
Elmer Y. Tu, MD; Megan E. Shoff, PhD; Weihua Gao, MS; Charlotte E. Joslin, OD, PhD

Importance: The significant antiacanthamoebal effect of benzalkonium chloride, at or below concentrations used for preservation of common ophthalmic preparations, should be understood both when choosing empiric antibiotic therapy for infectious keratitis and when assessing the persistent rise in Acanthamoeba cases in the United States since 2003.

Objective: To characterize the antiacanthamoebal efficacy of low concentrations of benzalkonium chloride (BAK) for drug preservation and therapeutic effect against Acanthamoeba.

Design: Experimental study with a review of the literature.

Setting: Laboratory.

Exposures: A concentration of 10^4 trophozoites of 3 well-characterized clinical strains of Acanthamoeba were exposed at 0.5, 2.0, 3.5, 5.0, and 6.5 hours to BAK (0.001%, 0.002%, and 0.003%), moxifloxacin hydrochloride (0.5%), and moxifloxacin (0.5%) + BAK (0.001% and 0.003%) with hydrogen peroxide (3%) and amoeba saline controls.

Main Outcomes and Measures: Amoeba survival was calculated using the most probable number method recorded as log kill values. The relationship of BAK concentration and exposure time as well as the relative effect of BAK and moxifloxacin on acanthamoebal survival were analyzed.

Results: Amoebicidal activity of BAK is both time dependent and concentration dependent in pooled and strain-stratified analyses (P < .001). Moxifloxacin demonstrated no significant independent inhibitory effect or additive effect to BAK efficacy on acanthamoebal survival. The profound antiacanthamoebal effect of BAK, 0.003%, was similar to that of hydrogen peroxide for certain strains.

Conclusions and Relevance: Low concentrations of BAK, previously demonstrated to concentrate and persist in ocular surface epithelium, exhibit significant antiacanthamoebal activity in vitro at or below concentrations found in commercially available ophthalmic anti-infectives. The unexplained persistence of the Acanthamoeba keratitis outbreak in the United States, clusters abroad, and clinical studies reporting resolution or modification of Acanthamoeba keratitis without specific antiacanthamoebal therapy suggests that other contributing factors should be considered, including changes in the formulations used for empirical therapy of presumed infectious keratitis occurring in the same period.


©2013 American Medical Association. All rights reserved.
specific antimicrobial spectrum extending far beyond the test organisms. Benzalkonium chloride (BAK), the predominant ophthalmic drug preservative for the last several decades, is a quaternary ammonium surfactant with this broad range of antimicrobial activity. The recent emphasis on its significant ocular surface toxicity has led, however, to the introduction of alternative preservative compounds as well as the reduction or elimination of its use in many ophthalmic preparations.13

Previous quantitative studies of the effect of BAK on Acanthamoeba are limited but have suggested a highly variable effect based on culture conditions and specific strain.14,15 The recent withdrawal and/or substitution for BAK in the marketplace provides an opportunity to study the potential clinical effects of adopting alternative preservation schemes, as an unintended reduction of the antiacanthamoebal activity of topical ophthalmic preparations not only would reduce the preservation effectiveness against an increasingly prevalent ophthalmic pathogen but could also alter the unrecognized effect of these formulations on the clinical course of Acanthamoeba infection. We therefore studied the antiacanthamoebal efficacy of BAK at concentrations at or below those levels found in current ophthalmic preparations using a standardized protocol in development for US Food and Drug Administration efficacy testing of contact lens disinfection systems to both quantify its preservation efficacy and assess its potential for therapeutic benefit in the context of the persistent Acanthamoeba keratitis outbreak in the United States and globally.

METHODS

ORGANISMS
Three organisms were chosen for comparison: Acanthamoeba castellanii (CDC V-568, isolate T4), Acanthamoeba polyphaga (CDC V-572, isolate T4), and Acanthamoeba hatchetti (CDC V-573, isolate T4), all obtained bacterized from Govinda Vivesvara, PhD, of the CDC.16 All 3 strains were originally isolated from patients with Acanthamoeba keratitis. On arrival, cultures were subcultured onto nonnutrient amoeba saline agar plates seeded with Enterobacter aerogenes and were incubated at room temperature. Trophozoites were produced for experiments by subculturing amoeba and, after 2 days, harvesting the amoeba from the plates and washing 3 times with 30 mL of amoeba saline; they were counted using a hemacytometer and adjusted to 10⁶ trophozoites by dilution or centrifugation.

PROCEDURE
Trophozoites were exposed to 3 concentrations of BAK (0.001%, 0.002%, and 0.003%), the lowest common concentration currently found in most ophthalmic formulations including topical antibacterial drugs) with amoeba saline and to 2 concentrations of BAK (0.001% and 0.003%) with moxifloxacin hydrochloride, 0.5%, to simulate the exposure of the ocular surface to BAK achieved with topical application using matched controls of moxifloxacin, 0.5%, alone, hydrogen peroxide, 3% (positive control), or amoeba saline (negative control). Parenteral moxifloxacin, identical in concentration to the ophthalmic preparation but differing primarily by the absence of small amounts of boric acid and buffers for topical ophthalmic use, was used in this study. Aliquots (100 μL) of trophozoite culture were added to 10 mL of the test solution or control in sterile glass tubes to 5 initial concentrations of 10⁶ trophozoites/mL. The tubes were vortexed to distribute the amoebae throughout the test material and were kept at room temperature throughout the test period.

The enumeration procedure was adapted from the most probable enumeration technique by Beattie et al.17 At predetermined intervals (0.5, 2.0, 3.5, 5.0, and 6.5 hours), 1-mL aliquots were removed from each test and control solution and placed in Dey-Engley broth for a minimum of 10 minutes for neutralization. At this time, the neutralization tablet was also added to the hydrogen peroxide solution for neutralization for the given time. From this 1:10 dilution, a 1:100 dilution was prepared in amoeba saline, and from this dilution, 5 aliquots each of 1, 0.1, and 0.01 mL were inoculated onto nonnutrient amoeba saline agar plates seeded with E aerogenes. The wells of 6-well and 12-well plates were used for the 0.1- and 0.01-mL aliquots. Plates were then sealed and incubated at 30°C for 7 to 14 days and then scored for growth.

For enumeration per the article by Beattie et al, a plate showing amoebic growth scored 1 and a plate showing no growth scored 0. The score for each of the 10-fold dilutions gave 1 value of a 3-digit number corresponding to a value on the most probable number table. The wells of 6-well and 12-well plates were used for the 0.1- and 0.01-mL aliquots. Plates were then sealed and incubated at 30°C for 7 to 14 days and then scored for growth.

Pooled results for the 3 strains of Acanthamoeba (Figure 1) demonstrate a significant antiacanthamoebal effect that is both time dependent (P < .001) and concentration dependent (P < .001). The mean log kill significantly increased with increasing concentrations of BAK; the mean log kill with BAK, 0.003%, was not significantly different from that with hydrogen peroxide (Figure 1). The mean log kill at 0.5 hour was significantly different from all other times tested but the mean log kill times were not significantly different between later times, reflecting the rapid onset of effect (Figure 1). There was no time × concentration interaction effect. Although stratification of individual strains showed interstrain differences in sensitivity to BAK.
The pattern of time of exposure and concentration-dependent killing remained statistically significant for all strains tested ($P < .001$). *A castellanii* was the least-sensitive amoebic species, only exceeding the bacterial standard of an organism reduction greater than 2 log units after 2.0 hours of exposure to BAK, 0.003%. The other pathogenic strains, *A hatchetti* and *A polyphaga*, were significantly more sensitive, effecting essentially equivalent kill rates of BAK, 0.003%, and hydrogen peroxide, 3%, plotted by time. Each time point represents a single strain performed in triplicate ($n=3$). Error bars indicate standard deviation.

Moxifloxacin alone showed no activity for any of the strains tested (Figure 5). The efficacy of BAK, 0.003%, with moxifloxacin was statistically greater than moxifloxacin alone at all times tested ($P > .001$), while BAK, 0.001%, with moxifloxacin was significantly different only at 2 times (Table). Conversely, there was no statistically significant difference between the antiacanthamoebal effect of BAK, 0.001%, and BAK, 0.001%, with moxifloxacin or between the antiacanthamoebal effect of BAK, 0.003%, and BAK, 0.003%, with moxifloxacin ($P > .99$).

**COMMENT**

Benzalkonium chloride, a cationic surfactant that exerts its antimicrobial effect through disruption of lipid cell membranes, is the most widely used preservative in ophthalmic products with known, broad antimicrobial ac-
tivity against bacteria and fungi. Using a reference test regimen developed for contact lens disinfection systems, we have demonstrated that even low concentrations of BAK exhibit significant antiacanthamoebal activity in both concentration- and time-dependent manners. While time-kill plots demonstrate significant inter-strain variability, antiacanthamoebal activity was present at concentrations at or below levels found in current commercial ophthalmic preparations at the earliest times tested for all strains. Combined with the long exposure times when included in an ophthalmic preparation, this strongly suggests that BAK is an effective antiacanthamoebal preservative. In fact, BAK, 0.003%, common to many ophthalmic antibacterials, approached the efficacy of hydrogen peroxide, 3%, a standard for known antiacanthamoebal activity. In this context and the recent surge of Acanthamoeba keratitis worldwide, the rapid, effective antiacanthamoebal efficacy demonstrated in this study also raises the potential for a therapeutic effect of BAK in Acanthamoeba keratitis, especially in early, superficial disease.

In such a rare disease with multiple risk factors, detection of a clinical antiacanthamoebal effect would be understandably difficult. Although there is a vast array of non-BAK-containing ophthalmic drugs, the anti-infectives are the most commonly prescribed agents for empirical treatment of infectious keratitis in sufficient frequency and therefore represent the drugs most likely to demonstrate a differential effect. Moxifloxacin ophthalmic solution (Vigamox) is unique among topical anti-infectives in that it is self-preserved, able to meet anti-infective efficacy remains controversial, especially because tear retention of BAK after topical administration appears transient, below detectable limits at 5 minutes. However, distribution studies of radioisotopelabeled BAK demonstrate that a single topical application is retained in corneal and conjunctival epithelium for several days and that multiple applications of a moderate frequency (4-5 times/day) are concentrated to levels exceeding the concentration of the administered formulation in these tissues. For Acanthamoeba, the most common commercial ophthalmic compounds usually contain BAK at levels between 0.003% and 0.01%, above levels shown in this model to result in a 1- to 4-log unit reduction of viable amoebae in as little as 30 minutes of exposure. Clinical observations and in vitro studies indicate that early Acanthamoeba keratitis primarily affects the epithelium, a stage at which the prognosis is good. These radioisotope studies strongly suggest that BAK saturation of the corneal epithelium with even moderate dosing can potentially achieve the concentrations.
and exposure times necessary to limit or possibly cure superficial acanthamoebal corneal disease.

Additionally, our own experience parallels recent reports of resolution of *Acanthamoeba*-positive keratitis with topical antibacterials alone before positive *Acanthamoeba* cultures were reported,\(^{31,32}\) suggesting that the isolates were nonpathogenic, were unable to survive axenically, or were cured by nonspecific therapeutic intervention, possibly debridement or the BAK component of a topical antibiotic. Similarly, BAK has been noted to have excellent minimum inhibitory concentrations against many ocular fungal pathogens at or below preservative levels.\(^{33-35}\) This has been previously proposed as a mechanism for the resolution of fungal keratitis with the use of topical antibacterials alone, but some fungal pathogens are also responsive in vitro and clinically to fluoroquinolones formulated without BAK.\(^{33,34,36,37}\) However, in our in vitro model, moxifloxacin alone had little or no antiacanthamoebal activity and the antiacanthamoebal activity of moxifloxacin + BAK is not significantly different from that of the BAK component alone, without evidence of synergy (Figure 5).

Nonetheless, the evidence for a clinical effect of BAK in *Acanthamoeba* keratitis is circumstantial and any conclusions are speculative. As we previously concluded, while *Complete MoisturePlus* was identified as a significant risk factor, it was unlikely to be the only factor because 38.8% of patients with *Acanthamoeba* keratitis had never used the solution.\(^{2,5,6}\) Similarly, the simple exclusion of those patients treated primarily for *Acanthamoeba* keratitis with moxifloxacin (27.3% of cases in our previous study) would still leave a substantial, statistically significant difference in the United States, further indicating that other factors, likely environmental, are also contributing.\(^{3}\) As always, a randomized trial for the efficacy of BAK would be ideal, but for *Acanthamoeba*, several factors make a clinical trial to test this hypothesis impractical, specifically the rarity of the infection, variable natural history, and limited knowledge of true incidence. It is also important to note that no standard for antiacanthamoebal efficacy is universally accepted. Because significant controversy over efficacy testing methods for *Acanthamoeba* has involved every aspect of preparation, performance, and assessment, we used the test regimen in development for contact lens disinfection systems standardized to 3 human pathogenic strains, nonaxenic cultivation, and the enumeration method. *Acanthamoeba* strains grown nonaxenically are harder and more pathogenic,\(^{10}\) potentially biasing our results, but are also more likely representative of the activity of human pathogens. Hydrogen peroxide, 3%, itself highly toxic to the ocular surface, was also noted to be rapidly amoebicidal, further supporting the validity of the model. It should also be noted that this model tests efficacy against trophozoites, which are far easier to kill than cysts, but the exposure to topical ophthalmic antibacterials is usually early in the course when trophozoites are likely predominant and are poorly established in the corneal stroma and when it would potentially limit their further proliferation if induced into a cyst form. This challenge model mirrors the current tests for both topical ophthalmic preservative efficacy and current contact lens disinfection systems and is easily reproducible.

In summary, our results indicate that BAK, which is concentrated and retained in both corneal and conjunctival epithelium, has a profound inhibitory effect on *Acanthamoeba* survival in vitro and that epidemiologic and clinical trends as well as clinical case studies raise the possibility that empirical antibacterial therapy may affect the disease course of *Acanthamoeba* keratitis. We have postulated that this effect would most likely be due to the BAK content of the formulation because the underlying fluoroquinolones have not been demonstrated to have antiacanthamoebal activity. It is clear that previously known risk factors cannot fully explain the coincident increases of *Acanthamoeba* keratitis worldwide. The rise in *Acanthamoeba* cases in some countries, including the United States, has temporally followed the introduction of a non–BAK-containing ophthalmic antibiotic to the marketplace and, when combined with the univariate relationship between the use of a non–BAK-containing antibiotic and a poorer outcome in *Acanthamoeba* keratitis, invites further exploration. Since their introduction, the topical fluoroquinolones have exhibited excellent efficacy and safety in the treatment of bacterial keratitis, and any relationship to the recent increase and persistence of *Acanthamoeba* keratitis worldwide remains exploratory. However, as has been found with contact lens disinfection systems, changes in purportedly inactive components of ophthalmic preparations may have profound, unpredictable effects on antimicrobial efficacy.\(^{38,39}\) Test regimens of antimicrobial efficacy should be periodically reconsidered not only as pathogens change with time but also if they are found not to serve as a rep-
representative proxy for pathogens involved in ocular disease. It is important to fully characterize the antimicrobial effects of ophthalmic preparations independent of mandated testing regimens to understand the potential clinical effects of their substitution.

Submitted for Publication: June 2, 2012; final revision received September 18, 2012; accepted October 3, 2012.

Published Online: March 21, 2013. doi:10.1001/jamaophthalmol.2013.1644

Correspondence: Elmer Y. Tu, MD, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W Taylor St, M/C 648, Room 3.164, Chicago, IL 60612 (etu@uic.edu).

Conflict of Interest Disclosures: None reported.

Role of the Sponsor: The Center for Devices and Radiological Health, US Food and Drug Administration provided material support and expertise in the design and conduct of the study as well as review and approval of the manuscript.

Disclaimer: The mention of commercial products, their sources, or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products by the US Department of Health and Human Services.

REFERENCES


10. Shoff M. Important variables and test methodology for the evaluation of efficacy of CLMPS against Acanthamoeba. Paper presented at: 2012 Contact Lens Association of Ophthalmologists Inc and CLAO Education and Research Foundation Symposium and Congress; January 26, 2012; Las Vegas, NV.


