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Toxicity of cosmetic preservatives on human ocular surface and adnexal cells

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ABSTRACT

Cosmetic products, such as mascara, eye shadow, eyeliner and eye makeup remover are used extensively to highlight the eyes or clean the eyelids, and typically contain preservatives to prevent microbial growth. These preservatives include benzalkonium chloride (BAK) and formaldehyde (FA)-releasing preservatives. We hypothesize that these preservatives, at concentrations (BAK = 1 mg/ml; FA = 0.74 mg/ml) approved for consumer use, are toxic to human ocular surface and adnexal cells. Accordingly, we tested the influence of BAK and FA on the morphology, survival, and proliferation and signaling ability of immortalized human meibomian gland (iHMGECs), corneal (iHCECs) and conjunctival (iHConjECs) epithelial cells. iHMGECs, iHCECs and iHConjECs were cultured with different concentrations of BAK (5 μg/ml to 0.005 μg/ml) or FA (1 mg/ml to 1 μg/ml) under basal, proliferating or differentiating conditions up to 7 days. We used low BAK levels, because we found that 0.5 mg/ml and 50 μg/ml BAK killed iHMGECs within 1 day after a 15 min exposure. Experimental procedures included analyses of cell appearance, cell number, and neutral lipid content (LipidTox), lysosome accumulation (LysoTracker) and AKT signaling in all 3 cell types. Our results demonstrate that BAK and FA cause dose-dependent changes in the morphology, survival, proliferation and AKT signaling of iHMGECs, iHCECs and iHConjECs. Many of the concentrations tested induced cell atrophy, poor adherence, decreased proliferation and death, after 5 days of exposure. Cellular signaling, as indicated by AKT phosphorylation after 15 (FA) or 30 (BAK) minutes of treatment, was also reduced in a dose-dependent fashion in all 3 cell types, irrespective of whether cells had been cultured under proliferating or differentiating conditions. Our results support our hypothesis and demonstrate that the cosmetic preservatives, BAK and FA, exert many toxic effects on cells of the ocular surface and adnexa.

1. Introduction

The United States has the largest cosmetics market in the world, with revenue exceeding $62 billion in 2016.(MarketResearch.com, 2016) It has been reported that the average woman in the United States uses 12 cosmetic products daily, and the average man uses six (Exposures add up - Survey results, 2017; O'Dell et al., 2016). However, few people pay attention to the dark side of the cosmetics. More than 12,000 chemicals are used in cosmetics. Many of these are synthetic and industrial chemicals, and less than 20% have been proven to be safe, (Anne Houtman, 2013; O'Dell et al., 2016). Once absorbed into the body through dermal penetration, these agents can act as carcinogens, endocrine disruptors, neurotoxins, mutagens and reproductive toxins (O'Dell et al., 2016; Rawlins, 2017).

Despite the prevalence of their use, United States law does not regulate cosmetic products or their ingredients, and FDA approval is not required before new cosmetics go on the market. In contrast, the Cosmetics Directive of the European Union (EU) sets limits to concentrations of ingredients in consumer products(Administration; EUROPEAN UNION, 2009).

Many of the chemicals used in cosmetics are added as preservatives (O'Dell et al., 2016). Because many consumers infrequently replace their cosmetic products, preservatives are widely added to prevent bacterial and fungal contamination. One of the most common

Abbreviations: BAK, benzalkonium chloride; FA, formaldehyde; iHMGECs, immortalized human meibomian gland epithelial cells; iHCECs, immortalized human corneal epithelial cells; iHConjECs, immortalized human conjunctival epithelial cells; EU, European Union; DED, Dry Eye Disease; IARC, International Agency for Research on Cancer; EGF, epidermal growth factor; BPE, bovine pituitary extract; AZM, Azithromycin; KSFM, keratinocyte serum-free medium; PVDF, polyvinylidene difluoride; PI3K, phosphoinositide 3-kinase; P-AKT, phospho-phosphoinositide 3-kinase-protein kinase B

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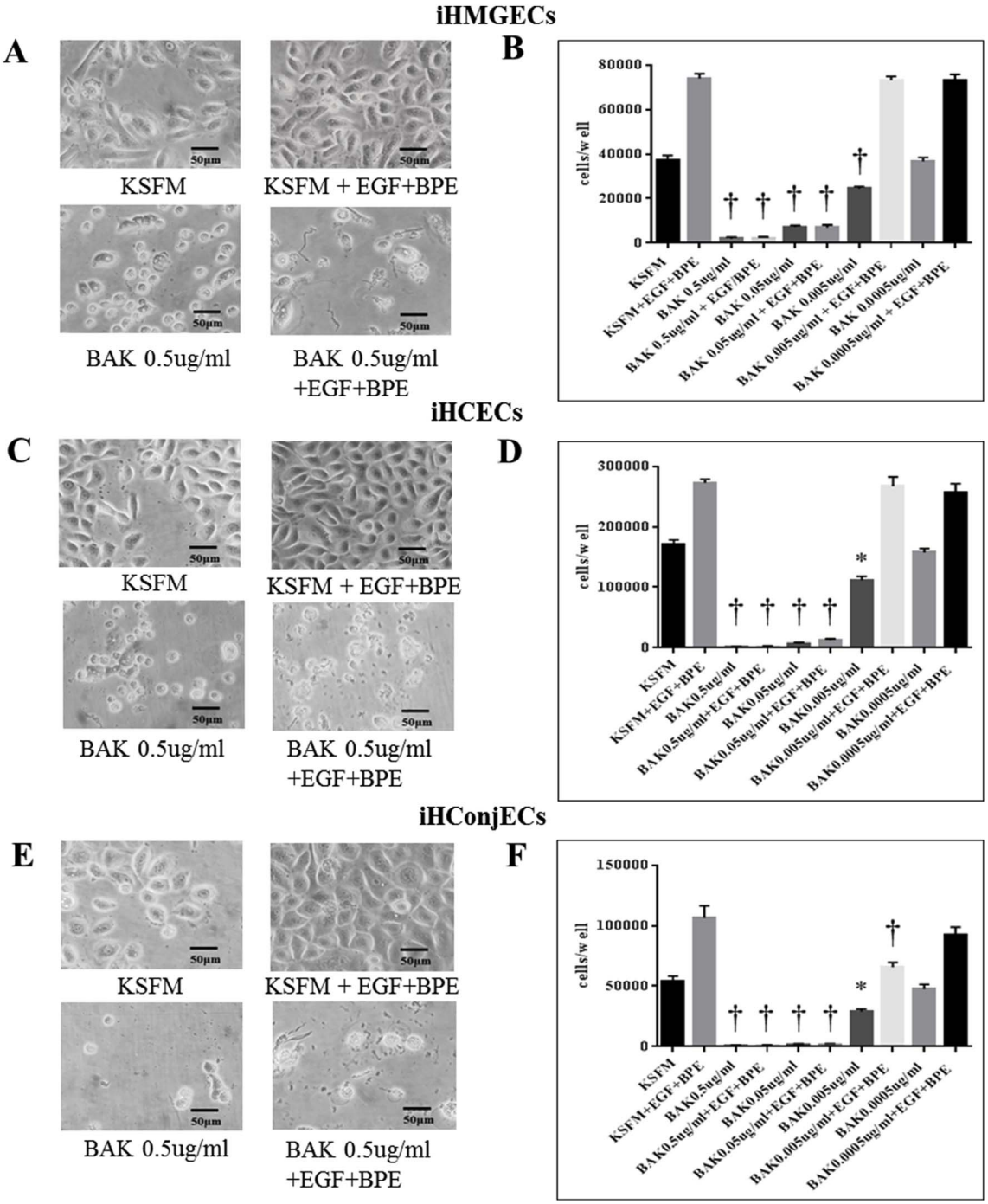

Fig. 1. Impact of BAK on the survival and proliferative ability of iHMGECs, iHCECs and iHConjECs. Cells were treated with vehicle or BAK in the presence or absence of growth supplements for 5 days before cell counting. BAK induced toxic morphological changes in iHMGECs, iHCECs and iHConjECs. All images are 200 × magnification. Scale bar is 50 μm (A, C, E). Results are reported as mean ± SE. *$p < 0.05$, †$p < 0.001$ (B, D, F). Significance signs reflect comparisons between basal conditions with or without BAK, or growth factor-containing cultures with or without BAK. Data from one experiment are shown as a representative of three studies performed under the same conditions.

preservatives used in cosmetics is benzalkonium chloride (BAK) (EUROPEAN UNION, 2009; Malik and Claoue, 2012; O'Dell et al., 2016; Review, 2012; Scheman, 2000). BAK is a quaternary ammonium, belonging to a family of detergent preservatives and cationic surfactants. Because of its antimicrobial activity, BAK is used as an active ingredient in many consumer products, including pharmaceutical products, cosmetics and some disinfectant solutions(Noecker, 2001). In addition to its role as an active ingredient, BAK is also the most commonly used preservative in topical ophthalmic solutions. However, it has been reported to be cytotoxic to corneal and conjunctival epithelial cells in numerous *in vitro* and *in vivo* models (Baudouin et al., 2008; Kahook and Noecker, 2008; Martone et al., 2009; Noecker et al., 2004; Pisella et al., 2004; Tressler et al., 2011). The 2017 TFOS DEWS II Report reported that BAK may cause or aggravate Dry Eye Disease (DED) through various mechanisms(Gomes et al., 2017). In the EU, BAK is authorized for use in cosmetic products at maximum concentrations of 0.1% (1 mg/ml)(EUROPEAN UNION, 2009). Administration of topical drugs containing BAK can lead to much higher prevalence of ocular surface disorders including irritation, burning, stinging, itching and foreign body sensation, as well as to conjunctival hyperaemia, blepharitis, and failure of glaucoma surgery(Boimer and Birt, 2013).

Formaldehyde (FA)-releasing compounds are another class of

preservatives widely used in cosmetic products(O'Dell et al., 2016; Scheman, 2000). These compounds have an easily detachable FA moiety, allowing them to gradually release small amounts of FA at room temperature. There are five FA-releasing preservatives commonly used in the United States: DMDM-hydantoin; quaternium-15; imidazolidinyl urea; diazolidinyl urea; and 2-bromo-2-nitropropane-1, 3-diol (O'Dell et al., 2016; Scheman, 2000). Despite its prevalence in cosmetics, most studies investigating FA exposure focus on occupational exposure in pathology and anatomy laboratories. The mutagenic, carcinogenic and pro-allergenic potential(Schmid and Speit, 2007; Speit et al., 2007) of FA has prompted increasing public health attention. The International Agency for Research on Cancer (IARC) classifies FA as carcinogenic to humans, based on evidence in humans and animals studies and "strong but not sufficient evidence for a causal association between leukemia and occupational exposure to formaldehyde."(CANCER, 2004) In studies *in vitro*, cytotoxic effects of FA have been identified in human bronchial epithelial cells, HUV-EC-C human endothelial cells, natural killer cells, lymphocytes (Li et al., 2013; Schmid and Speit, 2007; Tyihak et al., 2001; Yaqng et al., 2016), and rabbit corneal epithelial cells(Lai et al., 2013). The maximum limit of FA in EU is in cosmetics is 0.2% (2 mg/ml)(EUROPEAN UNION, 2009). More recently, a cosmetic ingredient review (CIR) panel deemed in 2012 that topical cosmetics should not contain FA more than 0.074% (0.74 mg/ml)(Review, 2012). Studies have shown that FA levels between 0.5 and 1.0 ppm (0.5–1.0 μg/ml, or 0.00005–0.0001%) can cause ocular irritation, including increased blinking frequency and conjunctival redness, and concentrations above 1.0 ppm can irritate the nose and throat(Lang et al., 2008).

Given the known ocular surface uptake of BAK following topical exposure(Champeau and Edelhauser, 1986), and the fact that FA is one of the most rapidly penetrating tissue fixatives(Burnett, 1982), we hypothesize that these cosmetic preservatives are harmful not only to corneal and conjunctival epithelial cells, but also to human meibomian gland epithelial cells. To begin to test our hypothesis, we investigated the effects of BAK and FA on the survival, proliferation, morphology, signaling, and/or lipid expression of immortalized human meibomian gland epithelial cells (iHMGECs). For comparative purposes, we also evaluated the effects of BAK and FA on immortalized human corneal (iHCECs) and conjunctival (iHConjECs) epithelial cells.

2. Materials and methods

2.1. Cell culture

iHCECs (Robertson et al., 2005)were provided by Dr. James V. Jester (Irvine, CA), iHConjECs (Gipson et al., 2003) were provided by Dr. Ilene Gipson (Boston, MA), and iHMGECs (Liu et al., 2010)were generated in our laboratory. Epithelial cells were cultured in keratinocyte serum-free medium (KSFM), supplemented with 5 ng/ml epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (BPE) (Thermo Fisher Scientific, Grand Island, NY). This supplemented medium has been shown to induce proliferation of iHMGECs (Liu et al., 2013), and to be effective in our laboratory in promoting growth of iHCECs and iHConjECs (see Fig. 1). Cells (n = 3 wells/treatment/experiment) were treated with varying concentrations of benzalkonium chloride (BAK, Sigma-Aldrich, St. Louis, MO) or formaldehyde (FA, Electron Microscopy Sciences, Hatfield, PA). Initial treatment concentrations were selected based on cosmetic preservative concentrations approved for consumer use (BAK = 1 mg/ml; FA = 0.74 mg/ml). We decreased BAK levels because we found in our preliminary studies that 0.5 mg/ml and 50 μg/ml BAK killed iHMGECs within 1 day after a single 15 min exposure (data not shown). The following doses were selected for all further studies: BAK, 0.5 ng/ml to 5 μg/ml; FA, 0.1 μg/ml to 1 mg/ml. In our pilot study we found that cellular exposure to high concentrations of FA led to FA vapor release from the treated wells and the death all of the cells in the culture plate within several hours. As a result, in subsequent studies, each treatment concentration group was seeded in a single plate. To determine the effects of each reagent on proliferation, cells were cultured for 5 days with BAK, FA or vehicle and counted using a hemocytometer. Cellular morphology was examined with a phase-contrast microscope.

2.2. Lipid analyses

To promote differentiation of iHMGECs, cells were cultured in a 50:50 mixture of DMEM/F12 (Mediatech, Inc., Manassas, VA), supplemented with 10% FBS (Thermo Fisher Scientific), as previously described(Sullivan et al., 2014) When indicated, Azithromycin (AZM, 10 μg/ml) was also added to the culture medium, because AZM is a potent stimulator of iHMGEC differentiation(Liu et al., 2014a). After treatment with BAK, FA, or vehicle, in the presence or absence of AZM for 7 days, cells were exposed to LysoTracker Red DND-99 (50 nM; Thermo Fisher Scientific) for 30 min, fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 30 min, stained with LipidTOX green neutral lipid stain (1:500, Thermo Fisher Scientific) for 2 h, and mounted using ProLong Gold antifade reagent (Thermo Fisher Scientific). Slides were viewed using an Eclipse E800 fluorescent microscope and images captured with NIS-Elements Basic Research software, version 4.2 (Nikon Instruments, Melville, NY) (Liu and Ding, 2014; Liu et al., 2014a, 2016, 2014b, 2015). Intensities were quantified using ImageJ.(http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD).

2.3. SDS-PAGE and immunoblot

Cells were cultured in KSFM with EGF and BPE to 80% confluence, then a) starved in KSFM without supplements overnight or b) grown in DMEM/F12 containing 10% FBS for 6 days, followed by starving in 1% FBS overnight, as previously described(Ding and Sullivan, 2014). Cells were subsequently treated with BAK for 30 min or FA for 15 min. After treatment, cells were lysed in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) supplemented with 1% protease inhibitor cocktail, 200 μM sodium orthovanadate and 5% β-mercaptoethanol (all from Sigma-Aldrich Corp.), denatured at 95 °C for 10 min, separated by SDS-PAGE on 4%–20% Tris-glycine gels (Thermo Fisher Scientific), and transferred to polyvinylidene difluoride (PVDF). Membranes were incubated with primary antibodies specific to phospho-phosphoinositide 3-kinase-protein kinase B (P-AKT) (1:4000, rabbit) or β-actin (1:10,000, mouse; both from Cell Signaling Technology, Danvers, MA), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (1:5000, Sigma-Aldrich Corp.). Blocking and antibody incubation were performed in Tris-buffered saline containing 5% bovine serum albumin and 0.1% Tween 20. Proteins were visualized with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) using a G-Box gel documentation station (Syngene, Frederick, MD). Image analysis and densitometry were performed by ImageJ.

2.4. Statistical analyses

One-way analysis of variance was performed using Prism 7 (GraphPad Software, Inc., La Jolla, CA). Each experiment was performed in triplicate under the same conditions and repeated at least 3 times.

3. Results

3.1. Influence of BAK and FA on proliferation of human ocular surface and adnexal cells

In order to test our hypothesis that BAK and FA are toxic to human ocular surface and adnexal cells at concentrations approved for

consumer use, we treated cells (n = 3 wells/treatment/experiment) with various doses of BAK or FA in KSFM with or without EGF and BPE for 5 days.

3.1.1. Benzalkonium chloride

In our pilot studies, we exposed iHMGECs to 0.5 mg/ml and 50 μg/ml BAK in supplemented KSFM for 15 min, then washed out the preservative and cultured the cells for 24 h (data not shown). After that time period, all iHMGECs were dead. We also exposed all 3 cell types to 5 μg/ml BAK in serum-containing medium, and all cells died by 2 h after treatment (data not shown). In order to mimic chronic exposure *in vivo*, we intended to treat the cells for a longer period; therefore, we decreased the concentration. As illustrated in Fig. 1, 0.005–0.5 μg/ml BAK in KSFM significantly decreased iHCEC, iHConjEC and iHMGEC survival, compared to KSFM alone. When cells were exposed to the growth factors EGF and BPE, they were slightly more resistant to the effects of BAK. Morphologically, 0.005–0.5 μg/ml BAK induced cell rounding, accumulation of perinuclear vesicles, cellular atrophy, poor adherence, and detachment in all 3 cell types.

3.1.2. Formaldehyde

At the EU-approved concentration of 1 mg/ml, FA fixed all of the ocular surface and adnexal cells tested. After 5 days of exposure, the cells were plasticized and could not be trypsinized within 60 min (data not shown). At lower concentrations, 0.1 mg/ml FA killed all the cells after 4 h of exposure; 0.01 mg/ml and 5 μg/ml FA killed the cells within 18 h (data not shown). As illustrated in Fig. 2, concentrations of 1.0–2.5 μg/ml FA significantly decreased iHMGEC suvival without, and proliferation with, growth factors. In iHCECs, however, supplementation with EGF and BPE protected cells from the toxic effects of 1 μg/ml FA. In iHConjECs, 2.5 μg/ml FA significantly impacted cell number with or without EGF and BPE, but lower concentrations had no effect. Morphologically, all 3 types of epithelial cells exhibited rounding, poor adherence, perinuclear vesicle accumulation, cellular atrophy and detachment when treated with 1.0–2.5 μg/ml FA.

3.2. Impact of BAK and FA on AKT signaling in human ocular surface and adnexal cells

To begin to identify the effects of BAK or FA on the activity of cell survival mediators, we explored whether these reagents alter phosphoinositide 3-kinase (PI3K)–protein kinase B (AKT) signaling. Activation of this pathway, as indicated by AKT phosphorylation, promotes cell growth, proliferation, and survival(Song et al., 2005). As illustrated in Figs. 4 and 5, we discovered that both BAK and FA caused a significant, dose-dependent decrease in the levels of phosphorylated AKT, as compared to controls. After 30 min exposure, 50 μg/ml and 5 μg/ml BAK, but not lower concentrations, significantly suppressed AKT phosphorylation, in both serum-free and serum-containing medium conditions (Fig. 3). Concentrations of 0.01–1 mg/ml FA resulted in a significant decrease in P-AKT in both medium conditions after 15 min exposure (Fig. 4).

3.3. Impact of BAK or FA on neutral lipid and lysosome accumulation in iHMGECs

To investigate whether BAK or FA influence iHMGEC differentiation, we cultured the cells (n = 2 wells/treatment/experiment) in serum-containing medium and treated them with preservatives, alone or in combination with AZM, for 7 days. We processed the cells for the analysis of neutral lipids and lysosomes, using LipidTox and LysoTracker, respectively. Neither BAK nor FA altered the neutral lipid or lysosome accumulation in iHMGECs. Moreover, these treatments did not affect the ability of cells to accumulate lipid in response to AZM treatment (Fig. 5).

4. Discussion

Our findings show that BAK and FA cause a dose-dependent decrease in cell survival, proliferation and AKT signaling in iHCECs, iHConjECs and iHMGECs. Our results support our hypothesis that BAK and FA are toxic to iHMGECs, as well as to iHCECs and iHConjECs, and affect their morphology, survival and proliferation capacity. The concentrations of these two cosmetic preservatives used in our study ranged from the approved levels to hundreds- or even thousands-fold lower concentrations, yet they led to cellular atrophy, poor adherence, and death. By contrast, exposure did not interfere with the ability of AZM to stimulate cellular neutral lipid and lysosome accumulation in iHMGECs.

The effects of topical drugs on corneal and conjunctiva epithelial cells have been investigated in short-term studies (≤30 min treatment) (Epstein et al., 2009; Lai et al., 2013; Noecker et al., 2004; Paimela et al., 2012), likely because it is thought that the compounds will be washed out within minutes. However, BAK has a long half-life retention in tissues (e.g. 20 h in the corneal and conjunctival epithelium and 11 h in deeper conjunctival structures) and can be found 168 h after a single 30 μl drop of 0.01% BAK in rabbits(Champeau and Edelhauser, 1986). Furthermore most cosmetics users apply these products, which consistently remain unless make-up remover is applied, at least once each day. A long-term study would be better to test the effects of cosmetic ingredients on the ocular surface.

In this study, we exposed cells to BAK and FA from the approved levels (1 mg/ml BAK, 0.74–2 mg/ml FA) to much lower concentrations (0.005 μg/ml BAK, 1 μg/ml FA) for an extended period (5–7 days), to mimic the daily use of preservative-containing cosmetics. Of particular interest, 1 mg/ml FA, which is 50% of the EU-approved limit, fixed the ocular surface and adnexal cells within a short period of time; this process is irreversible. Moreover, at concentrations hundreds-fold below the approved levels, BAK and FA killed all the cells within 18 h. Even at concentrations that are 20,000 fold lower (0.005 μg/ml vs 1 mg/ml BAK) and 740-2000 fold lower (1 μg/ml FA vs 0.74–2 mg/ml FA) than their limits in commercial products, BAK and FA are toxic to all 3 ocular cell lines tested.

No data about the bioaccumulation and tissue concentration of BAK or FA in humans have been published. However, because the doses that we used are hundreds-to thousands-fold lower than the approved commercial concentrations, it is more than likely that the tissue concentrations that accumulate in cosmetic users are included in our study. Given that our studies were limited to cellular exposure *in vitro*, pharmacokinetic studies *in vivo* are necessary to determine the extent to which these topical preservatives gain entry into the meibomian gland, as compared to the cornea and conjunctiva.

Our results are consistent with previous reports on the toxicity of these two reagents. In humans(Charnock, 2006; Ishibashi et al., 2003; Wilson et al., 1975), BAK has been reported to decrease tear film break-up time, and topical administration of 0.1% (1 mg/ml) BAK for 14 days or 0.2% (2 mg/ml) BAK for 7 days has been shown to induce dry eye signs in mice(Lin et al., 2011) and rabbits(Xiong et al., 2008), respectively. In rats(Pauly et al., 2007), ocular irritation was visible using macroscopic and slit lamp examinations in animals treated with 0.25% and 0.5% BAK. However, *in vivo* confocal microscopy and Sub-G1 assays revealed epithelial defects and apoptosis at lower doses (0.01% and 0.1%)(Pauly et al., 2007). Investigators have shown that incubation with BAK for 1 h at concentrations ranging from 10 μg/ml to 1 mg/ml caused 58%–82% toxicity in iHCECs and 54%–87% toxicity in iHConjECs(Epstein et al., 2009).

Other studies have investigated the effects of FA on the ocular surface. Investigators have shown that a 37% FA solution (370 mg/ml) directly contacting the eye can cause severe damage to the cornea and possible blindness(Maurer et al., 2001). Others have found that exposing rabbit corneal epithelial cells to up to 100 ppm (0.1 mg/ml) FA for 3 min decreased cell survival and increased the proportion of

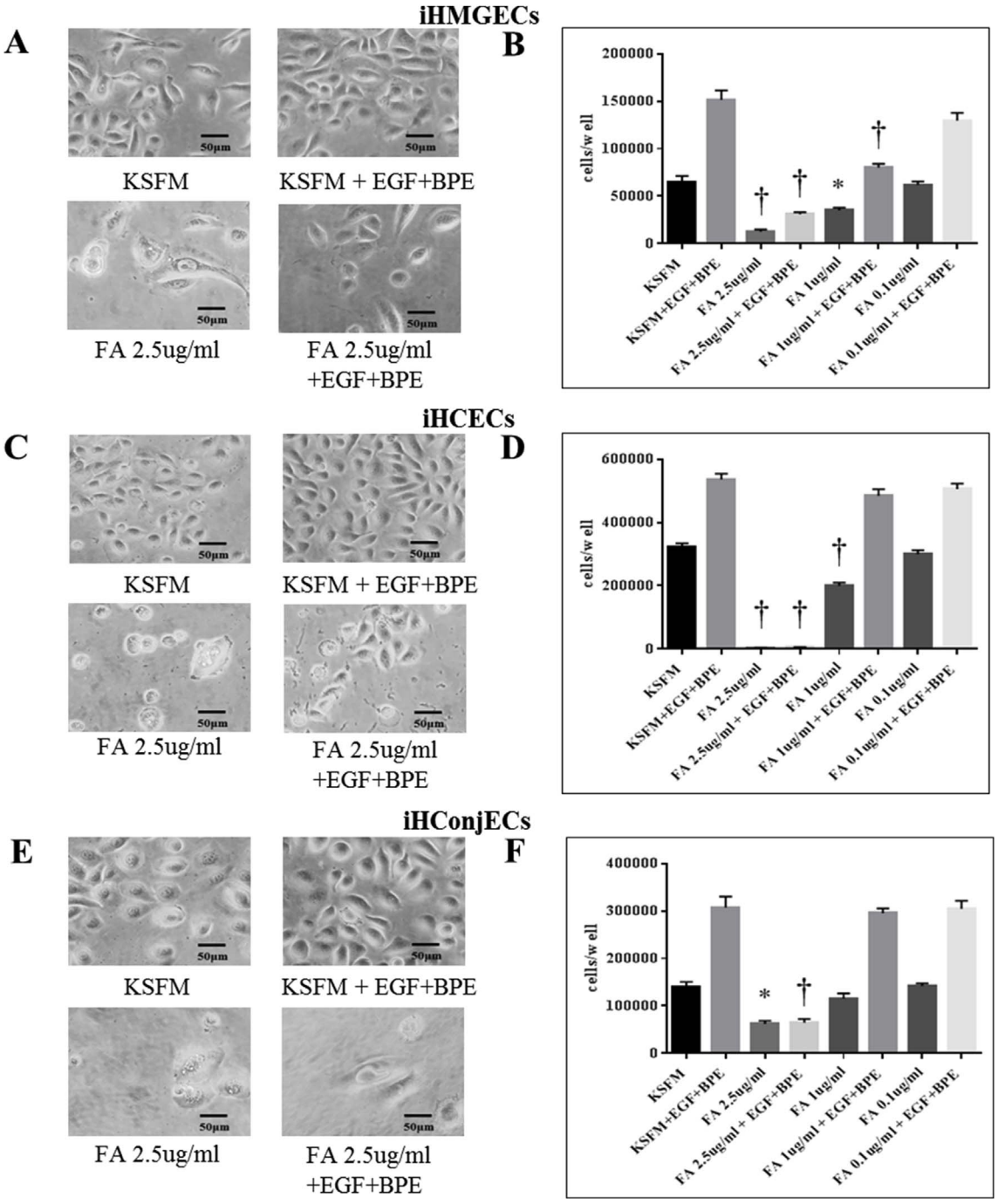

Fig. 2. Impact of FA on the survival and proliferative ability of iHMGECs, iHCECs and iHConjECs. Cells were treated with vehicle or FA in the presence or absence of growth supplements for 5 days before cell counting. FA induced toxic morphological changes in iHMGECs, iHCECs and iHConjECs. All images are 200× magnification. Scale bar is 50 μm (A, C, E). Results are reported as mean ± SE. *$p < 0.05$, †$p < 0.001$ (B, D, F). Significance signs reflect comparisons between basal conditions with or without FA, or growth factor-containing cultures with or without FA. Data from one experiment are shown as a representative of three studies performed under the same conditions.

apoptotic/necrotic cells and those in the sub-G1 phase of the cell cycle (Lai et al., 2013).

The mechanisms of the toxicity of BAK and FA are not fully understood. In our study, both BAK and FA reduced AKT phosphorylation in 3 ocular surface and adnexal epithelial cell lines after only 15 or 30 min exposure. The AKT pathway is an important regulator of cell cycle progression and cell survival(Song et al., 2005), and the effects of chronic exposure to these preservatives on p-AKT may contribute to their toxicity at low concentrations.

In addition to inhibiting the AKT pathway, others have reported that 0.001–0.5% (0.01–5 mg/ml) BAK can increase markers of apoptosis and inflammation in the cornea and conjunctiva of animals and humans *in vivo* and *in vitro*(Lin et al., 2011; Paimela et al., 2012; Pauly et al., 2007). FA can react with proteins, nucleic acids and amino acids, cause DNA-protein crosslinks(Cheng et al., 2003; Conaway et al., 1996; Merk and Speit, 1998) and induce chromosome changes(Kreiger and Garry, 1983; Orsiere et al., 2006; Ye et al., 2005). Treatment of mice with FA causes chromosome damage, oxidative stress, protein modifications and apoptosis(Matsuoka et al., 2010; Tang et al., 2011). Decreased extracellular signal-regulated kinase (pERK2) in cornea and increased c-Jun amino - terminal kinase (JNK) activation (pJNK) in cornea and conjunctiva, were reported in rabbits after exposure to 50–200 ppm

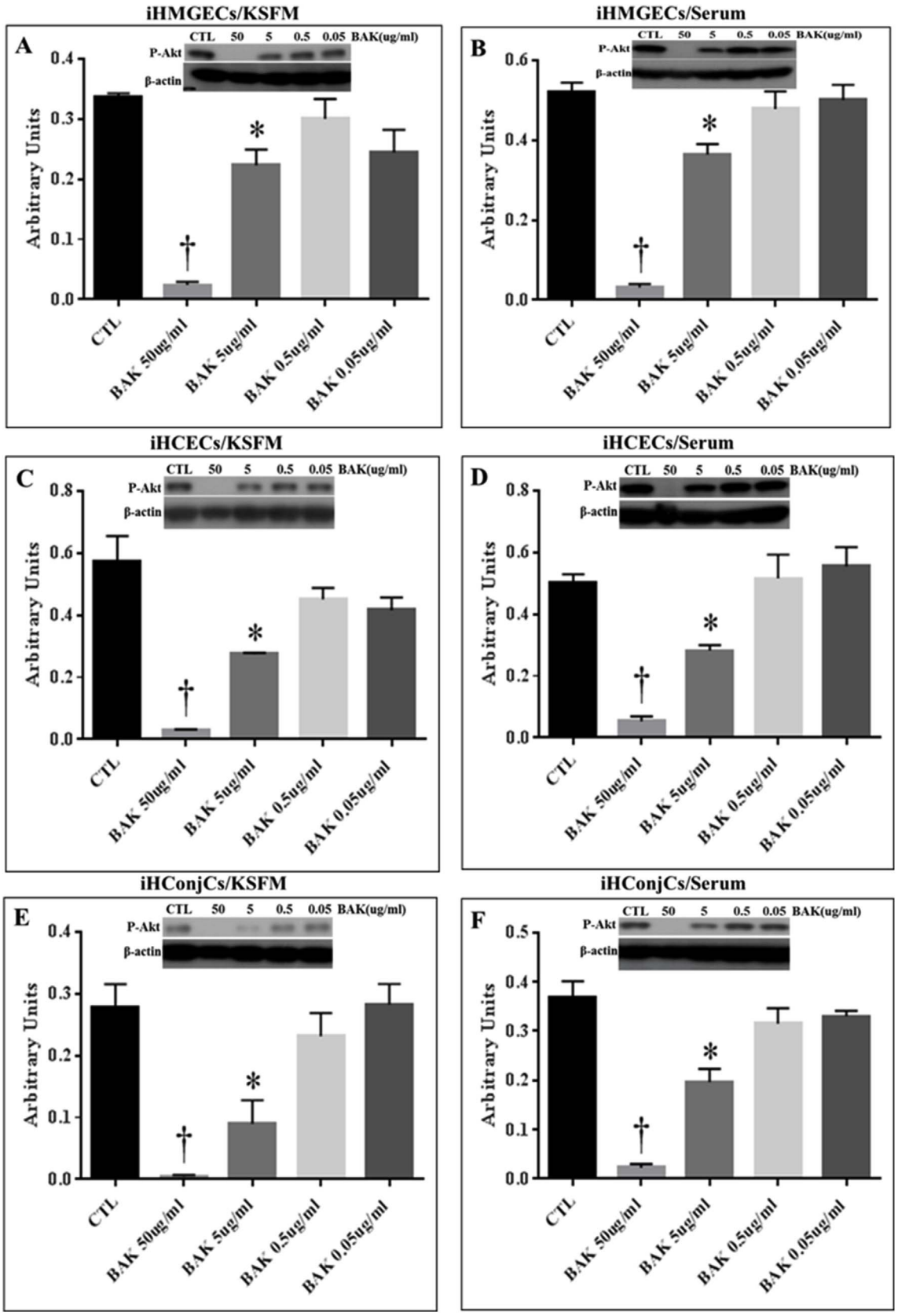

Fig. 3. BAK alters iHMGEC, iHCECs and iHConjECs signaling. Cells were treated with vehicle or BAK in serum-free (KSFM) or serum-containing medium for 30 min. BAK-treated cells showed a significant decrease in -AKT phosphorylation. Results are reported as mean ± SE. *$p < 0.05$, †$p < 0.001$. Data from one experiment are shown as a representative of three studies performed under the same conditions.

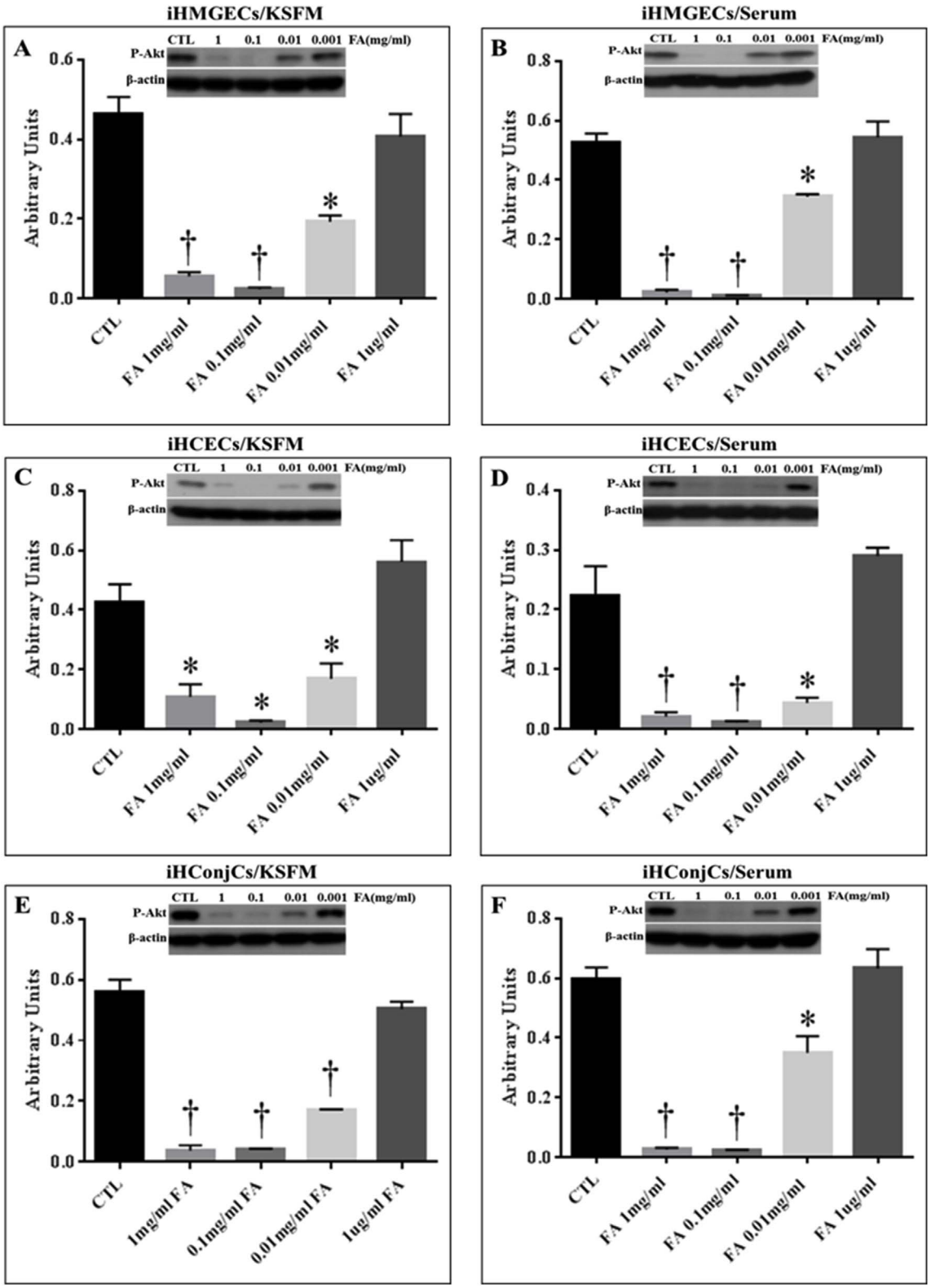

Fig. 4. FA alters iHMGEC, iHCECEs and iHConjECs signaling. Cells were treated with vehicle or FA in serum-free (KSFM) or serum-containing medium for 15 min before lysate and evaluated on immunoblotting for P-AKT. FA-treated cells exhibited a significant decrease in P-AKT, compared to controls. Results are reported as mean ± SE. *$p < 0.05$, †$p < 0.001$. Data from one experiment are shown as a representative of three studies performed under the same conditions.

(0.05–0.2 mg/ml) FA for 5 min. The ERK and JNK pathways are activators of apoptotic and necrotic pathways, indicating that FA exposure in rabbits may promote entrance of corneal and conjunctival tissues into these types of cell death(Lai et al., 2013).

An apparent confounding observation in our study was that while concentrations of FA (5 μg/ml) killed all, and BAK (0.5 μg/ml) significantly decreased the survival and proliferative ability of, iHMGECs, these preservative levels exerted no influence on cellular differentiation (i.e. neutral lipid and lysosome accumulation)? Two non-exclusive possibilities may explain these findings. First, cellular proliferation is

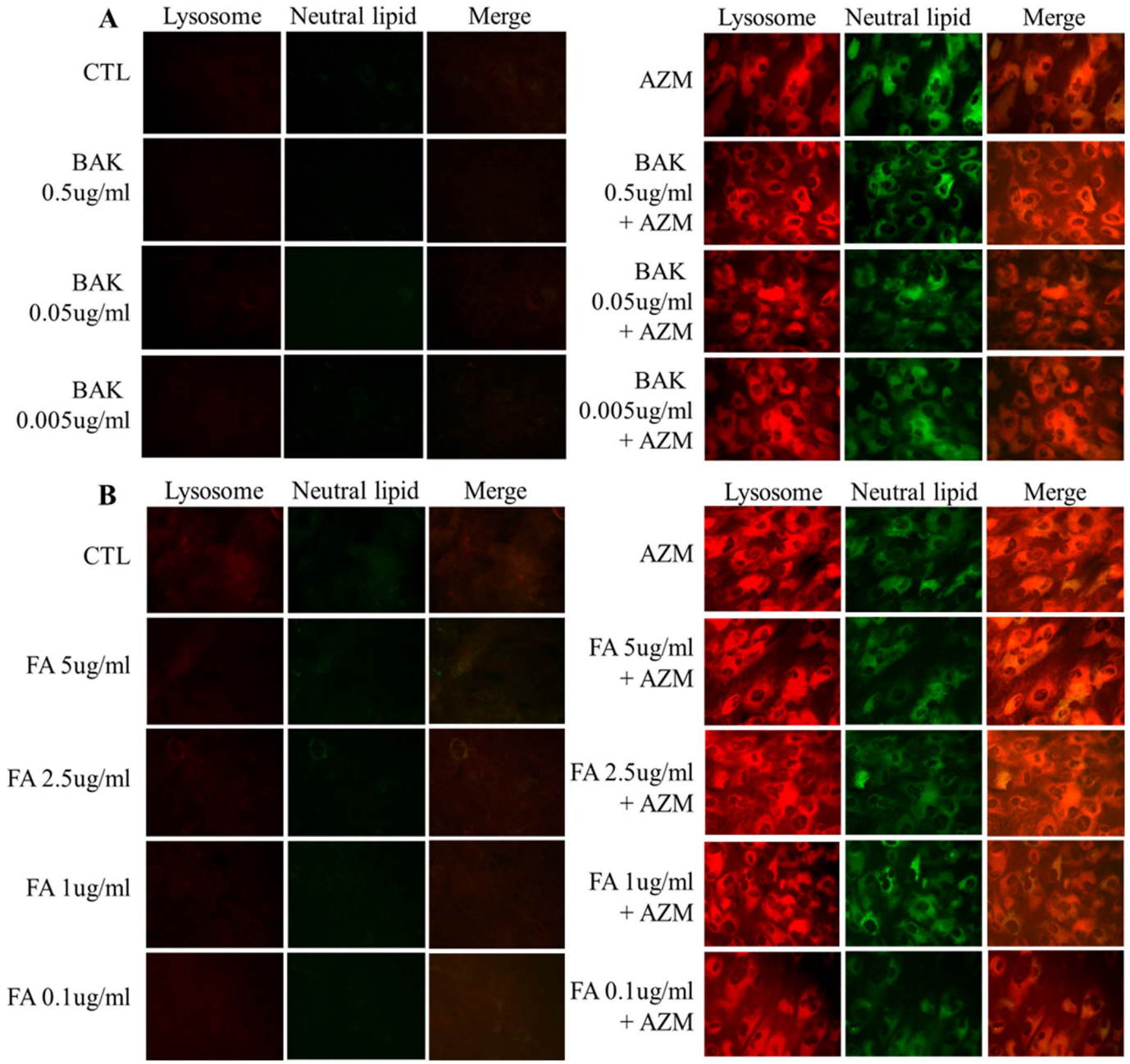

Fig. 5. Effect of BAK and FA on intracellular accumulation of neutral lipid and lysosomes in iHMGECs. Immortalized human meibomian gland epithelial cells were treated with BAK (A) or FA (B) in the presence (right side) or absence (left side) of AZM and stained for neutral lipids (LipidTox Neutral Green) and lysosomes (LysoTracker Red). All images are 400× magnification. Data from one experiment are shown as a representative of three studies performed under the same conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

quite distinct from differentiation, and toxic or non-toxic compounds that significantly reduce proliferation may have no effect on differentiation(Liu and Levy, 1997; Rajabalian et al., 2009). Indeed, these cellular processes appear to be controlled by different regulatory mechanisms(Liu and Levy, 1997). Second, we performed iHMGEC differentiation studies with culture media containing serum, which contains very high amounts of albumin. Albumin, in turn, is known to bind both BAK (Jono et al., 1986) and FA(Bogdanffy et al., 1987). This binding may have significantly decreased the effective media concentration, and the cellular uptake, of both BAK and FA. Thus, even though BAK and FA were added to culture media at levels 2000- and 400-fold less than permitted in cosmetics by the EU, the actual amounts impacting the iHMGECs were likely far less.

Because of the daily use of cosmetic products, those that contain BAK and/or FA may cause severe damage to the ocular surface and surrounding skin keratinocytes (Lee et al., 2016; Varani et al., 2007) as a result of chronic exposure. Considering their potential for ocular toxicity, consumers should be more aware of the ingredients in cosmetic products. However, it appears that few people are sufficiently educated and/or motivated to pay attention to the ingredients in cosmetics as they do in food. One survey showed that 70% of respondents (119/169) said they do not look at ingredients when deciding what cosmetics products to purchase(O'Dell et al., 2016). Research shows the use of multiple eye cosmetics is extensive and associated with the perception of ocular discomfort(Ng et al., 2012). Adverse reactions to eye cosmetics, including simple irritation, keratitis, corneal epithelial inflammation, eyelid dermatitis and dry eye symptoms have been reported(Coroneo et al., 2006; Gao and Kanengiser, 2004; Scheman,

2000). Further investigation into the possible effects of cosmetic ingredients on the ocular surface, as well as more patient education emphasizing that the delicate eyelids and ocular surface need thoughtful and special care, are extremely important.

5. Conclusions

In conclusion, BAK and FA, two commonly used preservatives in cosmetics, inhibit the survival and proliferative ability of ocular surface and adnexal epithelial cells, affect their morphology and reduce the activity of a cell survival mediator. These effects occur at much lower concentrations than allowed in consumer products. Individuals may be causing damage to their ocular surface with each application of these products, and especially with use over time(O'Dell et al., 2016). More study is needed to determine the full effects in cosmetic users.

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Conflicts of interest

SC, YL, WRK and DAS have no conflict of interests. AGS recently founded ESSIRI LLC.

Author contributions

Y.L, X.C., D.A.S, W.K. and A.G.S designed the experiments. Y.L did pilot study, X.C did cell culture, lipid analysis, SDS-PAGE and Immunoblot. X.C analyzed the data including statistics. X.C., Y.L., W.K., and D.A.S wrote the manuscript. A.G.S. critiqued the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2018.02.020.

References

Administration, U.F.D., FDA Authority Over Cosmetics: How Cosmetics Are Not FDA-Approved, but Are FDA-Regulated.
Anne Houtman, S.K.A.J.I., 2013. Toxic bottles? On the trail of chemicals in our every day lives. In: Correa, J. (Ed.), American Environmental Science for a Changing World, Kate Ahr paker, p. 54.
Baudouin, C., Liang, H., Hamard, P., Riancho, L., Creuzot-Garcher, C., Warnet, J.M., Brignole-Baudouin, F., 2008. The ocular surface of glaucoma patients treated over the long term expresses inflammatory markers related to both T-helper 1 and T-helper 2 pathways. Ophthalmology 115, 109–115.
Bogdanffy, M.S., Morgan, P.H., Starr, T.B., Morgan, K.T., 1987. Binding of formaldehyde to human and rat nasal mucus and bovine serum albumin. Toxicol. Lett. 38, 145–154.
Boimer, C., Birt, C.M., 2013. Preservative exposure and surgical outcomes in glaucoma patients: the PESO study. J. Glaucoma 22, 730–735.
Burnett, M.G., 1982. The mechanism of the formaldehyde clock reaction: methylene glycol dehydration. J Chem. Educ. 59 (2).
CANCER, W.H.O.I.A.F.R.O, 2004. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.
Champeau, E., Edelhauser, H., 1986. The Preocular Tear Film in Health, Disease, and Contact Lens Wear. DryEyeInstitute,Inc, Lubbock,TX.
Charnock, C., 2006. Are multidose over-the-counter artificial tears adequately preserved? Cornea 25, 432–437.
Cheng, G., Shi, Y., Sturla, S.J., Jalas, J.R., McIntee, E.J., Villalta, P.W., Wang, M., Hecht, S.S., 2003. Reactions of formaldehyde plus acetaldehyde with deoxyguanosine and DNA: formation of cyclic deoxyguanosine adducts and formaldehyde cross-links. Chem. Res. Toxicol. 16, 145–152.
Conaway, C.C., Whysner, J., Verna, L.K., Williams, G.M., 1996. Formaldehyde mechanistic data and risk assessment: endogenous protection from DNA adduct formation. Pharmacol. Therapeut. 71, 29–55.
Coroneo, M.T., Rosenberg, M.L., Cheung, L.M., 2006. Ocular effects of cosmetic products and procedures. Ocul. Surf. 4, 94–102.
Ding, J., Sullivan, D.A., 2014. The effects of insulin-like growth factor 1 and growth hormone on human meibomian gland epithelial cells. JAMA ophthalmology 132, 593–599.
Epstein, S.P., Ahdoot, M., Marcus, E., Asbell, P.A., 2009. Comparative toxicity of preservatives on immortalized corneal and conjunctival epithelial cells. Journal of ocular pharmacology and therapeutics. official J. Assoc. Ocul. Pharmacol. Therapeut. 25, 113–119.
EUROPEAN, T.E.P.A.T.C.O.T. UNION, 2009. Regulation (Ec) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products.
Exposures add up - Survey results. in: ewg.org. 2017 https://www.ewg.org/skindeep/2004/06/15/exposures-add-up-survey-results/#.WqK0CWrwa70.
Gao, Y., Kanengiser, B.E., 2004. Categorical evaluation of the ocular irritancy of cosmetic and consumer products by human ocular instillation procedures. J. Cosmet. Sci. 55, 317–325.
Gipson, I.K., Spurr-Michaud, S., Argueso, P., Tisdale, A., Ng, T.F., Russo, C.L., 2003. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. Investig. Ophthalmol. Vis. Sci. 44, 2496–2506.
Gomes, J.A.P., Azar, D.T., Baudouin, C., Efron, N., Hirayama, M., Horwath-Winter, J., Kim, T., Mehta, J.S., Messmer, E.M., Pepose, J.S., Sangwan, V.S., Weiner, A.L., Wilson, S.E., Wolffsohn, J.S., 2017. TFOS DEWS II iatrogenic report. Ocul. Surf. 15, 511–538.
Ishibashi, T., Yokoi, N., Kinoshita, S., 2003. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. J. Glaucoma 12, 486–490.
Jono, K., Takayama, T., Kuno, M., Higashide, E., 1986. Effect of alkyl chain length of benzalkonium chloride on the bactericidal activity and binding to organic materials. Chem. Pharmaceut. Bull. 34, 4215–4224.
Kahook, M.Y., Noecker, R.J., 2008. Comparison of corneal and conjunctival changes after dosing of travoprost preserved with sofZia, latanoprost with 0.02% benzalkonium chloride, and preservative-free artificial tears. Cornea 27, 339–343.
Kreiger, R.A., Garry, V.F., 1983. Formaldehyde-induced cytotoxicity and sister-chromatid exchanges in human lymphocyte cultures. Mutat. Res. 120, 51–55.
Lai, L.J., Hsu, W.H., Wu, A.M., Wu, J.H., 2013. Ocular injury by transient formaldehyde exposure in a rabbit eye model. PLoS One 8 e66649.
Lang, I., Bruckner, T., Triebig, G., 2008. Formaldehyde and chemosensory irritation in humans: a controlled human exposure study. Regulatory toxicology and pharmacology. RTP 50, 23–36.
Lee, E., Kim, H.J., Lee, M., Jin, S.H., Hong, S.H., Ahn, S., Kim, S.O., Shin, D.W., Lee, S.T., Noh, M., 2016. Cystathionine metabolic enzymes play a role in the inflammation resolution of human keratinocytes in response to sub-cytotoxic formaldehyde exposure. Toxicol. Appl. Pharmacol. 310, 185–194.
Li, Q., Mei, Q., Huyan, T., Xie, L., Che, S., Yang, H., Zhang, M., Huang, Q., 2013. Effects of formaldehyde exposure on human NK cells in vitro. Environ. Toxicol. Pharmacol. 36, 948–955.
Lin, Z., Liu, X., Zhou, T., Wang, Y., Bai, L., He, H., Liu, Z., 2011. A mouse dry eye model induced by topical administration of benzalkonium chloride. Mol. Vis. 17, 257–264.
Liu, Y., Ding, J., 2014. The combined effect of azithromycin and insulin-like growth factor-1 on cultured human meibomian gland epithelial cells. Investig. Ophthalmol. Vis. Sci. 55, 5596–5601.
Liu, Y., Levy, R., 1997. Phospholipase A2 has a role in proliferation but not in differentiation of HL-60 cells. Biochim. Biophys. Acta 1355, 270–280.
Liu, S., Hatton, M.P., Khandelwal, P., Sullivan, D.A., 2010. Culture, immortalization, and characterization of human meibomian gland epithelial cells. Investig. Ophthalmol. Vis. Sci. 51, 3993–4005.
Liu, S., Kam, W.R., Ding, J., Hatton, M.P., Sullivan, D.A., 2013. Effect of growth factors on the proliferation and gene expression of human meibomian gland epithelial cells. Investig. Ophthalmol. Vis. Sci. 54, 2541–2550.
Liu, Y., Kam, W.R., Ding, J., Sullivan, D.A., 2014a. Effect of azithromycin on lipid accumulation in immortalized human meibomian gland epithelial cells. JAMA ophthalmology 132, 226–228.
Liu, Y., Kam, W.R., Ding, J., Sullivan, D.A., 2014b. One man's poison is another man's meat: using azithromycin-induced phospholipidosis to promote ocular surface health. Toxicology 320, 1–5.
Liu, Y., Kam, W.R., Ding, J., Sullivan, D.A., 2015. Can tetracycline antibiotics duplicate the ability of azithromycin to stimulate human meibomian gland epithelial cell differentiation? Cornea 34, 342–346.
Liu, Y., Kam, W.R., Sullivan, D.A., 2016. Influence of omega 3 and 6 fatty acids on human meibomian gland epithelial cells. Cornea 35, 1122–1126.
Malik, A., Claoue, C., 2012. Transport and interaction of cosmetic product material within the ocular surface: beauty and the beastly symptoms of toxic tears. Contact lens & anterior eye. J. Br. Contact Lens Assoc. 35, 247–259.
MarketResearch.com: The U.S. Beauty and Cosmetics Market Expected to Exceed $62 Billion in 2016, MarketResearch.com.
Martone, G., Frezzotti, P., Tosi, G.M., Traversi, C., Mittica, V., Malandrini, A., Pichierri, P., Balestrazzi, A., Motolese, P.A., Motolese, I., Motolese, E., 2009. An in vivo confocal microscopy analysis of effects of topical antiglaucoma therapy with preservative on corneal innervation and morphology. Am. J. Ophthalmol. 147, 725–735 e721.
Matsuoka, T., Takaki, A., Ohtaki, H., Shioda, S., 2010. Early changes to oxidative stress levels following exposure to formaldehyde in ICR mice. J. Toxicol. Sci. 35, 721–730.
Maurer, J.K., Molai, A., Parker, R.D., Li, L.I., Carr, G.J., Petroll, W.M., Cavanagh, H.D.,

Jester, J.V., 2001. Pathology of ocular irritation with acetone, cyclohexanol, parafluoroaniline, and formaldehyde in the rabbit low-volume eye test. Toxicol. Pathol. 29, 187–199.
Merk, O., Speit, G., 1998. Significance of formaldehyde-induced DNA-protein crosslinks for mutagenesis. Environ. Mol. Mutagen. 32, 260–268.
Ng, A., Evans, K., North, R., Purslow, C., 2012. Eye cosmetic usage and associated ocular comfort. Ophthalmic & physiological optics. J. Br. Coll. Ophthalmic Opticians 32, 501–507.
Noecker, R., 2001. Effects of common ophthalmic preservatives on ocular health. Adv. Ther. 18, 205–215.
Noecker, R.J., Herrygers, L.A., Anwaruddin, R., 2004. Corneal and conjunctival changes caused by commonly used glaucoma medications. Cornea 23, 490–496.
O'Dell, L.E., Sullivan, A., Periman, L.M., 2016. Beauty does not have to hurt. Adv Ocul Care July/August 42–47.
Orsiere, T., Sari-Minodier, I., Iarmarcovai, G., Botta, A., 2006. Genotoxic risk assessment of pathology and anatomy laboratory workers exposed to formaldehyde by use of personal air sampling and analysis of DNA damage in peripheral lymphocytes. Mutat. Res. 605, 30–41.
Paimela, T., Ryhanen, T., Kauppinen, A., Marttila, L., Salminen, A., Kaarniranta, K., 2012. The preservative polyquaternium-1 increases cytoxicity and NF-kappaB linked inflammation in human corneal epithelial cells. Mol. Vis. 18, 1189–1196.
Pauly, A., Brignole-Baudouin, F., Labbe, A., Liang, H., Warnet, J.M., Baudouin, C., 2007. New tools for the evaluation of toxic ocular surface changes in the rat. Investig. Ophthalmol. Vis. Sci. 48, 5473–5483.
Pisella, P.J., Debbasch, C., Hamard, P., Creuzot-Garcher, C., Rat, P., Brignole, F., Baudouin, C., 2004. Conjunctival proinflammatory and proapoptotic effects of latanoprost and preserved and unpreserved timolol: an ex vivo and in vitro study. Investig. Ophthalmol. Vis. Sci. 45, 1360–1368.
Rajabalian, S., Meimandi, M.S., Badinloo, M., 2009. Diclofenac inhibits proliferation but not NGF-induced differentiation of PC12 cells. Pak. J. Pharm. Sci. 22, 259–262.
Rawlins, R., 2017. Fordham Environmental Law Review: Teething on Toxins: In Search of Regulatory Solutions for Toys and Cosmetics.
Review, C.I., 2012. Formaldehyde and methylene glycol. In: CIR Expert Panel Meeting.
Robertson, D.M., Li, L., Fisher, S., Pearce, V.P., Shay, J.W., Wright, W.E., Cavanagh, H.D., Jester, J.V., 2005. Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line. Investig. Ophthalmol. Vis. Sci. 46, 470–478.
Scheman, A., 2000. Adverse reactions to cosmetic ingredients. Dermatol. Clin. 18, 685–698.
Schmid, O., Speit, G., 2007. Genotoxic effects induced by formaldehyde in human blood and implications for the interpretation of biomonitoring studies. Mutagenesis 22, 69–74.
Song, G., Ouyang, G., Bao, S., 2005. The activation of Akt/PKB signaling pathway and cell survival. J. Cell Mol. Med. 9, 59–71.
Speit, G., Schutz, P., Hogel, J., Schmid, O., 2007. Characterization of the genotoxic potential of formaldehyde in V79 cells. Mutagenesis 22, 387–394.
Sullivan, D.A., Liu, Y., Kam, W.R., Ding, J., Green, K.M., Shaffer, S.A., Hatton, M.P., Liu, S., 2014. Serum-induced differentiation of human meibomian gland epithelial cells. Investig. Ophthalmol. Vis. Sci. 55, 3866–3877.
Tang, X.Q., Ren, Y.K., Chen, R.Q., Zhuang, Y.Y., Fang, H.R., Xu, J.H., Wang, C.Y., Hu, B., 2011. Formaldehyde induces neurotoxicity to PC12 cells involving inhibition of paraoxonase-1 expression and activity. Clin. Exp. Pharmacol. Physiol. 38, 208–214.
Tressler, C.S., Beatty, R., Lemp, M.A., 2011. Preservative use in topical glaucoma medications. Ocul. Surf. 9, 140–158.
Tyihak, E., Bocsi, J., Timar, F., Racz, G., Szende, B., 2001. Formaldehyde promotes and inhibits the proliferation of cultured tumour and endothelial cells. Cell Prolif 34, 135–141.
Varani, J., Perone, P., Spahlinger, D.M., Singer, L.M., Diegel, K.L., Bobrowski, W.F., Dunstan, R., 2007. Human skin in organ culture and human skin cells (keratinocytes and fibroblasts) in monolayer culture for assessment of chemically induced skin damage. Toxicol. Pathol. 35, 693–701.
Wilson, W.S., Duncan, A.J., Jay, J.L., 1975. Effect of benzalkonium chloride on the stability of the precorneal tear film in rabbit and man. Br. J. Ophthalmol. 59, 667–669.
Xiong, C., Chen, D., Liu, J., Liu, B., Li, N., Zhou, Y., Liang, X., Ma, P., Ye, C., Ge, J., Wang, Z., 2008. A rabbit dry eye model induced by topical medication of a preservative benzalkonium chloride. Investig. Ophthalmol. Vis. Sci. 49, 1850–1856.
Yaqng, F., Yu, Y., Wang, K., Zhang, H., Wang, H., Wang, R., Yi, J., 2016. Effect of formaldehyde exposure on the level of cytokines in human bronchial epitheial 16HBE cells. Zhonghua lao dong wei sheng zhi ye bing za zhi = Zhonghua laodong weisheng zhiyebing zazhi = Chinese journal of industrial hygiene and occupational diseases 34, 27–31.
Ye, X., Yan, W., Xie, H., Zhao, M., Ying, C., 2005. Cytogenetic analysis of nasal mucosa cells and lymphocytes from high-level long-term formaldehyde exposed workers and low-level short-term exposed waiters. Mutat. Res. 588, 22–27.