Title

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Riboflavin and ultraviolet-A as adjuvant treatment against *Acanthamoeba* cysts

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Abstract

Background—Experimental studies have shown that the standard dose of R or R+UVA as solo treatment are not able to exterminate *Acanthamoeba* cysts or even trophozoites. The purpose of this study is to determine whether the application of R+UVA can enhance the cysticidal effects of cationic antiseptic agents *in vitro*.

Methods—The log of either polyhexamethylene biguanide (PHMB) or chlorhexidine minimal cysticidal concentration (MCC) in solutions containing riboflavin (concentrations 0.1 %; 0.05% and 0.025 %) plus either *Acanthamoeba castellanii* cysts or *Acanthamoeba polyphaga* cysts was determined and compared in groups treated with UVA 30 mW/cm^2 for 30 min and in control groups (with no exposure to UVA). A permutation test was used to determine the P-value associated with treatment.

Results—Regardless of the riboflavin concentration and UVA treatment condition, no trophozoites were seen in plates where the cysts were previously exposed to cationic antiseptic agents concentrations   200 μg/mL for *Acanthamoeba castellanii* samples and   100 μg/mL for *Acanthamoeba polyphaga* samples. There was no statistical evidence that R+UVA treatment was associated with MCC (P = 0.82).

Conclusion—R+UVA in doses up to 10 times higher than recommended for corneal crosslinking does not enhance the cysticidal effect of either polyhexamethylene biguanide or chlorhexidine *in vitro*.

Keywords

riboflavin; *Acanthamoeba*; photochemotherapy; ultraviolet

INTRODUCTION

First described by Castellani in 1930, *Acanthamoeba* is a protist that is widely distributed in the environment and has two stages in its life cycle: an active trophozoite stage during which

Acanthamoeba reproduces; and a dormant, resistant cyst stage during which it remains inactive with little metabolic activity, but viable for years.[1] The two main diseases *Acanthamoeba* can cause in humans are a rare granulomatous encephalitis limited typically to immunocompromised patients and a vision-threatening keratitis in which contact lens wear is the main risk factor.[2,3] The first cases of *Acanthamoeba* keratitis (AK) were reported in the 1970s,[4,5] and in recent decades there has been a significant increase in the reported incidence of AK, apparently due to the growing popularity of soft contact lens wear.[6,7] AK cure is a challenge, and over the years multiple methods of treatment have been proposed.[8] Recently, the use of riboflavin (R) and ultraviolet-A radiation (UVA) in doses (5.4 J) similar to what is used for corneal collagen crosslinking (CXL) have been postulated as a possible adjuvant treatment for AK.[9] R+UVA has been successfully used in the inactivation of bacterial and virus isolates.[10–14] However, experimental studies have shown that the standard dose of R or R+UVA as solo treatment is not able to exterminate *Acanthamoeba* cysts or even trophozoites.[15–17] Sauer *et al.* showed that R+UVA had a significant effect against fungal colonies only if used in addition to amphotericin.[18] There are no experimental studies investigating the existence of additive effects when using R+UVA as an adjuvant therapy against *Acanthamoeba*. The objective of this study is to determine whether the application of riboflavin (R) + ultraviolet A radiation (UVA) can enhance the cysticidal effects of cationic antiseptic agents commonly used for AK treatment.

METHODS

Acanthamoeba solutions

Non-nutrient agar plates containing *Acanthamoeba castellanii* (ATCC 30011, genotype T4) or *Acanthamoeba polyphaga* (ATCC 50495, genotype T4)[19] were initially seeded with heat-killed *Escherichia coli* and then left starving for approximately two weeks until all trophozoites had encysted due to starvation. The cysts were washed from the plates with ¼ strength Ringer's solution and concentrated by centrifugation (326g, 5 minutes) to be used in the following preparations.

Stock cationic antiseptic solutions were prepared in in 0.2 mL PCR tubes (PGC Scientific, Frederick, MD) using 1/4 strength Ringer's. PHMB (Leiter's Compounding Pharmacy, San Jose, CA) was used at two different starting concentrations so that two-fold dilutions could be performed to generate solutions with the following concentrations: 300, 200, 150, 100, 75, 50, 37.5, 25, 18.75, 12.5, 9.37, 6.25, 4.67, 3.12 µg/mL. Chlorhexidine (Santa Cruz Biotechnology, Dallas, TX) was used at a starting concentration and diluted two-fold to generate the following concentrations: 200, 150, 100, 75, 50, 37.5, 25, 18.75, 12.5, 9.37, 6.25, 4.67, 3.12 µg/mL. Riboflavin (Sigma-Aldrich, Saint Louis, MO) and either *A. castellanii* or *A. polyphaga* were added such that the final solutions contained 3×10^5 cysts/mL, riboflavin 0.05%, and the multiple concentrations of either PHMB (from 300 µg/mL to 3.125 µg/mL) or chlorhexidine (from 200 to 3.125 µg/mL). The PCR tubes were agitated vigorously and left for 48h in an incubator at 37°C and protected from the light.

To determine whether different concentrations of riboflavin could influence the results, solutions containing *A. castellanii* cysts (3×10^5 cysts/mL) were prepared through the same

method described above, but changing the initial concentration of riboflavin in the stock solutions to achieve final concentrations of 0.1% or 0.025%.

Ultraviolet Radiation Treatment

A custom-made UV-A light source using a 365 nm LED (NC4U133, Nichia, Japan) attached to an optical fiber was used to deliver an irradiance of 30 mW/cm^2/s at 2 mm from the optical fiber tip. A spectrophotometer (Jaz, Ocean Optics, Dunedin, FL) was used to measure the irradiance. The optical fiber was connected to a custom-made autoclavable polyoxymethylene tray containing 30 wells (5 mm in diameter each) and specially designed for this experiment, allowing exchange of air and preventing the optical fiber tip from touching the solution at the bottom of the well.

The tubes containing the solutions previously described and left for 48 hours in an incubator were agitated vigorously, and two samples of 22 μL were withdrawn and placed into two wells of the sterile tray. The temperature of the tray was controlled and maintained between 36 and 38 °C. One well was irradiated with 30 mW/cm^2/s UVA for 30 minutes, and the other was left untreated as a control. Room light was kept reduced during the procedure. Due to evaporation, 8 μL of the same concentration riboflavin solution was added to each well after 15 minutes. After 30 minutes the remaining solution was removed from each well and processed separately, each one washed three times with 200 μL of ¼ strength Ringer's solution in order to be free of drugs. Following the final centrifugation (326 g, 10 minutes), 20 μL were left in the bottom of each sample. These tubes were agitated vigorously and two samples of 8 μL were removed from each tube and placed on two non-nutrient agar plates seeded with heat-killed *E. coli*.

Determination of the MCC

As previously described,[20] the inoculated plates were incubated at 30 °C and examined daily for seven days by at least two of the authors for the appearance of trophozoites. When present, live trophozoites were detected by observing contraction of their water expulsion vesicle. The MCC was defined as the lowest concentration of antiseptic agent that resulted in no excystment and growth of trophozoites of *Acanthamoeba* after seven days of incubation.

The experiments were repeated three to six times for each *Acanthamoeba* species, antiseptic agent, and each concentration of riboflavin. After determining the approximate MCC in the initial experiment, we decided to not repeat the concentrations below 12.5ug/ml.

Statistical Analysis

The log of MCC was modeled using linear regression with species and riboflavin as covariates along with UVA treatment and biocide agent. We used a permutation test (within the four riboflavin/species combinations) to determine the P-value associated with treatment. All computations were conducted in R v3.1 for Macintosh (R foundation for statistical computing, Vienna, Austria.)

RESULTS

The range of PHMB MCC was 150 – 200 μg/mL for *Acanthamoeba castellanii* samples and 75 – 100 μg/mL for *Acanthamoeba polyphaga* samples (Table 1). Regardless of the riboflavin concentration (0.1%, 0.05% or 0.025%) and UVA treatment condition, no trophozoites were seen in plates where the cysts were previously exposed to PHMB concentrations 200 μg/mL for *Acanthamoeba castellanii* samples and 100 μg/mL for *Acanthamoeba polyphaga* samples. The range of chlorhexidine MCC for *Acanthamoeba castellanii* was 25 – 200 μg/mL (Table 2). The geometric means of PHMB MCC and Chlorhexidine MCC are presented in Table 3. There was no statistical evidence that UVA treatment was associated with MCC (log transformation, permutation test P value = 0.82, 1000 replications). Similarly, subset analyses of each species did not change this finding, nor did using riboflavin concentration as a categorical factor rather than as a continuous variable.

Solutions containing initially riboflavin 0.1% had some residual yellow at the end of the treatment, whereas the ones containing riboflavin 0.025% were clear by the end, confirming that photolysis and degradation of the riboflavin had taken place with UV irradiation.[21]

DISCUSSION

In this study we sought to determine whether the application of riboflavin (R) + ultraviolet A radiation (UVA) could enhance the cysticidal effects of cationic antiseptic agents commonly used as monotherapy for AK. *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* are the two most common species to cause AK.[22] Several antibiotics have been shown to be effective against *Acanthamoeba* trophozoites, but the cyst form is highly resistant and challenging to inactivate.[23] Cysts can resist extremes of pH and temperature and survive more than 20 years.[24,25] Recently, in an online survey of worldwide ophthalmologists, Oldenburg *et al.* found that PHMB and chlorhexidine were the most common choices as monotherapy for treating AK.[26] Although the majority of patients achieve clinical cure, the duration of treatment for AK can be very long[27] and despite all the currently available drugs many patients develop severe corneal scarring or even perforation.[28]

The knowledge that ultraviolet radiation can be used to activate riboflavin and reduce pathogens *in vitro* and *ex vivo* such as in platelet concentrates,[12] together with the popularization of the CXL technique in which UVA is irradiated for 30 minutes at 3 mW/cm^2 (5.4 J) over a cornea treated with topical riboflavin,[29] led to the idea that the same parameters used for CXL could be used for reducing pathogens. Khan *et al.* reported three cases of AK in which the use of R+UVA (5.4 J) as an adjunctive treatment contributed to the improvement of patients' symptoms and signs, and ultimately brought about a cure.[9] However, experimental *in vitro* and *in vivo* studies[15,16] using one to two times the total 5.4 J UVA dose commonly used for keratoconus treatment showed no significant effect of R +UVA over *Acanthamoeba* trophozoites and cysts. Sauer *et al.*[18] showed that R+UVA had a significant effect against fungal colonies only if used in addition to amphotericin. We hypothesized that the reported good results in patients with AK treated with R+UVA could have resulted from the associated topical drug therapy administered to those patients. Since new protocols for CXL using devices capable of delivering higher UVA irradiance levels are

being studied, and in anticipation that this could raise future questions about whether a higher UVA irradiance would be effective against *Acanthamoeba*, we decided to use a total UVA dose of 54 J which is 10 times higher than the standard dose postulated by Wollensak *et al.*[29] and far above the safety limits reported by Spoerl *et al.*[30,31]

The effects of riboflavin upon *Acanthamoeba* are controversial; using a different experimental model, Makdoumi et al. suggested that the addition of 0.01% of riboflavin to a solution containing acanthamoeba did not amplify the antimicrobial effect of UV observed in their study.[32] We used riboflavin 0.05% as our standard concentration test because it is approximately the riboflavin concentration achieved in deep corneal stroma for CXL.[33] We also tested riboflavin 0.1% and 0.025% for *A.castellanii* to mitigate the risk that the results had been affected due to either excess or inadequate riboflavin. We opted not to add dextran to the riboflavin solution because it increases viscosity and could potentially interfere with the experimental method by sticking to the wells and pipette tips.

The MCC value is dependent upon the species, strain, cyst concentration and time in contact with antiseptic agent.[20,34–36] Narasimhan *et al.* reported a PHMB MCC ranging from 25 to 100 μg/mL over 19 *Acanthamoeba* strains (10^4 cysts/mL) and a chlorhexidine MCC ranging from 1.56 to 100 μg/mL.[20] In our experiment we tested solutions containing 10^5 cysts/mL, the PHMB MCC values ranged from 75 to 200 μg/mL, and the chlorhexidine MCC values ranged from 25 to 200 μg/mL. However, the purpose of this study was not to quantify MCC values but to determine whether the application of R+UVA would promote any additional cysticidal effect. Our *in vitro* results showed that even applying a UVA dose 10 times higher than that used in the standard CXL technique, there was no significant difference in the mean log of either PHMB or chlorhexidine MCC between *Acanthamoeba* cyst groups (UVA treated vs. control), suggesting that in the setting of using a cationic agent as primary treatment, R+UVA provides no additional cysticidal effect for *Acanthamoeba*.

There are limitations to using this in vitro model to estimate what will happen in vivo. Negative results can be related to methodological aspects, and other experimental models have been proposed leading to different conclusions. In a study treating *Acanthamoeba* over a gel dish and counting the number of cysts after 8 days, Letsch et al. suggested that treatment with only chlorhexidine was less effective than treatment with only R+UVA or with the combination of R+UVA+chlorhexidine. One main difference between their study and ours is that they applied chlorhexidine 0.02% 1 drop every 5 minutes for 30 minutes, whereas in our study we used the method proposed by Narasimhan et al[20] and left the cysts for 48h in contact with the solution containing chlorhexidine. We believe that our method better simulates what happens with patients that have been using chlorhexidine drops over a long term and for whom clinicians would question the possible benefit of a single application of R+UVA to kill the remaining cysts. Also, instead of treating the samples using one single concentration of the cationic antiseptic agents and counting the number cysts later, to measure the MCC one does not count the number of cysts but rather exposes the sample to multiple concentrations of the cationic antiseptic agent and then determines at what concentration there are no trophozoites. To mitigate the chance of error we also repeated the experiment up to six times in some instances, to ensure that we could have complete confidence in the data.

The clinicopathological correlation between *in vitro* sensitivities and the clinical outcome in AK is poor, and the reasons for *in vivo* resistance in face of good *in vitro* sensitivity remain incompletely understood.[28] Intrastromal concentrations of antiseptic agents that are suboptimal due to poor penetration or to binding tissue components are possible explanations. Of note is also the fact that in our experimental model the MCC observed was barely below the concentration that is commonly prescribed to patients, raising the hypothesis that some patients could benefit from increasing the concentration of the drug prescribed. Conversely, it is possible that treatments that do not show efficacy *in vitro* have some beneficial role in the treatment of AK. Based on our data, it seems likely that if there is any clinical advantage in using R+UVA as an adjunctive treatment, as suggested by Khan *et al.*,[9] it would probably result from indirect effects such as increasing the corneal collagen's resistance to the trophozoite's digestive enzymes and reducing its major nutritional supply (i.e. cells) through the cytotoxic effect upon stromal keratocytes.[37,38] As an example of the latter hypothesis, in the past the use of cryotherapy as an adjunctive treatment for AK was reported to have some success despite the fact that *Acanthamoeba* cysts are resistant to freezing temperatures.[39,40] Among the possible related reasons are that cryotherapy kills epithelial cells and keratocytes and affects osmotic and nutritional environmental factors. Similarly, the treatment with R+UVA is known to interfere with corneal hydration and to kill keratocytes[41–43]. Following this rationale, the debridement of the epithelial cells would not only directly remove the more superficial organisms but also contribute to the elimination of their nutritional source, and, in that line of reasoning, we could extrapolate that R+UVA transepithelial techniques would not be desirable when the aim is to eradicate this pathogen. Further studies should be conducted to investigate possible indirect effects of R+UVA treatment upon AK *in vivo*.

The high dose of UVA applied in this study is possibly harmful for clinical use, and it was applied in association with lower concentrations of riboflavin in order to maximize the consumption of riboflavin and reduce the risk of not having enough UVA reaching the bottom (or distal) part of the solution in our experiments. Theoretically the thickness of the solutions tested ranged from 1.1 to 0.7 mm inside the 5 mm wells (due to evaporation).

In conclusion, R + UVA in doses up to 10 times higher than recommended for corneal crosslinking does not enhance the cysticidal effect of either PHMB or chlorhexidine. If there is any beneficial role in using R+UVA as an adjunctive treatment against *Acanthamoeba*, it is likely not due to direct cysticidal effects.

Acknowledgments

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Table 1

Results of treatment with PHMB with and without UVA upon *Acanthamoeba* plates

Species	Riboflavin Concentration (%)	PHMB Concentration (μg/ml)	Fraction of Plates with Live Trophozoites		Number of Plates Tested (n)
			PHMB + UVA	PHMB only	
		100	12/12	12/12	24
	0.025	150	1/12	2/12	24
		200	0/12	0/12	24
		100	5/6	5/6	12
A.castelannii	0.05	150	6/6	4/6	12
		200	0/6	0/6	12
		100	8/8	8/8	16
	0.1	150	6/8	5/8	16
		200	0/8	0/8	16
		50	6/6	6/6	12
A.polyphaga	0.05	75	2/6	3/6	12
		100	0/6	0/6	12

PHMB = Polyhexamethylene biguanide; UVA = Ultraviolet A radiation; Samples of A. *castellanii* treated with PHMB concentrations 100 μg/ml (100, 75, 50, 37.5, 25,18.75, 12.5, 9.37, 6.25, 4.67, 3.12) were all positive for live trophozoites. Samples of A. *polyphaga* treated with PHMB concentrations 50 μg/ml were all positive for live trophozoites. All samples treated with PHMB concentrations 200 μg/ml (200,300) presented no live trophozoites.

Table 2

Results of treatment with Chlorhexidine with and without UVA upon *Acanthamoeba* plates

Species	Riboflavin Concentration (%)	Chlorexidine Concentration (µg/ml)	Fraction of Plates with Live Trophozoites		Number of Plates Tested (n)
			Chlorexidine + UVA	Chlorexidine only	
A.castelannii	0.05	18.75	10/10	10/10	20
		25	8/10	8/10	20
		37.5	8/10	8/10	20
		50	8/10	7/10	20
		75	8/10	7/10	20
		100	6/10	6/10	20
		150	6/10	6/10	20
		200	0/10	0/10	20

UVA = Ultraviolet A radiation; Samples of A. *castellanii* treated with chlorhexidine concentrations 18.75 µg/ml (18.75, 12.5, 9.37, 6.25, 4.67, 3.12) were all positive for live trophozoites. All samples treated with chlorhexidine concentrations 200 µg/ml (200,300) presented no live trophozoites.

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Table 3

Geometric means of PHMB MCC and Chlorhexidine MCC

Species	Riboflavin Concentration (%)	Cationic Antiseptic Agent	MCC (Geometrical Mean in µg/mL)		P Value
			UVA Treated	Control	
Acanthamoeba castellanii	0.05	Chlorhexidine	114.9	107.2	
	0.025	PHMB	153.6	157.4	0.82
	0.05		200	181.7	
	0.1		186.1	179.6	
Acanthamoeba polyphaga	0.05		82.5	86.6	

PHMB = Polyhexamethylene biguanide; MCC = Minimum cysticidal concentration ; UVA = Ultraviolet A radiation; P-value computed using permutation test.