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Comparative Toxicity of Preservatives on Immortalized Corneal and Conjunctival Epithelial Cells

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Abstract

Purpose: Nearly all eye drops contain preservatives to decrease contamination. Nonpreservatives such as disodium-ethylene diamine tetra-acetate (EDTA) and phosphate-buffered saline are also regularly added as buffering agents. These components can add to the toxicity of eye drops and cause ocular surface disease. To evaluate the potential toxicity of these common components and their comparative effects on the ocular surface, a tissue culture model utilizing immortalized corneal and conjunctival epithelial cells was utilized.

Methods: Immortalized human conjunctival and corneal epithelial cells were grown. At confluency, medium was replaced with 100 µL of varying concentrations of preservatives: benzalkonium chloride (BAK), methyl paraben (MP), sodium perborate (SP), chlorobutanol (Cbl), and stabilized thimerosal (Thi); varying concentrations of buffer: EDTA; media (viable control); and formalin (dead control). After 1 h, solutions were replaced with 150 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazonium bromide). After 4 h, solutions decanted, 100 µL of acid isopropanol added, and the optical density determined at 572 nm to evaluate cell viability.

Results: Conjunctival and corneal cell toxicity was seen with all preservatives. Depending upon concentration, BAK exhibited from 56% to 89% toxicity. In comparison, Cbl exhibited from 50% to 86%, MP from 30% to 76%, SP from 23% to 59%, and Thi from 70% to 95%. EDTA with minimal toxicity (from 6% to 59%) was indistinguishable from SP.

Conclusions: Generally, the order of decreasing toxicity at the most commonly used concentrations: Thi (0.0025%) > BAK (0.025%) > Cbl (0.25%) > MP (0.01%) > SP (0.0025%) ≈ EDTA (0.01%). Even at low concentration, these agents will cause some degree of ocular tissue damage.

Introduction

Most eye drops contain preservatives to provide a level of antimicrobial activity in the bottle, limiting secondary bacterial, mycotic, and amoebal ocular infections caused by contaminated solutions and prolong the half-life of the drug by preventing biodegradation and maintaining drug potency.[1] Preservatives can be classified into four main categories: detergents, oxidants, chelating agents, and metabolic inhibitors (pentavalent antimonials [Sb^V], quaternary ammoniums, and organomercurials).[2,3] Examples of such preservatives include: benzalkonium chloride (BAK; detergent), chlorobutanol (Cbl; detergent), methyl paraben (MP; chelating agent), sodium perborate (SP; oxidative agent), and stabilized thimerosal (Thi; organomercurial); although by far, the most common of the topical ophthalmic medication preservatives is BAK, typically used in concentrations varying from 0.015% to 0.05%. Disodium-ethylene diamine tetra-acetate (EDTA) and phosphate-buffered saline, while not preservatives, are added to most ophthalmic formulations as buffering agents. While stabilizing agents such as buffers are generally thought of as nontoxic, the potential for toxicity still exists. In fact any chemical added to eye drops, such as the preservative and buffering agents just mentioned, have the potential to harm the eye.[1] Toxicity from pharmaceutical agents can result in decreased visual acuity and/or patient comfort that can lead to decreased compliance.

Benzalkonium chloride (BAK) stabilizes drugs in solution and prevents spoilage by microbial growth; but it can also initiate ocular surface damage and subconjunctival inflammation.[1,4] BAK is a detergent preservative that can affect cell membrane permeability, interrupt the metabolic processes of the cell, cause lysis of cell contents, and allow

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vital substances to escape, eventually causing death of the microorganism.[5] As a cationic surfactant, BAK reduces surface tension at interfaces and attracts negatively charged surfaces, such as those of microorganisms. Cationic surfactants have been shown to have the ability to lyse cytoplasmic membranes and denature intracellular proteins.[5]

Chlorobutanol (Cbl) has a broad spectrum of antimicrobial action. Cbl, a detergent preservative, works by disorganizing the lipid structure of the cell membrane, which increases permeability of the cell, thus leading to cell lysis.[1] Cbl causes cell retraction and cessation of normal cytokinesis, cell movement, and mitotic activity. It disrupts the barrier and transport properties of the corneal epithelium as well as inhibits the utilization of oxygen by the cornea.

Methyl paraben (MP) is one of a homologous series of parabens used to exert the intended antimicrobial affect. Parabens are particularly useful against molds and yeasts. Nonetheless, MP can cause irritation to the eye if used at concentrations effective against microorganisms.[6]

Sodium perborate (SP) has cidal activity against bacteria, fungi, and viruses.[5] However, in solution, it can generate chloride dioxide free radicals that oxidize unsaturated lipids and glutathione in the cell, and hydrogen peroxide, which can disrupt protein synthesis, cellular functioning, and produce cell death.[1,4,5] SP is an oxidative preservative that kills by penetrating infectious organisms' cell membrane and then modifying lipids, proteins, and nucleic acids.[5]

Thimerosal (Thi) is a mercury-containing organic compound that helps prevent potentially life-threatening contamination with harmful microbes and is able to prevent the growth of fungi.[7,8] Nonetheless, a particularly noxious preservative, Thi has been eliminated from most ocular medications because of the severe damage it can cause to the nervous system.[8,9] In fact, with only minimal concentration and exposure time, it has been demonstrated to cause cell retraction, and cessation of mitotic activity.[10,11]

Disodium-ethylene diamine tetra-acetate (EDTA) is commonly used in ophthalmic solutions as a buffering agent. Buffers are needed in most ophthalmic preparations because the eye is very sensitive to noxious chemicals and changes in pH. Buffers stabilize pharmaceutical formulations keeping the active agent contained in them in their active form as well as controlling the drastic changes in pH that would otherwise be necessitated/induced by the active agent. Na-EDTA has been reported to promote corneal drug penetration presumably as the consequence of ultrastructural changes induced in the corneal epithelium.[12,13]

The purpose of this study was to evaluate the potential toxicity of the common components of eyedrops such as the preservatives added to decrease contamination of multiuse bottles utilizing a tissue culture model of immortalized corneal and conjunctival epithelial cells.

Materials and Methods

Conjunctival epithelial cell line

Wong-Kilbourne-derived human conjunctival epithelial cells, an established cell line[14,15] (Wong-Kilbourne derivative of conjunctiva, clone 1-5c-4, American Type Culture Collection [ATCC, Manassas, VA] certified cell line [CCL], 20.2), were cultured under standard conditions (humidified atmosphere of 5% CO_2 at 37°C) in Medium 199 in Hank's balanced salt solution (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 1% L-glutamine (Sigma Aldrich, St. Louis, MO), and 1% penicillin-streptomycin (Sigma Aldrich). Cells from passages 5 through 20 were used for the experiments. Normal culture cell development was assessed daily by phase-contrast microscopy. Confluent cultures were dissociated with cell dissociation solution (Sigma Aldrich) for 23 min and subcultured in ratios from 1:5 to 1:10 in 75 cm^2 tissue culture flasks with media renewal every 2 days. They were then seeded into 96-well culture plates to the equal ratios used in the tissue culture flasks, kept at 37°C for 24 h. After approximately 75%–80% of confluency was attained, they were used for experimentation.

Corneal epithelial cell line

Immortalized human corneal epithelial cells (HCEs), an established cell line[16,17] (10.014 pRSV-T, American Type Culture Collection [ATCC Manassas, VA] certified cell line [CCL], 11515), were cultured under standard conditions (humidified atmosphere of 5% CO_2 at 37°C) in precoated 25 cm^2 tissue culture flasks. The precoating solution contains a mixture of 0.01 mg/mL bovine fibronectin (Sigma Aldrich, St. Louis, MO), 0.03 mg/mL Collagen I (Pure-Col, Palo Alto, CA), and 0.01 mg/mL bovine serum albumin (Sigma Aldrich) diluted in balanced salt solution. The growth medium used was keratinocyte serum-free medium (Invitrogen, Grand Island, NY) supplemented with 0.05 mg/mL bovine pituitary extract (Invitrogen, Grand Island, NY), 5 ng/mL epidermal growth factor (Invitrogen, Grand Island, NY), 0.005 mg/mL human insulin (Sigma Aldrich), and 500 ng/mL hydrocortisone (Sigma Aldrich). Cells from passages 10 through 16 were used for the experiments. Normal culture cell development was assessed daily by phase-contrast microscopy. Eighty percent (80%) preconfluent cultures were dissociated with 0.025% trypsin–EDTA (Sigma Aldrich) for 2.45 min, then centrifuged at 125*g* for 10 min and, after discarding supernatant and resuspended cells in fresh medium, seeded in a ratio of 1:2 with media renewal every 2 days. They were then seeded into 96-well culture plates at the equivalent dilution ratios used in the tissue culture flasks and incubated (37°C) for 24 h. After subconfluence was attained (~75%–80%), they were used for experimentation.

Testing solutions

All testing solutions were prepared hours prior to each experiment and then pre-equilibrated (37°C, 5% CO_2). These solutions contained various concentrations of the previously mentioned representatives of the categories of the ophthalmic preservatives and stabilizing/buffering agents, as well as viable (pure cell media) and dead (formalin) controls (see Table 1). The ratios were 100 µL of solution to be tested (equivalent to two drops) per 17 µL of growth media (equivalent to twice the normal volume of tear film [7–10 µL]).

Experimental procedure

Each cell line was divided into the individual groups listed in Table 1 (12 wells each): (1–9) BAK 0.10%–0.001%; (10–18) Cbl 1%–0.01%; (19–27) MP 0.1%–0.001%; (28–36) SP 0.10%–0.001%; (37–45) Thi 0.01%–0.0001%; (46–62) EDTA 1%–0.0001%; (63)

Table 1. Groups

Groups in corneal and conjunctival cell lines
1) Benzalkonium chloride (BAK) + media (14.5%)
2) Methyl paraben (MP) + media (14.5%)
3) Sodium perborate (SP) + media (14.5%)
4) Chlorobutanol (Cbl) + media (14.5%)
5) Stabilized thimerosal (Thi) + media (14.5%)
6) Disodium-ethylene diamine tetra-acetate (EDTA) + media (14.5%)
7) Media (100%: total viable control)
8) Formalin + media (14.5%: dead control)

Experimental testing solutions used in tissue culture with immortalized human corneal (HCE: 10.014 pRSV-T) and conjunctival (CCC: Wong–Kilbourne derivative of conjunctiva) epithelial cells.

appropriate medium (total viable control) 100%; and (64) formalin (10% paraformaldehyde: dead control).

When the cells reached 75%–80% of confluency in the 96-well plates, the medium was removed and 117 µL of the appropriate pre-equilibrated (37°C, 5% CO_2) testing solution was added to each well. After 1 h incubation at 37°C, the test solutions were removed and 150 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazonium bromide; the most widely used assay for the measurement of cell proliferation and health[18]) was added to each well and incubated at 37°C for 4 h.[19] Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding blue/purple formazan crystals that are insoluble in aqueous solutions.[19] Later, after decanting off the solution, 150 µL of MTT solubilization solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) was added to each well to dissolve the formazan crystals. The resulting blue/purple solution was spectrophotometrically measured at a wavelength of 572 nm (with 690 nm used as a background) utilizing a Quart Reader (BioTek Instruments, Inc., Winooski, VT) and the results displayed with Kineticalc for Windows version #2.6, rev #3 software (BioTek Instruments, Inc.).

Data analysis

Parametric statistics. Optical densities were averaged first between samples within an experiment, then between experiments. Utilizing the controls (those receiving growth media solution [total viable control] toxicity = 0%; those receiving 10% formalin-containing test solution [total dead control] toxicity = 100%), the individual groups were converted to toxicities as a percentage of the controls and the individual groups analyzed for statistical differences, utilizing the mean ± standard deviation for each of the various groups in computer-generated two-tailed bivariant Student's *t*-test[18,20] (GB-STAT, New England Software, Inc., College Station, TX; SAS, SAS Institute Inc., Cary, NC; and SPSS, SPSS Inc., Chicago, IL). The parametric data were also analyzed for statistical differences, utilizing the mean ± standard deviation for each of the various study groups utilizing computer-generated contingency tables with the Monte Carlo randomization test (SPSS)[18,20] as well as computer-generated two-tailed bivariant Student's *t*-test (SPSS),[18,20] individual Fisher exact tests (SPSS),[18,20] overall chi-square analysis (SPSS),[18,20] Bonferroni Post-Hoc Comparisons (SPSS),[18,20] correlation coefficients,[18,20] and an One-Way Analysis of Variance (One-Way ANOVA: SPSS).[18,20] Normality and group independence/equivalency were confirmed with Shapiro-Wilk W (SPSS)[18,20] and Skewness/Kurtosis (SPSS)[18,20] tests for normality. Two-tailed significance was established at a confidence level of $0.05 > P > 0.95$.

Results

The optical densities of a total of 360 wells were measured for each study group in each of the two lines (2,880 total wells or fifteen 96-well plates for each line). As expected, the wells containing the media solution achieved the highest absorbance values in both lines, followed by the saline solution.

All the experimental compounds tested behaved in dose-dependent manner and proved more toxic than the total viable control (medium alone) and less toxic than the dead control (formalin [8.5%]; see Table 2 and Figure 1). In addition, all were statistically significant from the total viable control at their most commonly used concentrations. Of the tested compounds, in general, the nonpreservative EDTA exhibited the least toxicity (a maximum of ~60% at 1% EDTA [HCE: 57.86% ± 37.32%; CCC: 60.07% ± 25.27%]; ~28% at the most commonly used concentration of 0.01% [HCE: 24.74% ± 35.72%; CCC: 31.05% ± 23.11%]; see Table 2 and Figure 1), with all of the preservatives exhibiting at least a 10fold higher level of toxicity.

Of the preservatives tested, not surprisingly, thimerosal (Thi), presently out of favor in the ophthalmic pharmaceuticals although currently most commonly used at 0.0025%, was the most toxic of the preservatives tested (exhibiting a toxicity of ~95% at a concentration of 0.01% [HCE: 96.49% ± 21.59%; CCC: 92.62% ± 20.08%]; ~88% at the most commonly used concentration of 0.0025% [HCE: 91.17% ± 29.13%; CCC: 84.61% ± 17.90%]; see Table 2 and Figure 1). Much more of a surprise was the fact that BAK, by far the most common of the topical ophthalmic medication preservatives and typically used in concentrations varying from 0.015% to 0.05%, was actually the second most toxic of the ophthalmic preservatives tested, exhibiting a toxicity of ~80% at the most commonly used concentration of 0.025% ([HCE: 83.36% ± 32.29%; CCC: 77.08% ± 21.40%]; ~89% at a concentration of 0.1% [HCE: 91.98% ± 25.70%; CCC: 86.71% ± 22.38%]; see Table 2 and Figure 1).

Significantly less toxic than these were Cbl, exhibiting a toxicity of "only" as high as ~86% at a concentration of 1% ([HCE: 87.48% ± 33.22%; CCC: 83.65% ± 23.21%]; ~73% at the most commonly used concentration of 0.25% [HCE: 75.94% ± 34.56%; CCC: 70.35% ± 25.75%]; see Table 2 and Figure 1), and MP, exhibiting a toxicity of as high as ~76% at a concentration of 0.1% ([HCE: 77.30% ± 33.89%; CCC: 73.92% ± 26.24%]; ~55% at the most commonly used concentration of 0.01% [HCE: 57.09% ± 32.24%; CCC: 53.11% ± 24.83%]; see Table 2 and Figure 1).

The preservative SP was significantly the least toxic (a maximum of ~60% at 0.1% [HCE: 57.72% ± 32.65%; CCC: 60.18% ± 28.04%]; but "only" ~27% at the most commonly used concentration of 0.0025% [HCE: 21.77% ± 33.87%; CCC: 31.40% ± 26.60%]; see Table 2 and Figure 1).

Discussion

Our results showed that even at low concentrations all the preservatives and buffering agents tested cause some

Table 2. Toxicity

		HCE	*CCC*
		Average ± SD (σ)	*Average ± SD (σ)*
Controls			
Serum/medium (total viable control)		0.00% ± 28.32%	0.00% ± 21.14%
Formalin (dead control)	10%	100.00% ± 8.05%	100.00% ± 12.95%
Buffers			
Na-EDTA (buffer, nonpreservative)	1.00000%	57.86% ± 37.32%	60.07% ± 25.27%
Na-EDTA (buffer, nonpreservative)	0.75000%	55.87% ± 33.54%	55.47% ± 29.83%
Na-EDTA (buffer, nonpreservative)	0.50000%	53.40% ± 37.08%	50.62% ± 24.65%
Na-EDTA (buffer, nonpreservative)	0.25000%	47.98% ± 35.89%	47.40% ± 23.54%
Na-EDTA (buffer, nonpreservative)	0.10000%	42.96% ± 33.93%	41.70% ± 25.09%
Na-EDTA (buffer, nonpreservative)	0.07500%	39.74% ± 36.57%	37.36% ± 27.76%
Na-EDTA (buffer, nonpreservative)	0.05000%	37.33% ± 35.23%	34.79% ± 25.30%
Na-EDTA (buffer, nonpreservative)	0.02500%	30.96% ± 35.93%	33.04% ± 26.23%
Na-EDTA (buffer, nonpreservative)	0.01000%	24.74% ± 35.72%	31.05% ± 23.11%
Na-EDTA (buffer, nonpreservative)	0.00750%	21.95% ± 37.71%	29.83% ± 22.67%
Na-EDTA (buffer, nonpreservative)	0.00500%	19.88% ± 36.38%	26.87% ± 27.58%
Na-EDTA (buffer, nonpreservative)	0.00250%	18.25% ± 36.83%	24.60% ± 25.81%
Na-EDTA (buffer, nonpreservative)	0.00100%	16.02% ± 35.91%	22.57% ± 26.09%
Na-EDTA (buffer, nonpreservative)	0.00075%	14.21% ± 33.67%	19.27% ± 26.17%
Na-EDTA (buffer, nonpreservative)	0.00050%	12.39% ± 37.37%	16.01% ± 24.00%
Na-EDTA (buffer, nonpreservative)	0.00025%	9.87% ± 36.78%	10.09% ± 26.07%
Na-EDTA (buffer, nonpreservative)	0.00010%	5.03% ± 39.53%	6.03% ± 24.74%
Preservatives			
Sodium perborate (SP)	0.1000%	57.72% ± 32.65%	60.18% ± 28.04%
Sodium perborate (SP)	0.0750%	53.92% ± 37.49%	54.98% ± 28.70%
Sodium perborate (SP)	0.0500%	45.24% ± 34.88%	52.42% ± 22.45%
Sodium perborate (SP)	0.0250%	43.24% ± 38.85%	48.87% ± 26.46%
Sodium perborate (SP)	0.0100%	38.04% ± 32.90%	44.81% ± 23.77%
Sodium perborate (SP)	0.0075%	32.47% ± 34.88%	39.02% ± 21.95%
Sodium perborate (SP)	0.0050%	27.12% ± 37.56%	37.28% ± 25.21%
Sodium perborate (SP)	0.0025%	21.77% ± 33.87%	31.40% ± 26.60%
Sodium perborate (SP)	0.0010%	19.75% ± 32.91%	27.00% ± 32.52%
Methyl paraben (MP)	0.1000%	77.30% ± 33.89%	73.92% ± 26.24%
Methyl paraben (MP)	0.0750%	71.95% ± 34.74%	71.52% ± 26.58%
Methyl paraben (MP)	0.0500%	67.05% ± 35.52%	68.44% ± 25.67%
Methyl paraben (MP)	0.0250%	60.68% ± 32.93%	64.61% ± 25.91%
Methyl paraben (MP)	0.0100%	57.09% ± 33.24%	53.11% ± 24.83%
Methyl paraben (MP)	0.0075%	53.10% ± 33.81%	35.52% ± 24.64%
Methyl paraben (MP)	0.0050%	45.76% ± 33.88%	30.02% ± 28.87%
Methyl paraben (MP)	0.0025%	43.53% ± 33.78%	27.88% ± 25.51%
Methyl paraben (MP)	0.0010%	36.41% ± 33.95%	24.48% ± 23.24%
Chlorobutanol (Cbl)	1.000%	87.48% ± 33.22%	83.65% ± 23.21%
Chlorobutanol (Cbl)	0.750%	83.84% ± 33.01%	81.44% ± 21.58%
Chlorobutanol (Cbl)	0.500%	81.84% ± 36.83%	75.98% ± 24.27%
Chlorobutanol (Cbl)	0.250%	75.94% ± 34.56%	70.35% ± 25.75%
Chlorobutanol (Cbl)	0.100%	72.94% ± 33.58%	67.58% ± 24.20%
Chlorobutanol (Cbl)	0.075%	66.40% ± 35.63%	59.43% ± 29.66%
Chlorobutanol (Cbl)	0.050%	62.61% ± 36.26%	56.70% ± 29.05%
Chlorobutanol (Cbl)	0.025%	55.95% ± 35.67%	50.83% ± 25.27%
Chlorobutanol (Cbl)	0.010%	52.46% ± 35.71%	46.77% ± 27.39%
Benzalkonium chloride (BAK)	0.1000%	91.98% ± 25.70%	86.71% ± 22.38%
Benzalkonium chloride (BAK)	0.0750%	87.93% ± 28.02%	84.02% ± 21.85%
Benzalkonium chloride (BAK)	0.0500%	85.09% ± 33.27%	79.26% ± 22.93%
Benzalkonium chloride (BAK)	0.0250%	83.36% ± 32.29%	77.08% ± 21.40%
Benzalkonium chloride (BAK)	0.0100%	78.32% ± 32.67%	74.24% ± 24.16%
Benzalkonium chloride (BAK)	0.0075%	74.70% ± 34.27%	71.28% ± 23.50%
Benzalkonium chloride (BAK)	0.0050%	68.56% ± 29.46%	64.17% ± 24.46%
Benzalkonium chloride (BAK)	0.0025%	62.95% ± 30.37%	59.71% ± 25.77%

(*Continued*)

Table 2. *Continued*

		HCE	*CCC*
		Average ± SD (σ)	*Average ± SD (σ)*
Preservatives			
Benzalkonium chloride (BAK)	0.0010%	58.12% ± 31.56%	54.02% ± 25.99%
Thimerosal (Thi)	0.01000%	96.49% ± 21.59%	92.62% ± 20.08%
Thimerosal (Thi)	0.00750%	95.47% ± 20.18%	90.51% ± 18.64%
Thimerosal (Thi)	0.00500%	93.06% ± 23.87%	86.95% ± 18.60%
Thimerosal (Thi)	0.00250%	91.17% ± 29.13%	84.61% ± 17.90%
Thimerosal (Thi)	0.00100%	88.38% ± 30.09%	80.96% ± 21.78%
Thimerosal (Thi)	0.00075%	82.40% ± 32.65%	77.63% ± 21.86%
Thimerosal (Thi)	0.00050%	79.39% ± 27.49%	74.23% ± 20.34%
Thimerosal (Thi)	0.00025%	75.56% ± 29.30%	70.86% ± 22.75%
Thimerosal (Thi)	0.00010%	70.24% ± 33.92%	68.76% ± 22.85%

Relative toxicities of human corneal (HCE; 10.014 pRSV-T) and conjunctival epithelial cells (CCC; Wong–Kilbourne derivative of conjunctiva) as compared to controls after being put into contact with varying concentrations of each of the experimental testing solutions (media alone [total viable control], 10% formalin [dead control], benzalkonium chloride [BAK], methyl paraben [MP], sodium perborate [SP], chlorobutanol [Cbl], stabilized thimerosal [Thi], and EDTA) for 1 h.

degree of cell damage to ocular tissue as evaluated by corneal and conjunctival cells in tissue culture. With all agents, there was an increased toxicity with increasing concentration. The tested agents in order of decreasing toxicity at the concentrations most commonly used in ophthalmic preparations: thimerosal (Thi: 0.01%) > benzalkonium chloride (BAK: 0.01%) > chlorobutanol (Cbl: 0.5%) > methyl paraben (MP: 0.01%) > sodium perborate (SP: 0.02%) ≈ EDTA.

Recently, a variety of authors have raised the possibility of preservatives added to the formulations imparting toxic effects to the ocular surface.[1,3,7,9,21] Benzalkonium chloride (BAK) is the one most commonly mentioned, but toxicity

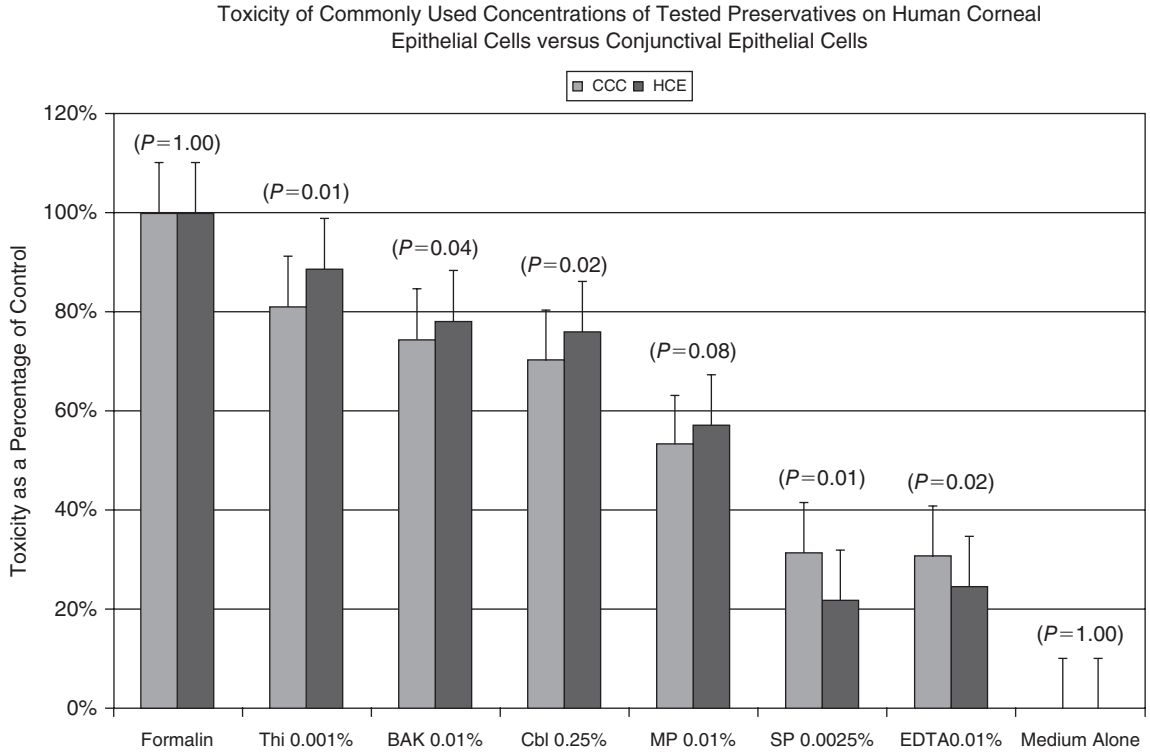

FIG. 1. Graphic representation of overall relative toxicities of human corneal (gray bars; 10.014 pRSV-T) and conjunctival epithelial cells (black; Wong–Kilbourne derivative of conjunctiva, clone 1-5c-4) after being put into contact with the most commonly used concentration of each of the experimental testing solutions (10% formalin [dead control], stabilized thimerosal [Thi: 0.0025%], benzalkonium chloride [BAK: 0.025%], chlorobutanol [Cbl: 0.25%], methyl paraben [MP: 0.01%], sodium perborate [SP: 0.0025%], disodium-ethylene diamine tetra-acetate [EDTA: 0.01%], and media alone [total viable control]) for 1 h.

secondary to other preservatives should be considered as well. Such toxicity of preservatives may cause ocular discomfort, changes in vision and may interfere with patient compliance with recommended dosages.[21] Examples of preservatives include: BAK, benzododecinium bromide (BDD), Cbl, MP, SP, stabilized oxychloro complex (SOC), and Thi.

For most multiuse aqueous ophthalmic, nasal, and otic products, BAK is the most commonly used preservative in topical ophthalmic medications and is typically used in concentrations varying from 0.015% to 0.05%, although the American College of Toxicology has concluded that BAK can be safely used as an antimicrobial agent at concentrations up to 0.1%. BAK comes from the quaternary ammoniums, which are detergent preservatives and cationic surfactants. The detergent properties of BAK have been shown to interfere with the integrity of the external lipid layer of the precorneal tear film, reduction of tear film breakup times, and exacerbation of dry eye symptoms.[1,4] In mammalian cells, which are unable to neutralize detergent preservatives,[5] the ocular surface cannot break down the preservative into basic components in which the eye can tolerate. BAK can accumulate in ocular tissue and remain there for extended periods of time, thus prolonging adverse reactions in the cornea.[1] Dose dependency can be seen in BAK because at low concentrations (0.0001%–0.01%), BAK may cause growth arrest or apoptotic mechanisms.[7,10,22] However, BAK at higher concentrations (0.05%–0.2%) may cause cell death by necrosis.[5,10,22]

Benzalkonium chloride has been extensively studied in nasal epithelium, as well. Of 18 toxicity studies of BAK on nasal epithelium identified, eight (all *in vivo* [seven human, one animal]) concluded that there were no toxic effects associated with BAK and 10 (human: four *in vivo*, three *in vitro*; animal: two *in vivo*, one *in vitro*) concluded that BAK was detrimental to nasal epithelium or exacerbated rhinitis medicamentosa at concentrations of BAK ranging from 0.1 mg/mL to 0.02%.[23] The studies that concluded there were no toxic effects associated with BAK studied BAK exposure to in concentrations ranging from 0.1% to 0.02%.[24] Overall, only two of the 10 studies that concluded BAK to be detrimental to nasal mucosa and/or exacerbated rhinitis medicamentosa via swelling of nasal tissues were supported by significantly different results from placebo or active controls.[24,25]

While BAK has been extensively studied in systemic pharmaceutical preparations, a relatively small number of authors have studied the relative toxicity of BAK applied topically in eyes. Labbé and colleagues studied BAK in an *in vivo* study (rats) and found that high doses of BAK were much more toxic than other preservatives at similar doses.[26] They found that BAK consistently and dramatically altered the corneoconjunctival surface as evaluated by slit-lamp examination, the fluorescein test, impression cytology, *in vivo* confocal microscopy, and histology.[26] Huhtala and colleagues evaluated the cytotoxicity of BAK utilizing an *in vitro* WST-1 test (cell viability assay).[27] They reported EC_{50} values in BAK-treated cells in the presence of serum ranged between 0.0650 and 0.0284 (WST-1), or an equivalent toxicity of 55%–85%.[27]

There are relatively few toxicity studies of other preservatives used in ophthalmic preparations. Benzododecinium bromide (BDD), like BAK, is from the family of preservatives, the quaternary ammoniums, which are detergent preservatives and cationic surfactants.[1] As a preservative, BDD can be used in gel-forming preparations that may prolong the contact of the preservative with the eye and in turn produce greater toxic effects to the cornea.[1,4,5]

Cbl at its most common concentration of 0.5% causes irritation in the eye, which is most likely due to cellular retraction and cessation of normal cytokinesis, cell movement, and mitotic activity.[1] Degeneration of HCEs, generation of conspicuous membranous blebs, cytoplasmic swelling, and occasional breaks in the external cell membrane have also been observed at 0.5%.[1] At a concentration of 0.1%, Cbl caused near depletion of the squamous layer.[1]

Stabilized oxychloro complex (SOC) has been found to have no *in vivo* or *in vitro* evidence of cytotoxicity.[1,4] It is effective at unusually low concentrations (0.005%),[1,4] which can be degraded into components normally found in tears, such as sodium ions, chloride ions, oxygen, and water.[9] Mammalian cells have oxidases, catalases, and antioxidants that readily neutralize the small amount of SOC generally utilized as a preservative.[8]

Sodium perborate (SP), like SOC, is readily neutralized by the oxidases, catalases, and antioxidants commonly found in mammalian cells.[1,5] Unlike SOC however, SP is readily converted into hydrogen peroxide, an efficient antimicrobial, in the presence of water.[1,4,5] However, hydrogen peroxide in even small amounts, such as 30 parts per million (0.003%), is known to be somewhat harmful to the eye.[1,5]

At clinical concentrations, thimerosal has been shown to directly cause cell death within 9 h, more slowly than BDD and BAK.[9] Within 5 h, thimerosal has been shown to cause severe cell damage in concentrations as low as 0.0005%.[9] It contains an extremely high concentration of mercury (49%).[8] Mercury has an extremely high penetration/absorption rate into the ocular tissues greatly potentiating the toxic effects.[9] In fact, thimerosal is so toxic; it is rarely used today in ocular preparations.

A special note however needs to taken, regarding the Wong–Kilbourne-derived human conjunctival epithelial cells utilized in these experiments. Recently, it has been reported that these cells have been contaminated with the HeLa cells utilized for the immortalization of the line.[28] The degree of "loss" of the epithelial nature of this line has not been determined and, as a result, the potential exists for their response to not completely mimic conjunctival epithelium. Nevertheless, Wong–Kilbourne-derived human conjunctival epithelial cells have long been used for ocular toxicity studies and numerous citations exist in the literature referencing their use for this purpose.[13–15,18]

There are relatively few comparative studies of the toxicity of preservatives used in ophthalmic preparations; nevertheless, despite the limited toxicity information available, our findings of the observed order of toxicity at the concentrations most usually used in ophthalmic preparations: thimerosal (Thi: 0.01%) > benzalkonium chloride (BAK: 0.01%) > chlorobutanol (Cbl: 0.5%) > methyl paraben (MP: 0.01%) > sodium perborate (SP: 0.02%) ≈ EDTA are consistent with what little is known. Our tissue culture model of toxicity looking at corneal and conjunctival epithelial cells demonstrates that use of preserved pharmaceutical agents can negatively affect the ocular surface. Even using a low concentration preservative for a long period of time may cause adverse reactions and high concentration of some preservatives can cause immediate damage and irritation to the ocular tissue.

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