescein solution. At 365 nm (ultraviolet-A), 0.1% riboflavin solution shows absorption of 100%, whereas 0.01% riboflavin absorbs at 40%. Fluorescein solution shows absorption of 64% at 365 nm and a concentration of 0.1%, whereas absorption is at 5% at a concentration of 0.01%.

DISCUSSION

Infectious keratitis represents one of the leading causes of blindness worldwide.[13] The increasing resistance of microbes to antimicrobial agents confronts the clinician with a rising number of keratitis cases that do not respond adequately to conventional antibiotic treatment.[14-18]

The antimicrobial effects of CXL have been demonstrated in vitro.[6,19] Several mechanisms may explain the antimicrobial effect of CXL. First, CXL induces the release of free radicals in corneal tissue[5,6] with potent antimicrobial properties.[20] Second, CXL increases tissue resistance to enzymatic digestion.[21] Finally, the phenomenon of cell apoptosis induced by CXL[22] possibly includes not only keratocytes but also pathogens, thus impeding the infectious process.

Several reports have documented the clinical efficacy of CXL in the treatment of severe infectious keratitis.[8,10-12,23-25] However, empirical data and anecdotal reports indicate that CXL is not always effective in the treatment of severe microbial keratitis. Multiple factors

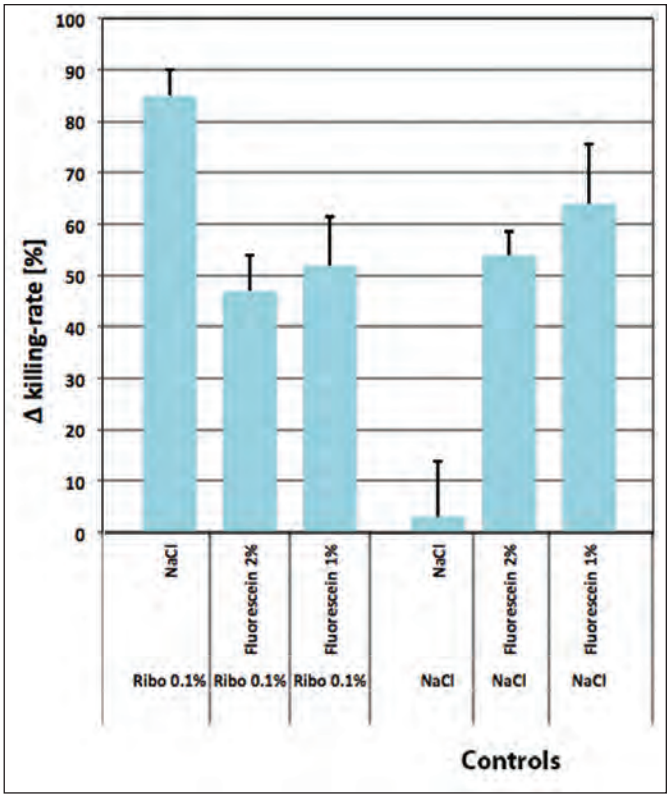

Figure 2. Killing rate of methicillin-resistant *Staphylococcus aureus* via photoactivated riboflavin/corneal collagen cross-linking after 30 minutes of ultraviolet-A irradiation. The killing rate was 85% for the suspension containing riboflavin only, 47% in the presence of 2% fluorescein, and 52% in the presence of 1% fluorescein. Solutions containing NaCl only and NaCl with fluorescein served as controls.

may account for this phenomenon, such as the microbial load of each individual patient, the susceptibility of the specific microorganism, the fluence of UV-A irradiation, the time of CXL application. We propose another factor that might have a negative impact on the efficacy of the CXL treatment: many ophthalmologists routinely stain the corneal ulceration with fluorescein prior to CXL to accurately estimate its size and to selectively remove corneal debris in the area of infiltration.

We investigated the absorption spectrum of fluorescein and the effect of fluorescein on the antimicrobial potency of CXL in vitro. Sodium fluorescein (molecular formula: $C_{20}H_{10}O_5Na$) is a phthalic indicator dye used in ophthalmology for the staining of corneal epithelial defects and for fluorescein angiography. Its molecular weight is 376. When excited, fluorescein emits light at a peak wavelength of 521 nm.[26] Fluorescein is not known in the literature for is ability to generate free radicals or to be a free radical scavenger. Riboflavin (molecular formula: C17H20N4O6), also known as vitamin B2, has a molecular weight of 376. Riboflavin occurs naturally in several products and is a photosensitizing agent with one absorption peak at 370 nm. Riboflavin carries a hydroxymethyl side chain that may be reduced to generate free radicals.[27] This mechanism is used during CXL to generate the free radicals needed for additional cross-links to occur.

Our experiments revealed that fluorescein absorbs UV-A to a significant extent and in a concentration-dependent manner. Even if our experiments demonstrate a certain killing rate for fluorescein only, this will have no effect in the clinical setting: first, the fluorescein-mediated killing rate is distinctly inferior to the killing rate mediated by riboflavin and, second, the fluorescein will be present in the superficial layers of the stroma only, preventing killing of microorganisms in the deeper layers of the stroma.

Consequently, addition of fluorescein in riboflavin solution leads to a significant reduction of the UV-A–mediated killing rate of MRSA strains in vitro.

A direct interaction between fluorescein and riboflavin might theoretically be another reason for the decreased ability of riboflavin to generate free radicals. No interaction (ie, precipitation between fluorescein and riboflavin or any other interaction) is known.

We believe that fluorescein directly competes with riboflavin for the absorption of UV-A irradiation during CXL, limiting the generation of free radicals and the therapeutic effect induced by the additional cross-links. The unfavorable clinical outcome in anecdotal cases of severe microbial keratitis treated unsuccessfully with CXL might be attributed, at least to some extent, to this competition effect of fluorescein. In view of these findings, we suggest that physicians intending to treat infectious ulcerative keratitis by CXL should not stain the cornea with fluorescein during the hours preceding a CXL procedure. Future comparative in vivo studies might further elucidate the clinical implications of our findings.

AUTHOR CONTRIBUTIONS

Study concept and design (OR, JS, FH); data collection (OR, ZG, FH); analysis and interpretation of data (OR, ZG, FH); drafting of the manuscript (OR, ZG, PF, JS, FH); critical revision of the manuscript (OR, ZG, PF, JS, FH)

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