Experimental Eye Research 120 (2014) 82–89

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Cationorm shows good tolerability on human HCE-2 corneal epithelial cell cultures

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ARTICLE INFO

Article history:
Received 20 September 2013
Accepted in revised form 7 January 2014
Available online 22 January 2014

Keywords:
corneal epithelium
dry eye syndrome
preservatives
cationic emulsion

ABSTRACT

Preservatives have been for a long time known to cause detrimental effects on ocular surface. Cationorm, a preservative-free compound with electrostatic properties is a novel way to solve the problems encountered with traditional benzalkonium chloride (BAK)-containing eye drops. The aim of this study was to evaluate tolerability of the preservative-free cationic emulsion Cationorm in vitro on corneal epithelial cells. The human corneal epithelial cell (HCE-2) culture line was used to study cellular morphology, cytotoxicity and inflammatory responses after Cationorm diluted 1/10 exposure for 5, 15 and 30 min. Exposures to Systane diluted 1/10 with polyquaternium-1/polidronium chloride 0.001% as preservative, BAK 0.001% or C16 (0.0002%) and normal cell culture medium served as positive and negative references. Cell viability was determined by measuring the release of lactate dehydrogenase (LDH) and mitochondrial dehydrogenase activity was evaluated using 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The possible induction of apoptosis was analyzed by measuring the activity of caspase-3, and Cell Counting Kit-8 (CCK-8) was used to evaluate the number of viable cells after the exposure to test compounds. Furthermore, the tendency of the test compounds to produce inflammatory reaction was determined by analyzing the production of proinflammatory cytokines IL-6 and IL-8, and DNA binding of the p65 subunit of transcription factor NF-κB was measured from cell lysates. HCE-2 cells showed no morphological changes after the exposure to Cationorm, but in cells exposed to BAK, clear cytoplasm vacuolization and loose cell–cell contacts were observed in transmission (TEM) or scanning (SEM) electron microscopic analyses. Cell viability, as measured with the release of LDH, indicated a time dependent increase in LDH expression after exposure to all test compounds but especially with BAK. Moreover, Cationorm and BAK time-dependently decreased the mitochondrial metabolism to 73% with Cationorm and 53% with BAK from that of the control cells after 30 min exposure in MTT assay. BAK was the only test compound having clear adverse effects on the cell number and metabolism in CCK-8 assay. The activity of caspase-3 did not show significant differences between the groups. Inflammatory response after exposure to Cationorm was significantly lower than after exposure to BAK. There were no significant differences in NF-κB activity between the groups. Diluted Cationorm and Systane with polyquaternium-1/polidronium chloride 0.001% showed good tolerability on HCE-2 cells and thereby provide a clear improvement when compared to BAK-containing eye drop formulations.

1. Introduction

Dry eye syndrome (DES), is one of the most common cause of ocular discomfort and red, irrigated eye (Lemp, 1995). It is defined as a multifactorial disease of the lacrimal system and ocular surface resulting in discomfort, visual disturbance and tear film instability, potentially damaging the corneal and conjunctival epithelial cells. Its pathology is usually idiopathic concerning aging, instability in

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http://dx.doi.org/10.1016/j.exer.2014.01.006

tear composition, and decreased tear secretion (Mishima et al., 1966; Scherz and Dohlman, 1975). Corneal and conjunctival epithelial cells as well as corneal endothelium have been shown in several studies to suffer from apoptosis, inflammation and decreased proliferation in DES (Ayaki et al., 2008; Baudouin, 2005; Burstein, 1980; Tripathi and Tripathi, 1989).

Since DES is a chronic condition, consistent medication with artificial tears is required. Topical eye-drop instillation of aqueous products from a multi-dose container is the traditionally used option to treat the disease. Problems with these compounds include rapid loss of drug due to reflexive tearing and drainage via the nasolacrimal system. It has been indicated that over 80% of the compound instilled onto an ocular surface is washed out via nasolacrimal drainage system (Shell, 1984). There has been lots of effort to prolong the exposure time of the topical drop on the ocular surface including changing of drop composition to more viscous or developing hydrogels. Limitations with these formulations include their limited applicability to hydrophilic drugs and their properties to cause visual disturbances (Lallemand et al., 2012). Furthermore, administration of a more viscous solution can be problematic since the drop is attached more tightly to a container wall and more muscle power is needed to squeeze the container. Challenges in the administration might also increase the risk of infectious contamination of the container tip (Lallemand et al., 2012).

Moreover, for protecting multi-dose containers from contaminations, preservatives, such as quaternary ammoniums have been used in clinical settings (Asbell, 2006). Benzalkonium chloride (BAK), the most widely used preservative in ophthalmic drugs, is a positively charged compound binding to a negatively charged surface of bacteria disrupting their cell membranes (Lallemand et al., 2012). Unfortunately, BAK is capable of damaging epithelial cell membranes of the eye tissues by the same mechanism (Gobbels and Spitznas, 1989; Pisella et al., 2002). Cell membrane damage may subsequently lead to nuclear factor kappa B (NF-κB)-mediated inflammation in the cells (Paimela et al., 2012). NF-κB, a widely expressed inducible transcription factor, is an important regulator of many genes involved in the inflammatory and immune responses, proliferation and apoptosis. BAK solution has been shown to cause cell death on the ocular surface via proinflammatory and proapoptotic routes (Cha et al., 2004; Durand-Cavagna et al., 1989).

Nanotechnology with bioadhesive properties is a new way to solve the problems of stability and preservation in traditional ocular drug delivery. Since bioadhesiveness enables electrostatic interactions with the ocular surface, residence time of the liquid becomes also prolonged. Cationorm is a novel preservative-free compound in a multi-dose container based on the Novasorb emulsion technology. The compound is a cationic oil-in-water emulsion containing positively charged nanodroplets. Cetalkonium chloride is used as the cationic agent to bring the positive charge to the oil droplets in the compound, and other ingredients include glycerol, mineral oil, poloxamer 188, tromethamine, tromethamine hydrochloride, and tyloxapol. The droplets create a large contact surface with the ocular surface cells enhancing the drug absorption (Daull et al. in press; Lallemand et al., 2012). Clinically the drops are indicated in mild and moderate dry eye disease therapy. Previously the tolerability and efficacy of Cationorm have been evaluated in animal models and clinical trials that revealed it to be efficient and safe (Daull et al. in press; Daull et al., 2012; Lallemand et al., 2012). The aim of this study was to evaluate effects of preservative-free cationic emulsion Cationorm on human corneal epithelial cell (HCE-2) morphology, cytotoxicity and inflammatory responses.

2. Methods

2.1. Cell culture and treatments

Human corneal epithelial cells (HCE-2) were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown on 12-well plates (Cellstar®; Greiner Bio-One GmbH, Frickenhausen, Germany) to confluency in a humidified 10% CO_2 atmosphere at 37 °C. Keratinocyte-Serum Free Medium (SFM, with bovine pituitary extract and epidermal growth factor; Life Technologies, Invitrogen, GIBCO®, Paisley, UK) containing insulin 0.005 mg/ml (Sigma–Aldrich, Steinheim, Germany), fetal bovine serum 10% (FBS; Thermo Scientific, Hyclone, Logan, UT) and penicillin 100 U/ml + streptomycin 100 μg/ml (Lonza, Basel, Switzerland) was used as the culture medium. For every well 100,000 cells were seeded and cultured for 48 h before of exposures. If not otherwise stated, cells were exposed to treatments (see below) in six parallel wells for 5, 15, and 30 min, and incubated in fresh medium for 24 h, except for NF-κB activity test for 6 h, before analyses. Cells were washed once with keratinocyte-SFM medium (without any supplements) before and after treatments to prevent protein precipitation caused by BAK. The treatments were: 0-control (normal cell culture medium), Systane (artificial tear drops, Polyquaternium-1/polidronium chloride 0.001% as preservative; Alcon, Fort Worth, TX), Cationorm (Novasorb, Novagali Pharma, Evry cedex, France), BAK (0.001% v/v aqueous solution; FeF Chemicals A/S, Køge, Denmark), and BAK homologue cetalalkonium chloride C16 (0.0002% v/v aqueous solution). No comparative screening between toxicity profiles of BAK and Cationorm was performed but the concentration used for BAK is based on in vitro data published with BAK solutions (Debbasch et al., 2001). Note that Cationorm and Systane drops were diluted in culture medium one to ten prior to exposures. Osmolality values of solutions were performed with an auto-osmometer (Osmostat, OM-6020, Daiichi Kagaku Co, Kyoto, Japan). Undiluted Cationorm showed clearly lower osmolality levels than Systane (Table 1). Osmolality levels were at the same levels, when drops were diluted one to ten. Therefore, diluted concentrations were used in further studies for drop comparison.

2.2. TEM and SEM morphological analyses

For transmission electron microscope (TEM) cell culture samples were prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 2 h at room temperature. After 15 min washing in 0.1 M phosphate buffer, the samples were post-fixed in 1% osmium tetraoxide and 0.1 M phosphate buffer for 1 h, and again washed with phosphate buffer for 15 min prior to standard ethanol dehydration. Subsequently, the samples were infiltrated and embedded in LX-112 resin. Polymerization was carried out at 37 °C for 24 h and at 60 °C for 48 h. The sections were examined with a JEM-2100F transmission electron microscope (Jeol, Tokyo, Japan) at 200 kV for 1500X magnification. For scanning electron microscopy (SEM), HCE-2 cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 2 h. Following fixation, samples were washed

Table 1
Osmolalities of used drugs in the experiments.

Drug	Undiluted (mOsm/kg)	Diluted (mOsm/kg)
SFM	287	–
BAK 0.001%	–	287
Cationorm	197	279
Systane	276	287

Dilutions 1:10 (Cationorm and Systane).

twice in 0.1 M phosphate buffer pH 7.4 for 10 min and then processed by standard dehydration protocol to critical point drying. Dried samples were coated with gold and imaged for 500× magnification using a XL 30 ESEM Philips (Fei Company, Netherlands) scanning electron microscope.

2.3. Cellular viability assays

Integrity of the cell membranes was determined by measuring lactate dehydrogenase (LDH) released to the cell culture medium using the commercial CytoTox 96®-kit (Promega, Fitchburg, WI) according to the manufacturer's instructions. Mitochondrial dehydrogenase activity of the cells was determined using 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Paimela et al., 2012). Moreover, cellular dehydrogenase activity was determined using the colorimetric Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD) which produces colored formazan from the highly water soluble tetrazolium salt (WST)-8. The response of HCE-2 cells to WST-8 was tested in twelve replicates (6000 cells/well) on flat-bottomed 96-well microtitre plates. Preceding and following the treatments, the wells were washed once with supplement-free medium. Cells were incubated with Systane® Ultra, Cationorm®, BAK, or its homologue C16 in a humidified CO_2 incubator at +37 °C for 5, 15 and 30 min. Cells incubated in cell culture medium without other supplements but insulin served as controls. After 24 h incubation in complete culture medium, the water-soluble tetrazolium salt WST-8 was added according to the instructions of the kit manufacturer. During two hours incubation in a humidified CO_2 incubator at +37 °C, the cells produced dehydrogenase enzymes which reduced WST-8 to formazan. The amount of yellow-colored end-product, proportional to the number of living cells, was measured as described below. For creating a calibration curve, 5000–25,000 cells/well were inoculated in ten replicate wells and allowed to settle down for three hours in the incubator. Thereafter, WST-8 was added and the results were measured after two hours incubation. The colorimetric measurements for LDH, MTT formazan, and WST-8 formazan were performed at the wavelengths of 490, 595, and 450 nm, respectively, using a BIO-RAD Model 550 microplate reader (BIO-RAD, Hercules, CA). The reference wavelength of 655 nm was used in the LDH and WST-8 measurements. Activity of the apoptosis marker Caspase-3 was measured from cell lysates using a colorimetric assay kit (Sigma–Aldrich, Steinheim, Germany) according to the instructions of the manufacturer. Assay Buffer provided with the kit served as a negative control. The absorbance values were measured at the wavelength of 405 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD, Hercules, CA).

2.4. Analysis of production and regulation of proinflammatory cytokines

Concentrations of soluble cytokines interleukin (IL)-6 and IL-8 were measured from the cell culture medium samples using commercial enzyme-linked immunosorbent assay (ELISA) reagents (OptEIA™ sets obtained from BD Pharmingen) according to the manufacturer's protocols. Absorbance values following the colorimetric reaction were measured at the wavelength of 450 nm with a reference wavelength of 655 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD, Hercules, CA). DNA binding of the p65 subunit of transcription factor NF-κB was measured from cell lysates using the colorimetric TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif, Rixensart, Belgium) following the instructions of the manufacturer. Absorbances were measured at the wavelength of 450 nm with a reference wavelength of 655 nm using a BIO-RAD Model 550 microplate reader (BIO-RAD, Hercules, CA).

2.5. Statistical analyses

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA). Statistical differences between groups were assessed using the Kruskall–Wallis test, and *post hoc* comparisons using the Mann–Whitney *U*-test. *P*-values of 0.05 or less were considered significant.

3. Results

In order to study morphological alterations on the cells, HCE-2 cells were exposed to fresh culture medium, Systane, Cationorm, BAK, and C16, for 5, 15 and 30 min and analyzed by TEM and SEM. Only in cells exposed to BAK clear cytoplasm vacuolization and swelling after 15 min and loose of cell–cell contacts after 30 min were observed (Fig. 1A and B).

In order to examine the effects of test compounds on the viability of HCE-2 cells, we measured the release of LDH enzyme, and determined the activity of cellular metabolism using two different tetrazolium substrates, *i.e.* MTT and WST-8. Results from the LDH assay were consistent with previous findings indicating BAK as the most harmful compound tested (Fig. 2). After 5 min exposure, BAK showed approximately 25% increase in the LDH expression in culture medium compared to the control group (Fig. 2). In addition, there was a time-dependent increase in the LDH concentration since after 30 min exposure the expression was approximately 45% higher in BAK-treated cells than in the control group. In later time points (15 min and 30 min) all treatment groups except for C16 at 30 min showed increased LDH release when compared to the control group (Fig. 2).

Subsequently, mitochondrial dehydrogenase activity of the cells was evaluated using MTT analysis. Cationorm and BAK time-dependently decreased the mitochondrial metabolism to 73% with Cationorm and 53% with BAK from that of the control cells after 30 min exposure (Fig. 3). Systane and C16 homologue of BAK did not show any significant difference when compared to control cells. In order to get a broader view on the cellular viability, we also used Cell Counting Kit-8 (CCK-8) which does not produce intracellular crystals and involves also other dehydrogenase activity in the cell than just mitochondrial. Color intensity of the WST-8 formazan is proportional to the number of viable cells, and our data indicated that BAK was the only test compound having clear adverse effects on the cell number and metabolism (Fig. 4). After 5 min exposure, the number of cells had decreased by approximately 25% in the BAK group. After 15 min exposure to BAK, the cell number remained at the same level, and at the 30 min timepoint, it dropped down close to 5000 cells/well, being 46% lower than in the control cells. The cell number/viability even increased with Systane, Cationorm and C16 after 15 min exposure, and the increase was still evident with Systane and Cationorm after 30 min exposure.

In addition to positive cell viability tests, we also determined the possible induction of apoptosis by measuring the activity of caspase-3. As shown in Fig. 5, there was an increasing trend in the caspase-3 activity in all groups but the differences were not significant in any time points evaluated. After 30 min of exposure, the trend was even decreasing with Cationorm.

For analyzing the tendency of test compounds to induce inflammation in HCE-2 cells, we measured the production of proinflammatory cytokines IL-6 and IL-8 from the cell culture medium samples. The production of IL-6 was increased after 5 min exposure only in the BAK group (Fig. 6). When compared to the

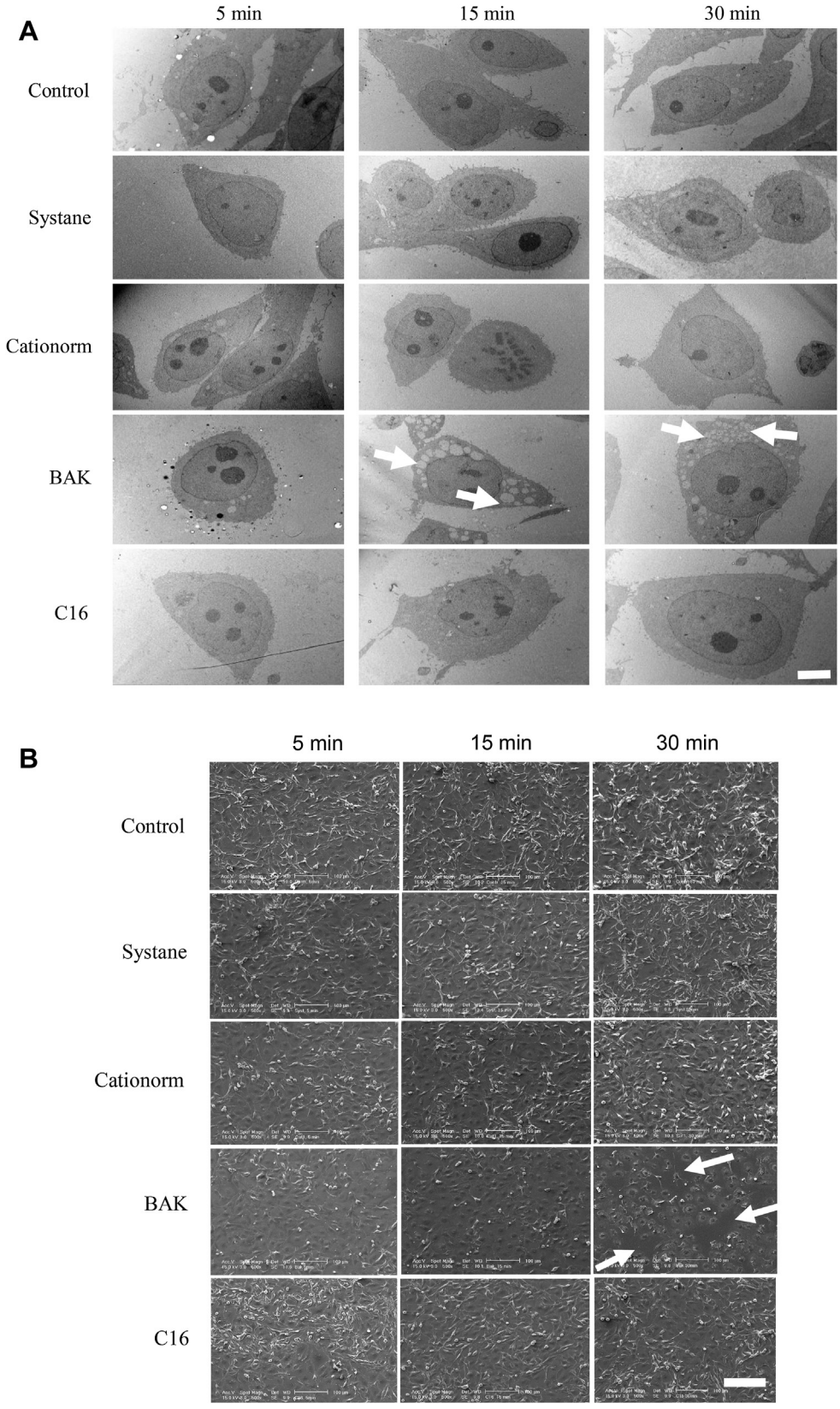

Fig. 1. Morphological alterations on the human corneal epithelial cells (HCE-2). HCE-2 cells were exposed to fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min and subsequently allowed to recover in fresh medium for 24 h. Transmission electron microscope (TEM) micrographs of HCE-2 cells show cytoplasmic vacuolization in the cells after the exposure to BAK (arrows, A). Scanning electron microscope (SEM) micrographs of HCE-2 cells show loose of cell–cell contacts after the exposure to BAK (arrows, B). The scale bar is 5 μm in A, and 100 μm in B.

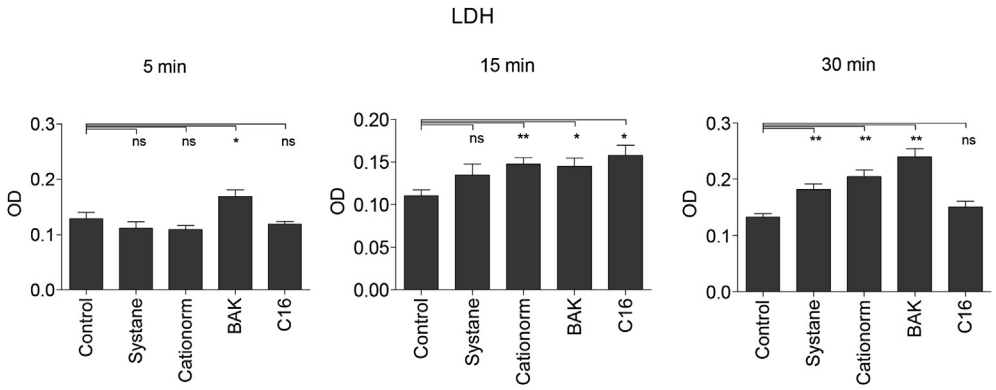

Fig. 2. Release of lactate dehydrogenase (LDH). Human corneal epithelial cells (HCE-2) were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. For statistical analysis, treatments with compounds were compared to untreated controls. Results are presented as mean OD ± SEM from six parallel samples. An asterix indicates $p < 0.05$, ns denotes "not significant" (Mann–Whitney *U*-test).

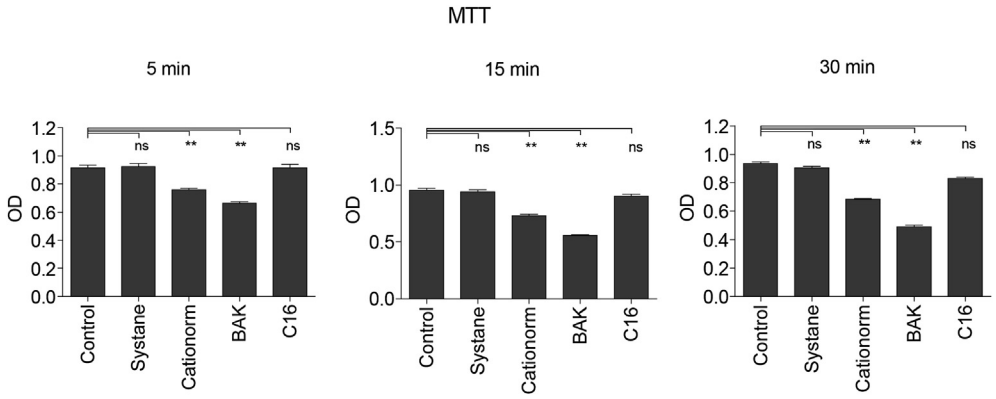

Fig. 3. Cell viability assessed using the MTT assay. Human corneal epithelial cells (HCE-2) were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. For statistical analysis, treatments with compounds were compared to untreated controls. Results of mitochondrial dehydrogenase activity are presented as mean OD ± SEM from six parallel samples. An asterix indicates $p < 0.05$, a double asterix $p < 0.01$, and ns "not significant" (Mann–Whitney *U*-test).

control group, the increase was 57%. After 15 min, also other groups showed a slight but statistically significant increase in the IL-6 expression. However, the increase in the IL-6 levels was over five times higher with BAK exposure than with Systane, Cationorm, or C16. After 30 min, the trend was consistent but the increase in IL-6 levels with BAK was even higher being now eight times higher than with Systane or Cationorm. Concentrations of IL-8 in the culture medium were significantly increased already after 5 min exposure in all other groups than C16 (Fig. 7). The increase was approximately 15% in the Systane and Cationorm groups, and 38% in the BAK group when compared to the control group. After 15 min of exposure, the differences in the expression levels of IL-8 were not significant between the control group and Systane group or C16 group, whereas the increase in the Cationorm group was 20%. However, in the BAK group, the expression was increased by 155% when compared to untreated control cells. After 30 min of

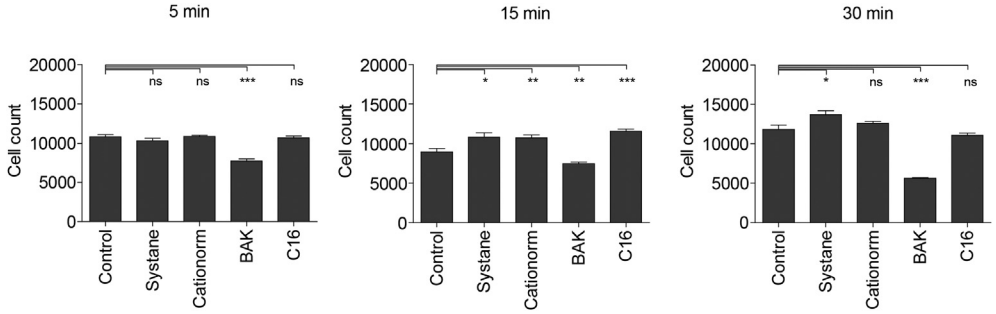

Fig. 4. Cell viability determined using the CCK-8 test. Human corneal epithelial cells (HCE-2) were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. For statistical analysis, treatments with compounds were compared to untreated controls. Results are presented as cell numbers ± SEM from twelve parallel samples. An asterix indicates $p < 0.05$, and a double asterix $p < 0.01$, a triple asterix $p < 0.001$, and ns "not significant" (Mann–Whitney *U*-test).

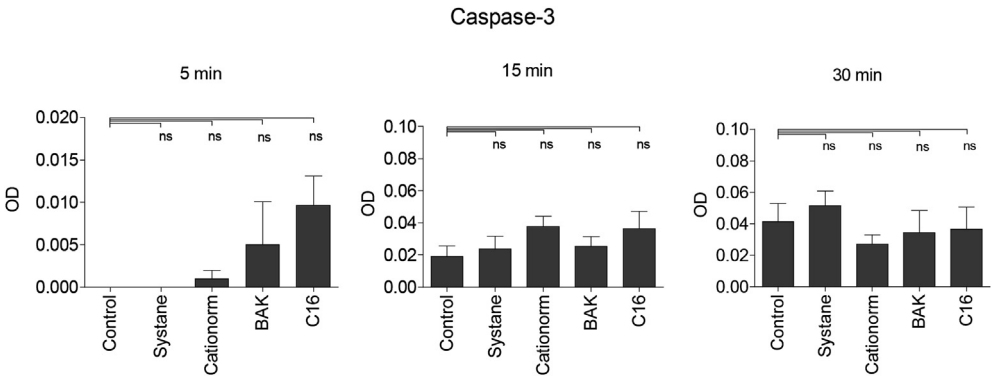

Fig. 5. Activity of caspase-3. Human corneal epithelial cells (HCE-2) were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. Results are presented as mean OD ± SEM from six parallel samples. For statistical analysis, treatments with compounds were compared to untreated controls. ns indicates "not significant" (Mann–Whitney *U*-test).

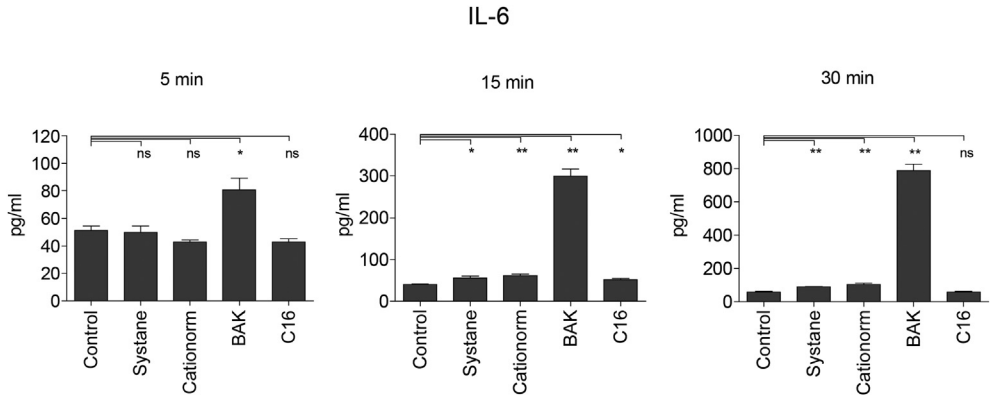

Fig. 6. Interleukin-6 secretion by human corneal epithelial cells (HCE-2). HCE-2 cells were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. Cytokine concentrations measured from six parallel medium samples are presented as mean pg/ml ± SEM. Treatments with eye drops and preservatives were compared to untreated controls. Statistical significance is shown as an asterix $p < 0.05$, a double asterix $p < 0.01$, or ns denoting "not significant" (Mann–Whitney *U*-test).

exposure, the increase in the IL-8 production was 20%, 34%, and 405% higher in Systane, Cationorm, and BAK groups, respectively, when compared to the control group. It should be noted that increased IL-levels for Cationorm and Systane are very low, although statistical significance can be observed.

Since both IL-6 and IL-8 could be produced through NF-κB signaling, we determined the DNA binding of the active subunit p65. Our results show that there were no significant differences in the NF-κB activity between the groups (Fig. 8).

4. Discussion

BAK is generally considered to be a toxic and proinflammatory compound for the ocular surface (Cha et al., 2004; Durand-Cavagna

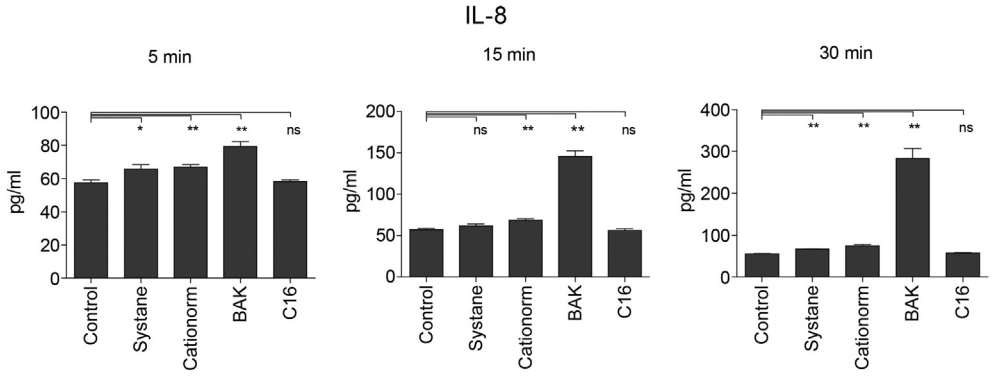

Fig. 7. Interleukin-8 secretion by human corneal epithelial cells (HCE-2). HCE-2 cells were incubated either with fresh cell culture medium (control) or different eye drops (Systane, Cationorm) or preservatives (benzalchoniumchloride (BAK), C16 homologue of BAK) for 5, 15, and 30 min. IL-8 concentrations measured from six parallel medium samples are presented as mean pg/ml ± SEM. Treatments with eye drops and preservatives were compared to untreated controls. Statistical significance is indicated as an asterix $p < 0.05$, a double asterix $p < 0.01$, or ns denoting "not significant" (Mann–Whitney *U*-test).

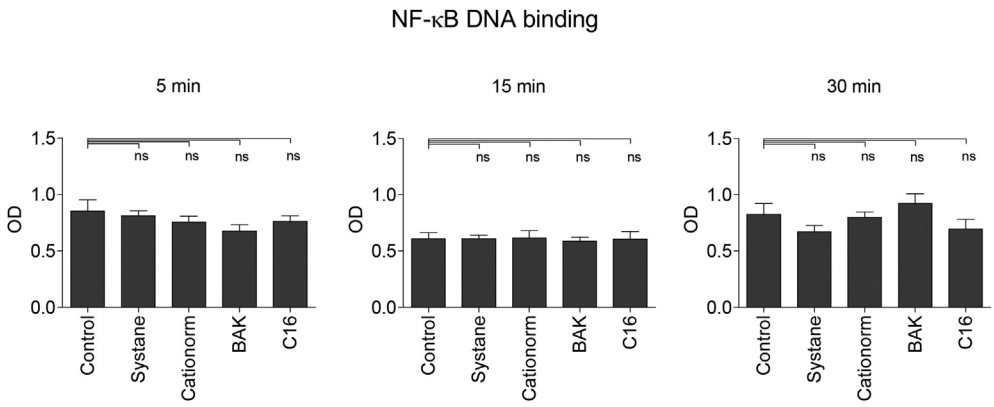

Fig. 8. Binding of active subunit p65 of NF-κB to DNA 6 h after the exposure of human corneal epithelial cells (HCE-2) to eye drops and preservatives. The results were normalized to protein concentrations and compared to the results of untreated controls. The results are presented as mean OD ± SEM. ns indicates "not significant" (Mann–Whitney *U*-test).

et al., 1989). The phenomenon was obtained also in this study although the concentration of BAK in assays was significantly lower (0.001%) than in most of the commercial eye drops containing BAK. Present results suggest that higher concentrations of BAK are even more detrimental to epithelial cells as recently showed (Paimela et al., 2012). Especially in DES, these detrimental reactions can be potentiated in epithelial cells due to the stress caused by disease pathology itself. In previous studies, it has been shown that toxicity of BAK is dependent on the lengths of alkyl chains of the molecule. Consistent with previous findings (Pellinen et al., 2012; Uematsu et al., 2010), C16 seemed to cause less inflammation and cell death on corneal epithelial cells than BAK. However, commercial preparations of BAK contain a mixture of alkyl chains of different lengths, the most toxic of them constituting a major proportion of the compound (Pellinen et al., 2012).

Alternative preservatives for BAK have been generated, but many of them still induce adverse effects on the eye surface cells (Ammar et al., 2010; Paimela et al., 2012). Preservative-free eye drops have proven to be the safest therapy, although stability of the medication has been a problem with eye drops in single-use containers (Jaenen et al., 2007). Moreover, due to lacrimal system, most of the instilled drug is washed away with tears. More viscous compounds have a longer residence time on the ocular surface but side effects may include visual disturbances (due to the viscosity of the drop) (Daull et al. in press; Lallemand et al., 2012). To avoid these disadvantages, nanotechnologies with bioadhesive properties have been developed (Lallemand et al., 2012).

This is the first study evaluating the effects of Cationorm exposure on corneal HCE-2 epithelial cells in vitro. We showed that Cationorm and Systane evoked a very mild upregulation of IL-6 and IL-8 secretion but the inflammatory response was significantly weaker than after the exposure of the cells to BAK. Both proinflammatory cytokines might be regulated by the transcription factor NF-κB (Blackwell and Christman, 1997). We did not, however, see any change in the DNA binding capacity of active subunit of NF-κB which suggests that the cytokines were regulated through a different signaling system, or that the transcription factor is not activated due to the minor and obviously physiological inflammatory response in the present conditions. One signaling factor for IL production might be the stress-activated protein kinase/c-Jun N-terminal kinase which we have been previously shown to become activated by UVB-irradiation in HCE-2 cells (Jauhonen et al., 2011). Our recent publication reveals that NF-κB has been activated in response to the undiluted polyquaternium-1 containing eye drops (Paimela et al., 2012). However, this does not necessarily simulate the situation in vivo since the concentrations of the compounds are rapidly diluted by tear fluid on the eye surface. In vitro, repeating short exposition is difficult to manage repeatedly. Thus to approximate in vitro the same exposition as the one existing in vivo, it is reasonable to increase the exposition time and concomitantly reduce the concentration of the eye drop. This strategy has been used also by other groups (Ayaki et al., 2012a, 2012b; Liang et al., 2012). Therefore, we used Cationorm and Systane in this study that were diluted one to ten to more closely mimic the in vivo situation. Furthermore, exposure times in vivo are generally short even with the most sustainable agents. Therefore, the time points of 15 min and 30 min in the present study are certainly too long for simulating the in vivo situation directly on the ocular surface. Note that dilution has a clear effect on inflammation and cytotoxicity results when compared to pure drug exposure in HCE-2 cell cultures (Paimela et al., 2012). This should be thought when severe dry eye patients with a weak tear film are treated with lubricative drops.

The activity of mitochondrial dehydrogenase was decreased after the treatment with Cationorm but a greater decrease was seen with BAK. Importantly, the overall cellular dehydrogenase capacity was not decreased with Cationorm, although a significant decrease was seen with BAK. Our results suggest that Cationorm could be a safer option than BAK-containing compounds also in clinical use, which was supported by TEM and SEM morphological analyses. Constancy in the activity of caspase-3 alludes that none of the compounds tested induces apoptosis in corneal epithelial cells. Transient increase in the production of IL-6 and IL-8 may even protect from apoptosis since both cytokines have been shown to inhibit apoptosis via several mechanisms (Abdollahi et al., 2005; Biffl et al., 1995). BAK-induced morphological and biochemical changes in this study revealed more necrotic cell death rather than apoptotic cell death type in the HCE-2 cells.

In conclusion, Cationorm and Systane were well tolerated on HCE-2 cells even at longer time exposures than would be realistic in vivo. Therefore, both of them might be a good choice for a long time use on dry eye syndrome.

Acknowledgments

This work was supported by the EVO grants of Kuopio University Hospital, the Finnish Eye Foundation, the Academy of Finland, and the Päivikki and Sakari Sohlberg Foundation.

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