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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.

SINCE 2003, A SUBSTANTIAL INCREASE IN THE NUMBER OF Acanthamoeba keratitis cases has been reported in the United States and in countries around the world. Despite the identification and recall of Complete Moisture-Plus (Advanced Medical Optics), whose use was associated with a significantly greater risk of developing Acanthamoeba keratitis, the outbreak has persisted, compelling both a second multicenter study of Acanthamoeba keratitis initiated by the Centers for Disease Control and Prevention (CDC) in 2011 and the development of new testing regimens for the antiacanthamoebal efficacy of contact lens disinfection systems by the US Food and Drug Administration. Despite these efforts, a unique, primary risk factor for the sudden increase in cases remains undiscovered, suggesting that multiple risk factors may be involved.

Ophthalmic formulations, specifically multidose commercial preparations, are required to resist contamination as demonstrated by a standard test regimen against a small number of stock bacteria and fungi, usually achieved by the addition of a preservative compound with a non-
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 Vishvesvara, PhD, of the CDC. All 3 strains were originally isolated from patients with 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 10⁴ 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. 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 checked 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 to give the most probable number of amoebae per milliliter of solution. Each test solution was tested in triplicate. Resulting values were expressed in units of log kill for each individual strain at each individual time for each drug compared with the negative control (amoeba saline).

STATISTICAL ANALYSIS

General linear models were used for comparisons of time difference and concentration difference as well as their interaction effect. The F test was used to evaluate the overall effect of time and concentration as well as interaction effect. To further investigate which pair groups were different, multiple comparisons with Tukey adjustments were used. The BAK data were analyzed both pooled and stratified by individual strain to identify differences in sensitivity to BAK. These analyses were also applied to test for differences between BAK vs BAK + moxifloxacin groups and pairwise comparisons for moxifloxacin vs moxifloxacin + BAK (0.001% or 0.003%) at each time and each concentration to identify any potential additive effects of the fluoroquinolone to the antiacanthamoebal efficacy of BAK and to simulate the effect of a preserved vs nonpreserved fluoroquinolone. Means and corresponding standard deviations were reported. P < .05 was considered statistically significant. All analyses were performed using SAS version 9.2 statistical software (SAS Institute, Inc), primarily the general linear model procedure.

RESULTS

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...
(Figures 2, 3, and 4), 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 anticanthamoebal effect of BAK, 0.001%, and BAK, 0.001%, with moxifloxacin or between the anticanthamoebal 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-
The use of ophthalmic moxifloxacin prior to presentation and specific treatment for Acanthamoeba keratitis was potentially associated with a poorer outcome in univariate analysis (odds ratio = 3.50; 95% CI, 1.02-12.03), although it was not independently associated in a multivariable analysis. Any suggestion of effect should be approached cautiously because the study of prognostic factors was not sufficiently powered to demonstrate a difference, but in a rare disorder such as Acanthamoeba keratitis, these factors may provide a useful path of investigation where other options are limited. Despite the recall of Complete MoisturePlus in 2007, the number of Acanthamoeba keratitis cases has not decreased to baseline levels identified prior to 2003-2004, indicating that additional risk factors introduced during this time are involved. Since the introduction of topical ophthalmic ciprofloxacin hydrochloride in the early 1990s, initial community-based therapy for presumed infectious keratitis had predominantly been BAK-containing fluoroquinolones until the introduction of moxifloxacin to the US market in 2003. Introduction to other countries also reporting Acanthamoeba outbreaks was staged—Singapore in November 2004, Japan in 2006, and later in Europe. Although the Acanthamoeba outbreaks temporally followed the introduction of commercial unpreserved moxifloxacin in the United States and Singapore, the early Acanthamoeba keratitis outbreak in Singapore was postulated secondary to an increase (from 24% to 46%) in the use of Complete MoisturePlus after the 2004 Fusarium outbreak associated with ReNu with MoistureLoc (Bausch & Lomb). This was reported, however, before the full extent of the persistence of the outbreak in the United States was well known after the recall of Complete MoisturePlus. This increased market share for Complete MoisturePlus after the recall of ReNu with MoistureLoc appeared more muted in the United States (5%-10.7%), making it unlikely that this simple increase in use was a significant factor despite the higher risk of Acanthamoeba keratitis when using it. The magnitude and rapidity of the increase as well as reports worldwide suggest other modifying risk factors.

The therapeutic role of BAK in topical antibiotics has been strongly debated. The loosening of corneal epithelial junctions by BAK has been shown to improve the efficacy of pilocarpine hydrochloride, but its effect on 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 radioisotope-labeled 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

Figure 5. Pooled results for the log kill results of 3 strains of Acanthamoeba (Acanthamoeba castellanii, Acanthamoeba hatchetti, and Acanthamoeba polyphaga) when exposed to moxifloxacin alone, moxifloxacin + benzalkonium chloride (BAK), 0.001%, and moxifloxacin + BAK, 0.003%. Each time point represents 3 different strains each performed in triplicate (n=9). Error bars indicate standard deviation.
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 antibiotics 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 antibiotics 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 anticanthamoebal activity and the anticanthamoebal 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 anticanthamoebal 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 anticanthamoebal 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-

### Table. Pairwise Comparisons of Log Kill Rates for Moxifloxacin, 0.5%, Moxifloxacin, 0.5% + Benzalkonium Chloride, 0.001%, and Moxifloxacin, 0.5% + Benzalkonium Chloride, 0.003% at Each Time

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Moxifloxacin, 0.5%, Log Kill, Mean (SD)</th>
<th>Moxifloxacin, 0.5% + BAK, 0.001% Log Kill, Mean (SD)</th>
<th>Moxifloxacin, 0.5% + BAK, 0.003% Log Kill, Mean (SD)</th>
<th>Valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.01 (0.22)</td>
<td>0.62 (0.48)</td>
<td>2.72 (0.83)</td>
<td>.92</td>
</tr>
<tr>
<td>2.0</td>
<td>0.04 (0.30)</td>
<td>1.04 (0.82)</td>
<td>3.70 (0.53)</td>
<td>.23</td>
</tr>
<tr>
<td>3.5</td>
<td>−0.05 (0.19)</td>
<td>1.23 (1.06)</td>
<td>3.35 (1.05)</td>
<td>.03</td>
</tr>
<tr>
<td>5.0</td>
<td>−0.15 (0.31)</td>
<td>1.22 (0.97)</td>
<td>3.37 (1.00)</td>
<td>.01</td>
</tr>
<tr>
<td>6.5</td>
<td>0.01 (0.19)</td>
<td>1.03 (1.02)</td>
<td>3.40 (0.97)</td>
<td>.21</td>
</tr>
</tbody>
</table>

Abbreviation: BAK, benzalkonium chloride.

*For both moxifloxacin, 0.5% + BAK, 0.001%, and moxifloxacin, 0.5% + BAK, 0.003%, the comparison was with moxifloxacin, 0.5%, alone. P < .05 indicates statistical significance.*
respresentative 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.

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