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ORIGINAL ARTICLE

Evaluation of Biomarkers of Inflammation in Response to Benzalkonium Chloride on Corneal and Conjunctival Epithelial Cells

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Abstract

Purpose: Most eye drops contain preservatives; benzalkonium chloride (BAK) is most common. Recent data demonstrated BAK adding to toxicity. BAK is degraded into hydrogen peroxide (H_2O_2), which in even small amounts is known to be an irritant. Increased toxicity should cause localized inflammation with increased elaboration of inflammatory biomarkers. To evaluate the inflammation BAK causes to the ocular surface, enzyme linked immunosorbant assays (ELISAs) were utilized to quantify the levels of inflammatory biomarkers in response to BAK and/or H_2O_2.

Methods: Immortalized human conjunctival and corneal epithelial cells were exposed to: BAK (0.001%–0.1%), hydrogen peroxide (H_2O_2) (0.01%–0.1%), and cell media for 1 h. Cytokine quantification was performed via enzyme-linked immunosorbent assays [ELISAs]). Additional experimentation was performed in which testing solutions were replaced with media after 1 h and the resulting supernatants quantified after 24 h.

Results: BAK induced significant amounts of interleukin (IL-) 1 and tumor necrosis factor (TNF), but only moderate amounts of C-reactive protein (CRP), IL- 10 and 12, and H_2O_2. Lower concentrations of BAK induced proportionally less elaboration. Replacing the test solutions with media and providing 23 h for cytokine elaboration significantly increased TNF, but not IL-1. Lipopolysaccharide (LPS) positive controls induced substantial elaboration/release of both IL-1 and TNF as did in increasing the exposure to the full 24 h.

Conclusions: After 1 h of exposure, BAK increased quantities of all biomarkers. The biomarkers in decreasing order of induction/upregulation were: TNF ≥ IL-1 ≥ IL-12 ≥ IL-10 ≥ CRP. Even low concentrations caused some degree of inflammation. Replacing the testing solution with media and providing 23 h for cytokine elaboration, significantly increased the elaboration/release of TNF, but not IL-1, as compared to the 1-h BAK exposure. Whereas increasing the exposure to the full 24 h by not removing the testing solution at the 1-h time point significantly increased the elaboration/release of both IL-1 and TNF.

Introduction

Most eye drops contain preservatives that provide a level of antimicrobial activity in multiuse bottles, limiting secondary bacterial, mycotic, and amoebal ocular infections caused by contaminated solutions and prolong the shelf 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.[2,3] Metabolic inhibitors have been further subdivided into three subcategories: pentavalent antimonials (Sb^V), quartenary ammoniums, and organomercurials.[2,3] The most common preservative currently used in topical ophthalmic medications is BAK.[4] It is typically used in concentrations varying from 0.01% to 0.05%.[4] Recently, multiple authors have reported BAK as adding to the toxicity of eye drops and as potentially causing ocular surface disease.[1,2,5,6] Although it (BAK) stabilizes drugs in solution, and prevents spoilage by microbial growth; it also has the potential of initiating ocular surface damage and subconjunctival inflammation.[1,4] Although BAK is classified as a quaternary ammonium compound composed of a mixture of alkylbenzyl-dimethylammonium chloride homologues,[7] it exhibits many detergent-like actions. Among them, it can affect cell membrane permeability, interrupt the metabolic processes of the cell, cause lysis of cell contents, and allow vital substances to escape, eventually causing death of the

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microorganism.[8] BAK is also a cationic surfactant, reducing surface tension at interfaces. As such it also is attracted to negatively charged surfaces, including those of microorganisms having the ability to lyze cytoplasmic membranes and denature intracellular proteins.[8]

BAK, is known to be degraded into hydrogen peroxide (H_2O_2),[8] which, in even small amounts as low as 30 parts per million (0.003%), is known to be an ophthalmic irritant.[9,10]

C-reactive protein (CRP),[11] fibrinogen,[12] and multiple cytokines,[13,14] specifically interleukin (IL-)1α and β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-23, and tumor necrosis factor (TNF-)α, have long been described as conventional biomarkers of inflammation. Increased toxicity should cause a localized inflammatory response with concomitant increased elaboration of these inflammatory biomarkers. To evaluate the potential toxicity of BAK to the ocular surface and the secondary resultant inflammatory response, enzyme linked immunosorbant assays (ELISAs) were utilized to quantify the resultant levels of the above-mentioned biomarkers of inflammation in response to varying concentrations of BAK and/or H_2O_2 on a corneal and conjunctival epithelial cell models.

Methods

Conjunctival epithelial cell line

Wong-Kilbourne-derived conjunctival cells, an established cell line[15,16] [Wong-Kilbourne derivative of conjunctiva, clone 1-5c-4, American Type Culture Collection (ATCC, Manasas, VA)] were cultured under standard conditions (humidified atmosphere of 5% CO_2 at 37°C) in Medium 199 in Hank's balanced salt solution (Gibco, 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, St. Louis, MO). 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, St. Louis, MO) 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, an established cell line[17,18] [10.014 pRSV-T, American Type Culture Collection (ATCC Manasas, VA)] 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, St. Louis, MO) diluted in balanced salt solution. The growth medium used was keratinocyte serum-free medium (Gibco, Grand Island, NY) supplemented with 0.05 mg/mL bovine pituitary extract (Gibco, Grand Island, NY), 5 ng/mL epidermal growth factor (Gibco, Grand Island, NY), 0.005 mg/mL human insulin (Sigma Aldrich, St. Louis, MO), and 500 ng/mL hydrocortisone (Sigma Aldrich, St. Louis, MO). Cells from passages 12 through 19 were used for the experiments. Normal culture cell development was assessed daily by phase-contrast microscopy. Eighty percent preconfluent cultures were dissociated with 0.025% trypsin-EDTA (Sigma Aldrich, St. Louis, MO) for 2.45 min, then centrifuged 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 (approximately 75%–80%) they were used for experimentation.

Testing solutions

All testing solutions were prepared hours prior to each experiment and then pre-equilibrated to (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 the pure cell media control (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

Initial experimentation. Each cell line was divided into the individual groups listed in Table 1/Initial experimentation (12 wells each): (1–9) BAK 0.10%–0.001%; (10–20) H_2O_2: 0.10%–0.01%; (21) appropriate medium. Ten standards of varying known concentrations for each of the enzyme-linked immunosorbent assays (ELISAs) were also run.

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 (see Table 1/Initial experimentation) was added to each well. After a 1-h incubation at 37°C, the testing solutions were removed for CRP (American Diagnostica, Inc., Stamford, CT), IL-1α/IL-β (Becton, Dickinson and Company Biosciences, San Jose, CA), IL-10 (Becton, Dickinson and Company Biosciences), IL12 (Becton, Dickinson and Company Biosciences), TNFα (Becton, Dickinson and Company Biosciences), and H_2O_2 (Ozone Services, Yanco Ltd, Burton, B.C., Canada) quantification via ELISA. After following the staining procedure as per the manufacturers' instructions, the resulting solutions were spectrophotometrically measured at a wavelength of 450 nm (with 570 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 (Bio Tek Instruments, Inc.). CRP and cytokine ELISAs give results in pg/mL to four significant figures (three decimal places). H_2O_2 ELISA gives results in mg/mL to 2 significant figures (one decimal place).

Follow-up experimentation. As a 1-h incubation is sufficient for cytokine release but insufficient for cytokine elaboration, additional experiments were run in which each cell line was divided into the individual groups listed in Table 1/Follow-up (three wells each): (1) BAK: 0.1%; (2) BAK: 0.02%; (3) BAK: 0.002%; (4) lypopolysaccharide (LPS: 100 ng/mL); (5) lypopolysaccharide (LPS: 10 ng/mL); and (6) appropriate

Table 1. Groups: Initial and Follow-Up Experimentation

Groups in corneal and conjunctival cell lines	
Initial experimentation	*Follow-up experimentation*
1. Benzalkonium chloride (0.100%–0.001) + media (14.5%); 2. Hydrogen peroxide (0.100%–0.010) + media (14.5%); 3. Media (100%: total viable control).	1. Benzalkonium chloride (0.1%) + media (14.5%); 2. Benzalkonium chloride (0.02%) + media (14.5%); 3. Benzalkonium chloride (0.002%) + media (14.5%); 4. Lypopolysaccharide (100 ng/mL) + media (14.5%); 5. Lypopolysaccharide (10 ng/mL) + media (14.5%); 6. Media (100%: total viable 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 in the initial and follow-up phases of the experimentation.

cell medium. As in the initial experimentation, 10 standards of varying known concentrations for each of the enzyme-linked immunosorbent assays (ELISAs) were also run. In these experiments, after the cells reached 75%–80% of confluency in the 96-well plates, the medium was removed and the 117 µL of the appropriate pre-equilibrated (37°C, 5% CO_2) testing solutions (Table 1/Follow-up) were incubated for 1 h, the testing solutions were discarded and replaced with the appropriate cell media, which was then allowed to incubate for an additional 23 h. The resulting cell supernatants after the 23-h incubation were then removed for IL-1 and TNFα (cytokines most markedly increased following 1-h incubation) quantification via enzyme-linked immunosorbent assays [ELISAs; CRP (American Diagnostica, Inc., Stamford, CT), IL-1α/IL-β (Becton, Dickinson and Company Biosciences, San Jose, CA), IL-10 (Becton, Dickinson and Company Biosciences), IL-12 (Becton, Dickinson and Company Biosciences), TNFα (Becton, Dickinson and Company Biosciences), and H_2O_2 (Ozone Services, Yanco Ltd, Burton, B.C., Canada)] and subsequent spectrophotometrical measurement at 450 nm (and 570 nm for background), as described above. Each cell line was divided into the individual groups listed in Table 1/Initial experimentation (12 wells each): (1–9) BAK 0.10%–0.001%; (10–20) H_2O_2: 0.10%–0.01%; (21) appropriate medium. Ten standards of varying known concentrations for each of the enzyme-linked immunosorbent assays (ELISAs) were also run.

Data analysis

Parametric statistics. Utilizing the standards, the optical density to CRP/cytokine concentration conversions were calculated for each ELISA via linear regression of power (log-log) plots. The concentrations of CRP (pg/mL), IL-1α/IL-β (pg/mL), IL-10 (pg/mL), IL12 (pg/mL), TNFα (pg/mL), and H_2O_2 (mg/mL) of each test solution were then calculated. The resulting concentrations were averaged first between samples within an experiment, then between experiments. The individual groups were analyzed for statistical differences, against the control (appropriate medium), utilizing the mean ± standard deviation for each of the various groups in computer-generated two-tailed bivariant Student's *t*-tests[19,20] (GB-STAT, New England Software, Inc., College Station, TX, USA; SAS, SAS Institute Inc., Cary, NC, USA; and SPSS, SPSS Inc., Chicago, IL, USA). The parametric data was 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)[19–21] as well as individual Fisher exact tests (SPSS),[19–21] overall chi-square analysis (SPSS),[19,20] Bonferroni Post-Hoc Comparisons (SPSS),[19–21] correlation coefficients,[19,20] and a One Way Analysis of Variance (One Way ANOVA: SPSS).[21,22] Normality and group independence/equivalency were confirmed with Shapiro-Wilk W (SPSS)[21,22] and Skewness/Kurtosis (SPSS)[21,22] 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 456 wells were measured for each study group in each of the two lines (5,472 total wells or 57 96-well plates). Media alone induced very small amounts of CRP (CCC: 0.256 ± 0.060 pg/mL; HCE: 0.275 ± 0.088 pg/mL), while levels of IL-1 (CCC: 0.006 ± 0.001 pg/mL; HCE: 0.013 ± 0.0007 pg/ml), IL-10 (CCC: 0.000 ± 0.000 pg/mL; HCE: 0.000 ± 0.000 pg/mL), IL-12 (CCC: 0.001 ± 0.001 pg/mL; HCE: 0.003 ± 0.001 pg/mL), TNF-α (CCC: 0.000 ± 0.000 pg/mL; HCE: 0.000 ± 0.001 pg/mL), and H_2O_2 (CCC: 0.00 ± 0.00 mg/mL; HCE: 0.00 ± 0.00 mg/mL) remained imperceptible in both corneal and conjunctival epithelial cells [see: Tables 2 and 3, Fig. 1 (BAK-induced cytokines in CCC) and Fig. 2 (BAK-induced cytokines in HCE), Fig. 3 (BAK-induced H_2O_2 in CCC and HCE)].

In both cell lines, BAK: 0.1% induced, in a dose-dependent fashion, markedly increased quantities of: CRP (50-fold increase: ≤13.147 ± 0.768 pg/mL), IL-1 (10^4-fold increase: ≤145.329 ± 5.687 pg/mL), and TNF-α (10^4-fold increase: ≤316.442 ± 12.304 pg/mL) over media (CRP: 0.256 ± 0.001 pg/mL; IL-1: 0.006 ± 0.001 pg/mL; TNF: 0.001 ± 0.001 pg/mL). Elaborations of IL-10 and 12 were only marginally increased (IL-10: ≤19.903 ± 0.413 pg/mL; IL-12: ≤16.286 ±

Table 2. Conjunctival Cells

	CRP (pg/mL)	*IL-1 (pg/mL)*	*IL-10 (pg/mL)*	*IL-12 (pg/mL)*	*TNF-α (pg/mL)*	*H_2O_2 (mg/mL)*
	Average ± σ	*Average ± σ*	*Average ± σ*	*Average ± σ*	*Average ± σ*	*Average ± σ*
Media	0.26 ± 0.06	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
BAK 0.1000%	13.15 ± 0.77	145.33 ± 5.69	19.90 ± 0.41	16.29 ± 0.71	316.44 ± 12.30	6.5 ± 1.64
BAK 0.0750%	10.16 ± 0.72	70.63 ± 3.27	3.14 ± 0.17	11.78 ± 0.54	123.23 ± 3.73	5 ± 0.00
BAK 0.0500%	9.31 ± 0.52	29.90 ± 1.90	2.28 ± 0.26	8.25 ± 0.43	32.19 ± 3.06	3 ± 0.00
BAK 0.0250%	7.65 ± 0.62	8.27 ± 0.61	1.05 ± 0.11	4.87 ± 0.30	6.80 ± 0.36	3 ± 0.00
BAK 0.0200%	6.32 ± 1.32	6.13 ± 0.79	0.94 ± 0.10	3.95 ± 0.31	3.93 ± 0.14	3 ± 0.00
BAK 0.0150%	7.11 ± 0.38	3.62 ± 0.26	0.70 ± 0.10	3.16 ± 0.21	2.07 ± 0.15	1 ± 0.00
BAK 0.0100%	6.83 ± 0.32	2.59 ± 0.33	0.60 ± 0.11	2.49 ± 0.17	1.15 ± 0.09	1 ± 0.00
BAK 0.0075%	4.98 ± 0.31	0.70 ± 0.03	0.21 ± 0.03	1.22 ± 0.22	0.12 ± 0.01	0.5 ± 0.55
BAK 0.0050%	3.49 ± 0.21	0.09 ± 0.01	0.07 ± 0.02	0.44 ± 0.07	0.01 ± 0.00	0.05 ± 0.55
BAK 0.0025%	2.26 ± 0.11	0.04 ± 0.00	0.01 ± 0.00	0.29 ± 0.05	0.00 ± 0.00	0 ± 0.00
BAK 0.0010%	1.74 ± 0.12	0.02 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0 ± 0.00
H_2O_2 0.1000%	3.08 ± 0.14	9.28 ± 0.89	7.07 ± 0.39	4.59 ± 0.26	1.50 ± 0.07	80 ± 0.00
H_2O_2 0.0750%	1.12 ± 0.08	5.84 ± 0.51	3.01 ± 0.18	2.53 ± 0.21	0.30 ± 0.03	70 ± 5.16
H_2O_2 0.0500%	1.01 ± 0.07	2.65 ± 0.36	0.67 ± 0.12	0.72 ± 0.12	0.03 ± 0.01	50 ± 5.16
H_2O_2 0.0250%	0.94 ± 0.04	0.68 ± 0.08	0.12 ± 0.01	0.40 ± 0.05	0.00 ± 0.00	15 ± 5.89
H_2O_2 0.0100%	0.79 ± 0.03	0.26 ± 0.03	0.00 ± 0.00	0.16 ± 0.03	0.00 ± 0.00	5 ± 0.00

Concentrations of inflammatory biomarkers [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by conjunctival epithelial cells (CCC: Wong-Kilbourne derivative of conjunctiva) after being put in contact with varying concentrations of each of the initial experimental testing solutions [media alone, benzalkonium chloride (BAK: 0.001%–0.1%), and hydrogen peroxide (H_2O_2: 0.01%–0.1%) for 1 h.

0.712 pg/mL) from that of media (IL-10: ≤0.001 ± 0.001 pg/mL; IL-12: ≤0.001 ± 0.001 pg/mL; see: Tables 2 and 3, Figs. 1 and 2). Production of H_2O_2 was induced by BAK (≤6.5 ± 0.01 mg/mL) as compared to media alone (0.01 ± 0.01 mg/mL; see: Tables 2 and 3, Fig. 3). Lower concentrations of BAK induced the elaboration of proportionally lower amounts of all studied biomarkers in a dose-dependent fashion (see: Tables 2 and 3, Fig. 1 (BAK-induced cytokines in CCC) and Fig. 2 (BAK-induced cytokines in HCE), Fig. 3 (BAK-induced H_2O_2 in CCC and HCE)].

The addition of H_2O_2 (0.1%) induced increased quantities of all cytokines studied in both conjunctival (CRP:

Table 3. Human Corneal Epithelial Cells

	CRP (pg/mL)	*IL-1 (pg/mL)*	*IL-10 (pg/mL)*	*IL-12 (pg/mL)*	*TNF-α (pg/mL)*	*H_2O_2 (mg/mL)*
	Average ± σ	*Average ± σ*	*Average ± σ*	*Average ± σ*	*Average ± σ*	*Median ± "σ"*
Media	0.28 ± 0.09	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0 ± 0.00
BAK 0.1000%	11.84 ± 0.62	133.73 ± 6.49	17.95 ± 1.36	38.02 ± 1.19	272.79 ± 18.82	10 ± 0.00
BAK 0.0750%	9.28 ± 0.45	62.79 ± 4.76	3.05 ± 0.18	28.59 ± 0.94	77.75 ± 1.92	8 ± 1.55
BAK 0.0500%	7.45 ± 0.42	27.37 ± 0.91	0.63 ± 0.13	19.12 ± 0.60	33.33 ± 1.62	5 ± 0.00
BAK 0.0250%	5.94 ± 0.37	5.68 ± 0.41	0.92 ± 0.07	11.47 ± 0.33	9.21 ± 1.50	5 ± 0.00
BAK 0.0200%	5.49 ± 0.27	5.37 ± 0.20	0.81 ± 0.10	9.25 ± 0.29	5.18 ± 0.69	5 ± 0.00
BAK 0.0150%	5.09 ± 0.18	4.48 ± 0.13	0.69 ± 0.05	7.40 ± 0.30	2.83 ± 0.28	3 ± 0.41
BAK 0.0100%	4.73 ± 0.34	3.31 ± 0.17	0.63 ± 0.04	5.78 ± 0.25	1.52 ± 0.11	3 ± 0.00
BAK 0.0075%	3.15 ± 0.21	0.58 ± 0.03	0.19 ± 0.02	3.20 ± 0.13	0.19 ± 0.05	2 ± 0.89
BAK 0.0050%	2.33 ± 0.24	0.08 ± 0.01	0.10 ± 0.01	1.23 ± 0.07	0.01 ± 0.00	1 ± 0.00
BAK 0.0025%	1.63 ± 0.12	0.04 ± 0.01	0.01 ± 0.00	0.54 ± 0.00	0.00 ± 0.00	0 ± 0.00
BAK 0.0010%	1.02 ± 0.12	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0 ± 0.00
H_2O_2 0.1000%	2.48 ± 0.90	10.61 ± 0.29	6.03 ± 0.39	13.01 ± 0.54	4.11 ± 0.15	90 ± 0.00
H_2O_2 0.0750%	0.92 ± 0.08	6.65 ± 0.29	2.67 ± 0.13	8.29 ± 0.25	0.92 ± 0.17	70 ± 0.00
H_2O_2 0.0500%	0.82 ± 0.06	2.45 ± 0.26	0.64 ± 0.07	2.18 ± 0.11	0.15 ± 0.03	50 ± 5.16
H_2O_2 0.0250%	0.78 ± 0.06	0.65 ± 0.04	0.10 ± 0.01	0.87 ± 0.06	0.00 ± 0.00	25 ± 2.58
H_2O_2 0.0100%	0.72 ± 0.12	0.29 ± 0.03	0.00 ± 0.00	0.28 ± 0.04	0.00 ± 0.00	7.5 ± 2.74

Concentrations of inflammatory biomarkers [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by human corneal epithelial cells (HCE; 10.014 pRSV-T) after being put in contact with varying concentrations of each of the initial experimental testing solutions [media alone, benzalkonium chloride (BAK: 0.001%–0.1%), and hydrogen peroxide (H_2O_2: 0.01%–0.1%) for 1 h].

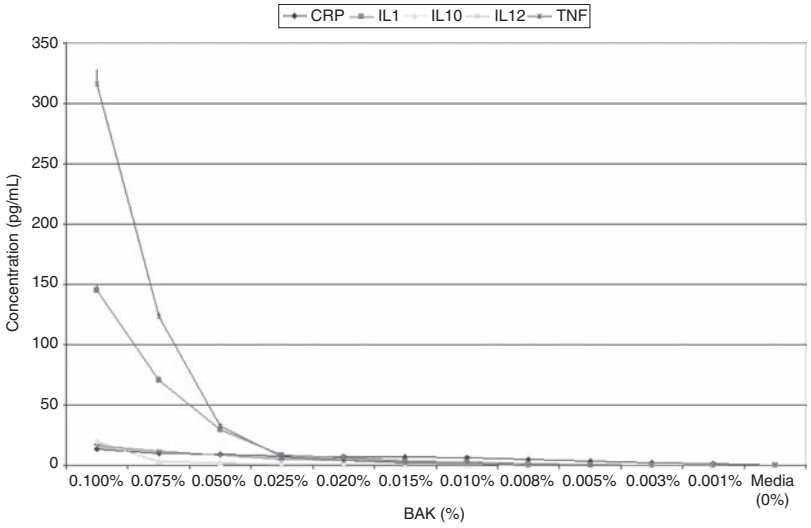

FIG. 1. Graphic representation of concentrations of the various inflammatory cytokines studied [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by conjunctival cells (Wong-Kilbourne derivative of conjunctiva, clone 1–5c) after being put into contact for 1 h with varying concentrations of benzalkonium chloride (BAK) as compared to media alone. All depicted values are statistically significant as compared to controls.

≤3.084 ± 0.140 pg/mL; IL-1: ≤9.261 ± 0.889 pg/mL; IL-10: ≤7.071 ± 0.389 pg/mL; IL-12: ≤4.590 ± 0.262 pg/mL; TNFα: ≤1.504 ± 0.070 pg/mL) and corneal (CRP: ≤2.481 ± 0.897 pg/mL; IL-1: ≤10.608 ± 0.287 pg/mL; IL-10: ≤6.033 ± 0.389 pg/mL; IL-12: ≤13.014 ± 0.535 pg/mL; TNFα: ≤4.112 ± 0.150 pg/mL) cells over media (CCC: CRP: 0.256 ± 0.001 pg/mL; IL-1: 0.006 ± 0.001 pg/mL; IL-10: ≤0.001 ± 0.001 pg/mL; IL-12: ≤0.001 ± 0.001 pg/mL; TNF: 0.001 ± 0.001 pg/mL; HCE: CRP: 0.275 ± 0.088 pg/mL; IL-1: 0.013 ± 0.007 pg/mL; IL-10: ≤0.001 ± 0.001 pg/mL; IL-12: ≤0.003 ± 0.001 pg/mL; TNF: 0.000 ± 0.001 pg/mL; see: Tables 2 and 3, Figs. 4 and 5). Lower concentrations of H_2O_2 induced the elaboration of proportionally lower amounts of all studied biomarkers in a dose-dependent fashion (see: Tables 2 and 3, Figs. 4 and 5).

Replacing the testing solution with media and providing 23 h for cytokine elaboration, significantly increased the elaboration/release of TNF (12.456 ± 1.701 ug/mL), but not IL-1 (267.639 ± 71.502 pg/mL) as compared to the 1-h BAK exposure (see: Table 4, Figs. 6 and 7). Of course, both IL-1 and TNF were significantly increased as compared to the negative controls (IL-1: 267.639 ± 71.502 pg/mL; TNF: 2.909 ± 2.454 pg/mL; see: Table 4, Figs. 6 and 7). Lipopolysaccharide (LPS) positive controls at 100 and 10 ng/mL, respectively, induced substantial elaboration/release of both IL-1 (100 ng/mL: 38.541 ± 9.112 pg/mL; 10 ng/mL: 1.449 ± 0.339 pg/mL; see: Table 4, Figs. 6 and 7) and TNF (100 ng/mL: 1.925 ± 0.859 ug/mL; 10 ng/mL: 12.456 ± 1.701 ug/mL; see: Table 4, Figs. 6 and 7). Increasing the exposure to the full 24 h by not removing the testing solution at the 1-h time point significantly increased the elaboration/release of both IL-1 (100 ng/mL: 617.985 ± 100.057 pg/mL; 10 ng/mL: 274.115 ± 99.892 pg/mL) and TNF (100 ng/mL: 12.655 ± 2.400 ug/mL; 10 ng/mL: 7.516 ± 1.343 ug/mL).

Discussion

Our results showed that even at low concentrations BAK induced markedly increased quantities of IL-1 and TNFα (see: Tables 2 and 3, Figs. 1 and 2). Elaboration of CRP, IL-10 and -12 was only marginally increased (see: Tables 2 and 3, Figs. 1 and 2). The addition of H_2O_2 induced increased quantities of all cytokines studied in both conjunctival and corneal epithelial cells (see: Tables 2 and 3, Figs. 1 and 2). Production of H_2O_2 was induced by BAK as well (see: Tables

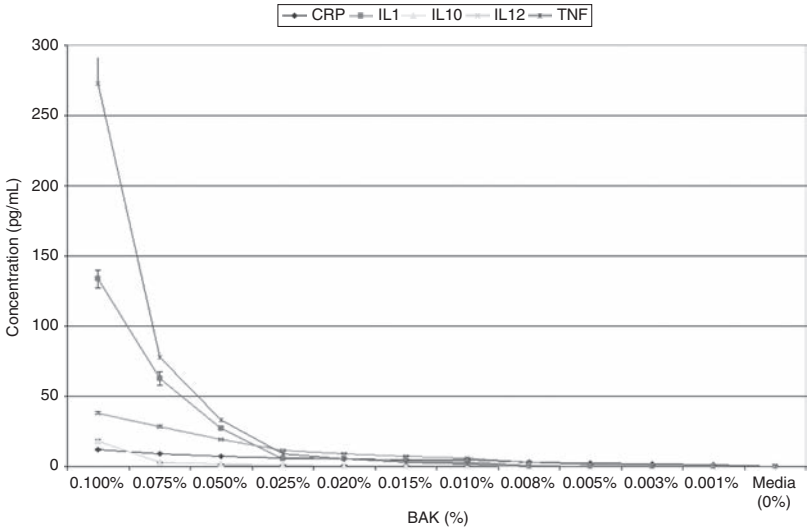

FIG. 2. Graphic representation of concentrations of the various inflammatory cytokines studied [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by human corneal epithelial cells (HCE; 10.014 pRSV-T) after being put into contact for 1 h with varying concentrations of benzalkonium chloride (BAK) as compared to media alone. All depicted values are statistically significant as compared to control.

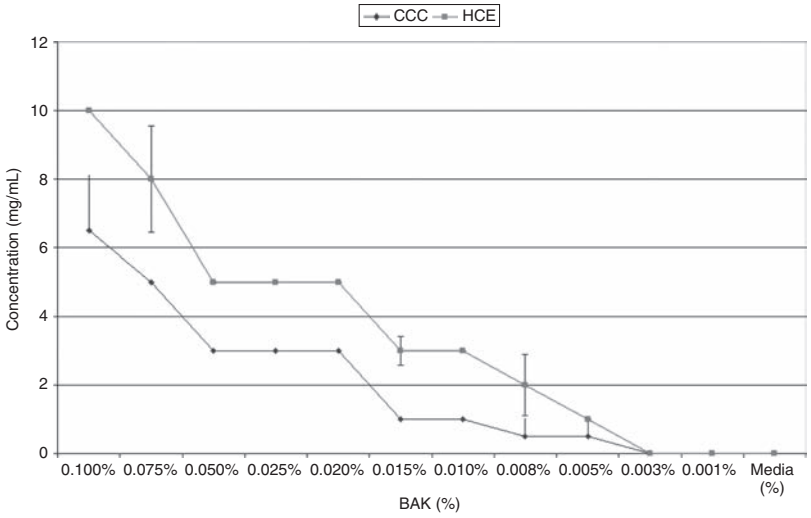

FIG. 3. Graphic representation of concentrations of hydrogen peroxide (H_2O_2) as produced by conjunctival (CCC; Wong-Kilbourne derivative of conjunctiva, clone 1–5c) and human corneal (HCE; 10.014 pRSV-T) epithelial cells after being put into contact for 1 h with varying concentrations of benzalkonium chloride (BAK) as compared to media alone. Probability (*P*) values of statistical comparisons of BAK concentrations from 0.01 to 0.005 are significantly different, lower concentrations are not.

2 and 3, Figs. 1 and 2). Lower concentrations of BAK and/or H_2O_2 induced the elaboration of proportionally lower amounts of all studied biomarkers in a dose-dependent fashion (see: Tables 2 and 3, Figs. 1 and 2). Media alone induced very small amounts of CRP, while levels of IL-1, IL-10, IL-12, TNF-α, and H_2O_2 remained imperceptible in both corneal and conjunctival epithelial cells (see: Tables 2 and 3, Figs. 1 and 2). In general, we found that even low-concentrations of BAK and/or H_2O_2 caused some degree of inflammation/inflammatory response by corneal and conjunctival cells in tissue culture.

Our results also showed that with all agents, there was an increased elaboration of cytokines and/or H_2O_2 with increasing concentration. We found the biomarkers in decreasing order of induction/upregulation in response to BAK to be: TNF-α ≥ IL-1 ≥ IL-12 ≥ IL-10 ≥ CRP, while in response to H_2O_2 the order was: IL1 ≥ IL-12 ≥ IL-10 ≥ TNF-α ≥ CRP. The only discrepancy is with respect to TNF-α, which was induced in the highest amounts after BAK stimulation, while the second to lowest in response to H_2O_2, seeming to indicate that the primary mechanism of BAK-induced toxicity is not secondary to H_2O_2.

Although this does not correlate with the findings of authors studying the levels of inflammatory cytokines in the tear film of humans and animal models of Dry Eye Disease,[23–31] this is not surprising as the induction of inflammation is believed to be primarily mechanical in dry eye (secondary to lack of lubrication), as compared to chemical (BAK, H_2O_2) induction, here.

As mentioned, most eye drops contain preservatives that provide a level of antimicrobial activity in the bottle, limiting secondary bacterial, mycotic, and amoebal-ocular infections caused by contaminated solutions and prolong the shelf life of the drug by preventing biodegradation and maintaining drug potency.[1] As previously discussed, preservatives can be classified in four main categories: detergents, oxidants, chelating agents, and metabolic inhibitors, which are further subdivided into three subgroups: pentavalent antimonials (Sb^V), quartenary ammoniums, and ethyl mercurials.[2,3] Preservatives added to eye drops can add to the toxicity of the pharmaceuticals and can cause ocular surface disease with a resulting inflammatory response.[1,32]

By far, the most common of the topical ophthalmic medication preservatives, and the best studied, is BAK, typically

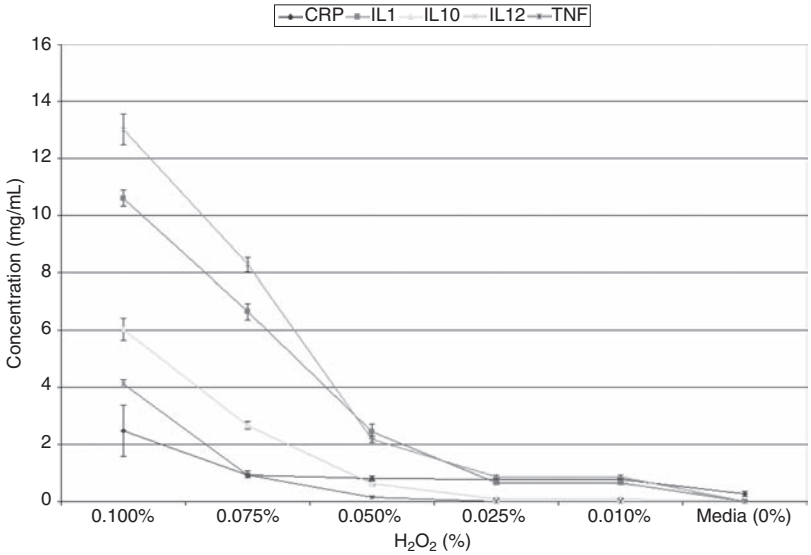

FIG. 4. Graphic representation of concentrations of the various inflammatory cytokines studied [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by human corneal epithelial cells (HCE; 10.014 pRSV-T) after being put into contact for 1 h with varying concentrations of hydrogen peroxide (H_2O_2) as compared to media alone. Probability (*P*) values of statistical comparisons of quantities of IL-10 and TNF elaborated in response to exposure to 0.01% H_2O_2 are not statistically significant as compared to controls. All other depicted values are statistically significant as compared to controls.

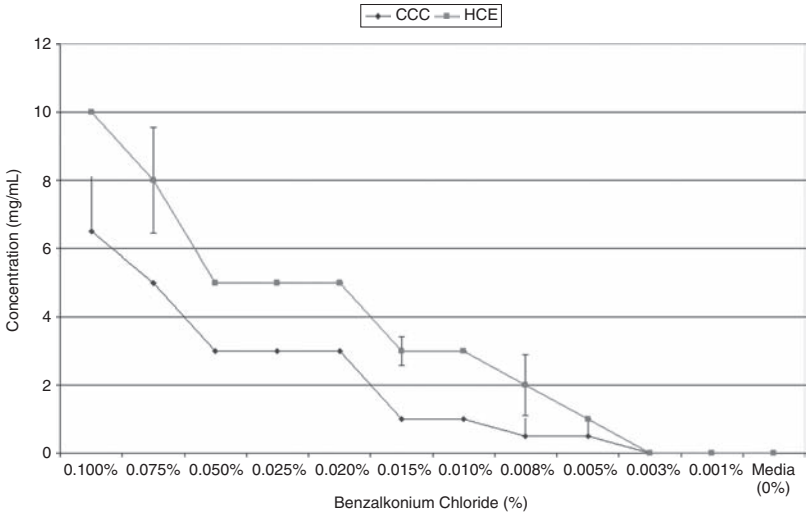

FIG. 5. Graphic representation of concentrations of the various inflammatory cytokines studied [C reactive protein (CRP), interleukin (IL)-1, interleukin (IL)-10, interleukin (IL)-12, and tumor necrosis factor (TNF)] elaborated by conjunctival cells (Wong-Kilbourne derivative of conjunctiva, clone 1–5c) after being put into contact for 1 h with varying concentrations of hydrogen peroxide (H_2O_2) as compared to media alone. Probability (*P*) values of statistical comparisons of quantities of IL-10 and TNF elaborated in response to exposure to 0.01% H_2O_2 are not statistically significant as compared to controls. All other depicted values are statistically significant as compared to controls.

used in concentrations varying from 0.015% to 0.05%,[4] although the American College of Toxicology has concluded that BAK can be safely used as an antimicrobial agent at concentrations up to 0.1%.[33] In addition, BAK stabilizes drugs in solution and prevents spoilage by microbial growth; but has a markedly low-pH in aqueous solutions (ie, is highly acidic)[34–36] and can initiate ocular surface damage and subconjunctival inflammation.[1,4] BAK comes from the quaternary ammoniums and is a detergent preservative, which can affect cell membrane permeability, interrupt the metabolic processes of the cell, cause lysis of cell contents, and allow vital substances to escape, eventually causing death of the microorganism.[8] The detergent properties of BAK have also been shown to interfere with the integrity of the external lipid layer of the precorneal tear film, reduce tear film break-up times, and exacerbate dry eye symptoms.[1,37] As previously mentioned, BAK is also a cationic surfactant, which reduces surface tension at interfaces and attracts to negatively charged surfaces, including those of microorganisms. However, cationic surfactants have the ability to lyze cytoplasmic membranes and denature intracellular proteins.[8]

Table 4. Follow-Up Experimentation

	IL-1 (pg/mL)	*TNF-α pg/mL)*
	Average ± σ	*Average* ± σ
Media	0.007 ± 0.01	0.00 ± 0.00
BAK 0.100%	145.33 ± 5.69	316.44 ± 12.30
BAK 0.020%	48.26 ± 2.87	5.37 ± 0.71
BAK 0.002%	18.59 ± 5.31	0.59 ± 0.19
LPS 100 ng/mL	38.54 ± 9.11	1.93 ± 1.86
LPS 10 ng/mL	1.45 ± 0.34	0.51 ± 0.17

Concentrations of inflammatory biomarkers [interleukin (IL)-1 and tumor necrosis factor (TNF)] elaborated by conjunctival epithelial cells (CCC: Wong-Kilbourne derivative of conjunctiva) after being put in contact with varying concentrations of each of the follow-up experimental testing solutions [media alone, benzalkonium chloride (BAK: 0.1%, 0.02%, 0.002%), and lypopolysaccharide (LPS: 100 ng/mL, 10 ng/mL) for 1 h, the solutions replaced with media and then incubated for 23 h.

A variety of authors have raised the possibility of preservatives adding to the formulations imparting toxic effects to the ocular surface[1,3,6,38,39] and recent data have demonstrated BAK as adding to the toxicity of eye drops and as potentially causing ocular surface disease.[1,2,5,6] Such toxicity of preservatives such as BAK may cause ocular discomfort, changes in vision, and may interfere with patient compliance with recommended dosages.[6] Dose-dependency has been previously reported with BAK[2,3,5]; at low concentrations (0.0001%–0.01%) BAK has been reported as having the potential to cause growth arrest or apoptotic mechanisms,[38,40,41] while at higher concentrations (0.05%–0.2%) it has been reported as being able to cause cell death by necrosis.[40–42]

Mammalian cells are unable to neutralize preservatives BAK, and most of the detergent preservatives, in general, as the human ocular surface cannot break them down into more basic components, which the eye can better tolerate.[1,42,43] Such preservatives can thus accumulate in ocular tissue and remain there for extended periods of time,[1,42,43] prolonging adverse reactions and explaining "chronic-like" appearances to the ocular surface (ie, an inflammatory response).[44–47]

The detergents/surfactant preservatives such as BAK generate chloride dioxide free radicals that oxidize unsaturated lipids and glutathione in the cell, and H_2O_2.[1,37,42] These preservatives all attribute their antimicrobial activity to their production of H_2O_2,[1,37,42] an efficient antimicrobial,[48] but also a known ophthalmic irritant.[9,10] However, there has yet to be shown a link between the production of H_2O_2 and ocular inflammation and/or ocular surface disease following BAK administration.

There is evidence, however, that ocular surface disease is an immunopathological state with increased release of inflammatory cytokines and increased elaboration of inflammatory markers in tear film.[13,14] In fact, tear samples from glaucoma patients have been shown to have greater amounts of inflammatory cytokines compared to their normal counterparts.[49] Although fibrinogen is suspected as being upregulated indirectly (secondary to the inflammatory cytokines),[50] it (ie, fibrinogen),[12] CRP,[11] and multiple cytokines,[13,14] specifically IL-1α and β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-23, and TNFα, have long been described as conventional markers of inflammation.

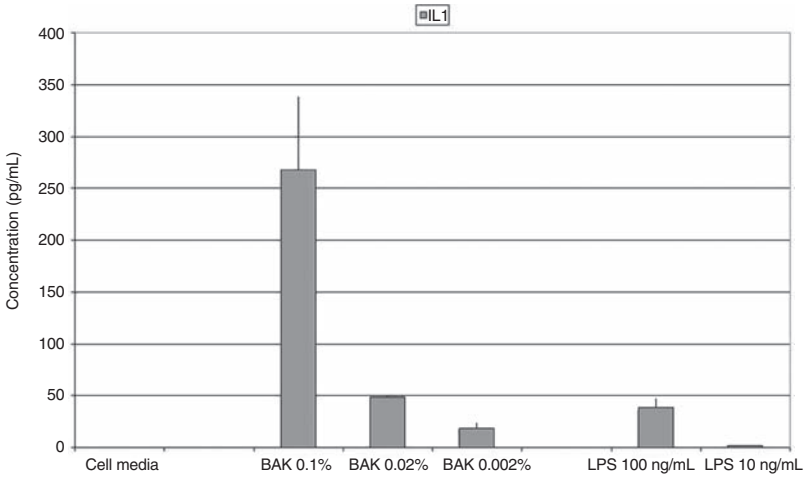

FIG. 6. Graphic representation of concentrations of the inflammatory biomarker interleukin (IL)-1 elaborated by conjunctival epithelial cells (CCC: Wong-Kilbourne derivative of conjunctiva) after being put in contact with varying concentrations of each of the follow-up experimental testing solutions [media alone, benzalkonium chloride (BAK: 0.1%, 0.02%, 0.002%), and lypopolysaccharide (LPS: 100 ng/mL, 10 ng/mL) for 1 h, the solutions replaced with media and then incubated for 23 h. All depicted values are statistically significant as compared to control.

Yet, despite BAK being the most common, and the best studied, of the topical ophthalmic medication preservatives,[4] there have been no studies published regarding BAK and inflammation or quantification of inflammatory cytokines specifically in response to BAK. As previously mentioned, a variety of authors have studied the levels of inflammatory cytokines (IL-1, IL-6, IL-8, and tumor necrosis factor TNF-α) in the tear film and conjunctival epithelium in humans as well as animal models, mostly in relation to Dry Eye Disease.[23–31] In addition, a few similar studies comparing the concentrations of inflammatory cytokines in tears of patients with Sjögren syndrome, in particular, to normal patients have also been reported.[28,30,31] However, nothing directly dealing with inflammatory cytokine levels with respect to BAK appear to have been published.

Here, we have demonstrated that cell media alone induces release of minimal to no amounts of inflammatory cytokines or H_2O_2. BAK in concentrations from 0.1% to 0.001%. induces release of high to moderate amounts of CRP and IL-12, release of very high to minimal amounts of IL-1 and TNF, responses are dose-dependent, but in an irritational, rather than truly "toxic," fashion (quickly drops off). BAK induces the release of IL-10 and the production of H_2O_2, at higher concentrations (through 0.005%), but not at lower concentrations (0 pg/cell). H_2O_2 in concentrations from 0.1% to 0.01% induces release of small amounts of CRP and IL-10, moderate to small amounts of IL-1 and IL-12, and small to minimal amounts of Tumor Necrosis Factor. H_2O_2 also induces the production of high amounts of H_2O_2 (~700 µg/cell). All responses are dose-dependent, although many are not truly "toxic," fashion (quickly drops off). Thus, based on the data presented here, if a link between the production of H_2O_2 and ocular inflammation and/or ocular surface disease following BAK administration does exist, it does not seem to be of a direct "cause and effect" etiology.

Recently, a great deal of attention has been made regarding the potential contamination of the Wong-Kilbourne-derived human conjunctival cells utilized in these experiments with the HeLa cells utilized for the immortalization of the line.[51] 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. In addition, while epithelial cells are a major inhabitant of the ocular surface, small numbers of inflammatory cells may also inhabit it and will certainly become involved in the ocular surface response to BAK toxicity through cell-cell interactions or cytokines.

While a multitude of authors have published regarding the potential toxicity of BAK used in ophthalmic preparations as a preservative, our tissue culture model of toxicity

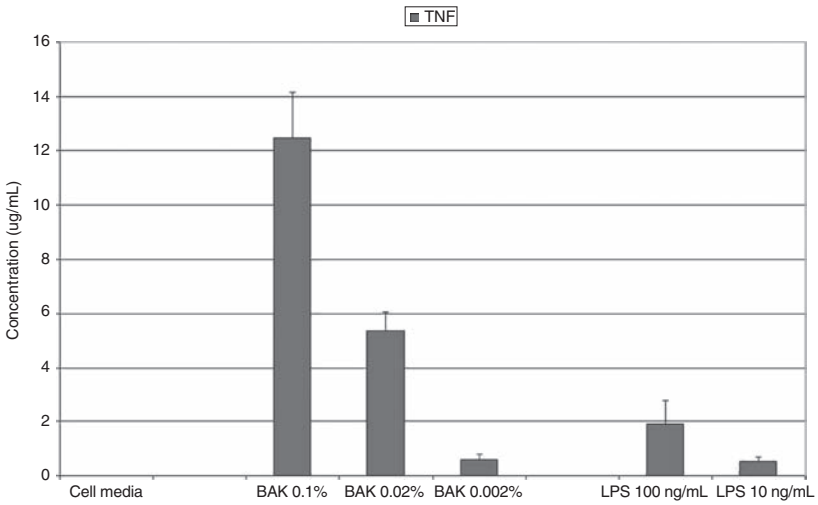

FIG. 7. Graphic representation of concentrations of the inflammatory biomarker tumor necrosis factor (TNF) elaborated by conjunctival epithelial cells (CCC: Wong-Kilbourne derivative of conjunctiva) after being put in contact with varying concentrations of each of the follow-up experimental testing solutions [media alone, benzalkonium chloride (BAK: 0.1%, 0.02%, 0.002%), and lypopolysaccharide (LPS: 100 ng/mL, 10 ng/mL) for 1 h, the solutions replaced with media and then incubated for 23 h. All depicted values are statistically significant as compared to control.

looking at corneal and conjunctival epithelial cells demonstrates that, regardless of the preservative, chronic, long-term use of any preserved pharmaceutical agent can negatively affect the ocular surface. Even using a low-concentration preservative for a long period of time can cause adverse reactions and high concentration of some preservatives can cause immediate damage and irritation to the ocular tissue. The inflammatory responses observed with pharmaceutical agents could be related to the preservative used in the different formulations (BAK) and might be decreased with the use of a different, less toxic, preservative. Use of a BAK-preserved pharmaceutical agent can negatively affect the ocular surface, although whether or not this reflects upon *in vivo* immunological responses remains to be determined.

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