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High-Frequency Application of Cationic Agents Containing Lubricant Eye Drops Causes Cumulative Corneal Toxicity in an *Ex Vivo* Eye Irritation Test Model

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Abstract

Purpose: High-frequency applied cetalkonium chloride (CAC) and benzalkonium chloride (BAC) 0.02% did not hamper corneal healing in a living rabbit model of induced corneal erosion. In contrast, the *ex vivo* eye irritation test (EVEIT) shows inhibition of healing for these substances. In a systematic *ex vivo* reproduction of the *in vivo* experiments, we discuss the background of these differences.
Methods: Excised rabbit corneas ($n = 5$ per group) were cultured in artificial anterior chambers (EVEIT). Four erosions were induced for each cornea before starting regular 21 installations/day over 3 days of (1) CAC containing eye drops (Cationorm®), (2) 0.02% BAC. Corneal fluorescein staining, quantification of glucose-/lactate consumption, and histology were performed.
Results: BAC 0.02% treated corneas showed increased epithelial lesions from $10.13 \pm 0.65\,mm^2$ to $10 \pm 0.8\,mm^2$ on day 0, to $86.82 \pm 5.18\,mm^2$ ($P < 0.0001$) by day 3. After a trend toward smaller lesions for CAC on day 1, erosion sizes increased significantly by day 3 from $9.82 \pm 0.30\,mm^2$ to $29.51 \pm 16.87\,mm^2$ ($P < 0.05$). For 1 cornea, corneal erosions nearly disappeared on day 3 ($0.89\,mm^2$). Corneal lactate increased significantly for BAC and CAC, whereas glucose concentrations were unchanged. Histology revealed disintegration of the corneal structures for both compounds.
Conclusions: The data underline the EVEIT as a predictive toxicity test to show side effects in a time-compressed manner. The consistency of these predictions was previously demonstrated by the EVEIT for BAC, phosphate buffer, and others. The EVEIT is suited for a chronic application prediction of tolerability and toxic side effects of eye drops in particular, and other chemicals in general.

Keywords: EVEIT, ocular toxicity prediction, epithelial healing, ophthalmic preservatives tolerability

Introduction

According to the 2017 International Dry Eye Workshop II report, dry eye is ''a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film. This is accompanied by ocular symptoms. The instability and hyperosmolarity of the tear film and ocular surface results in inflammation and damage.''[1] The prevalence of dry eye syndrome (DES) ranges from 7% in the United States to 30% in East Asia.[2,3]

Two sub-pathologies, tear deficiency and evaporative DES, as well as a mixed form, have been described. Tear deficiency is caused by lacrimal gland dysfunction, whereas evaporative DES is caused by Meibomian gland dysfunction leading to enhanced tear evaporation leaking from the surface, due to missing protection by Meibomian oils. Clinically, patients suffer from itching, burning, light sensitivity, and blurred vision.[4,5] In mild to moderate DES, topical application of lubricant eye drops is the first-line therapy. A review analyzing the treatment outcomes of peer-reviewed studies from 1947 until 2007 found an overall improvement of factors such as tear film stability and corneal surface damage by an overall 25%.[6] Improvement depended on the type of lubricant applied. Although the worst results have been shown for saline and intermediate results for hypromellose, carbomers, and polyacrylic formulations, hyaluronic acid presented the best results over a 30-day period of treatment. Aside from treating aqueous tear deficiency, today lubricants are marketed with active ingredients that address tear deficiency (carbomers) as well as enhanced tear evaporation (oils). Examples of such products are Optive® (carboxymethylcellulose, glycerol), Cationorm® (mineral oils, glycerol), Genteal Moderate®

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(dextran, hypromellose, glycerin), and Soothe Lubricant Eye Drops Preservative Free® (propylene glycol, glycerin); whereas products such as Oasis tears® (glycerin) and Evo-Tears® [perfluorohexyloctane (F6H8)] solely aim at replacing Meibomian oils.

Given the prevalence of DES and the large number of lubricants available, no large double-masked study has been rolled out to address the possible side effects of treatment. With regards to preservatives in ophthalmological solutions, multiple studies have addressed the inflammatory, epithelial toxic, and proapoptotic effects of benzalconium chloride (BAC).[7–9] Today, BAC toxicity is widely accepted; therefore, in novel formulations, BAC has mostly been replaced by so called ''soft preservatives.'' Nevertheless, for other diseases such as glaucoma, in 48% of cases, patients applying BAC-preserved antiglaucoma eye drops suffer from ocular pain or discomfort, compared with 19% for unpreserved eye drops.[10] This is especially since BAC itself is known to induce DES.[11,12] Looking at alternative preservatives, we demonstrated corneal epitheliopathy and metabolic stress by applying cetalkonium chloride (CAC, Cationorm), a quaternary ammonium compound and an oxidative-type preservative formulated in lubricant eye drops.[13] For these experiments, we made use of the e*x vivo* eye irritation test (EVEIT) system, a non-animal test that simulates the anterior ocular chamber with a physiological corneal barrier for studying corneal drug toxicity and permeability.[14,15]

The EVEIT system was established according to the 3R principles[16]: to (R)educe animal consumption, (R)efine animal research by avoiding animal suffering, and, more recently, to (R)eplace common animal models. Regarding our results on BAC and CAC, our results, and the whole EVEIT system in particular, were questioned by a recent study by Daull et al.[17] In their study, Daull's group applied the compounds CAC and BAC to the abraded corneas of living rabbits, similar to our EVEIT experiment published by Pinheiro et al.[13] In contrast to the EVEIT system, they detected no interference of BAC and CAC with corneal healing in live rabbits.

The current study was designed in the same manner to question the validity of our EVEIT model.

Methods

Ex vivo *eye irritation test*

The experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research.

In brief, the EVEIT system is composed of a culture of rabbit corneas whereby rabbit eyes are enucleated from slaughterhouse rabbits and the corneas are excised and placed on top of an artificial anterior ocular chamber for long-term nutrition within 8 h postmortem. For nutrition, the chamber is constantly supplied with a culture medium containing Earle's salts and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer [Minimal Essential Medium Eagle (MEM), HEPES buffer 5.8 g/L, both Biochrome GmbH, Germany].

In these current experiments, the medium was constantly replenished by using a micropump (Ismatec IPC, IDEX Health & Science GmbH, Wertheim, Germany) with a starting pH-value of 7.4 ± 0.2 and a flow rate of 6.44 µL/min, which imitates the physiological conditions of the eye. In the experiments 5 corneas were used per test substance. The corneas were incubated at a temperature of 32°C and a humidity of more than 95% throughout all the experiments.

All experiments were performed in accordance with the Code of Ethics of the World Medical Association.

Test substances and experimental procedure

We compared the influence of 2 quaternary ammonium compounds on corneal healing efficacy: benzalkonium chloride (BAC), 0.02% solved in Ringer solution from a 50% stock solution [BatchN° 63581, Molekula, UK, BAC: contains varying amounts of $C_9H_{13}ClNR$ ($R = C_8H_{17}$ till $C_{18}H_{37}$)] and the CAC containing formulation Cationorm, which is a lubricant eye medication (batches: SS282, 2019-09, ST444, 2020-12; Santen SAS., CAC: $C_{25}H_{46}ClN$, $M_r = 396.1$ g/mol).

Both test formulations were used in quintuple for each treatment group. In accordance with the *in vivo* experimental study design of Daull et al.,[17] 19 ± 1 µL of the respective test substance (BAC or Cationorm) was applied 45 min apart, for up to 21 installations per day. The exception is day 1, which started 1 h after the completion of the corneal abrasion procedure and had 16 installations (Fig. 1).

Corneal abrasions

Corneas were evaluated by microscopy after 24 h of stabilization within the EVEIT culturing system. Corneas with

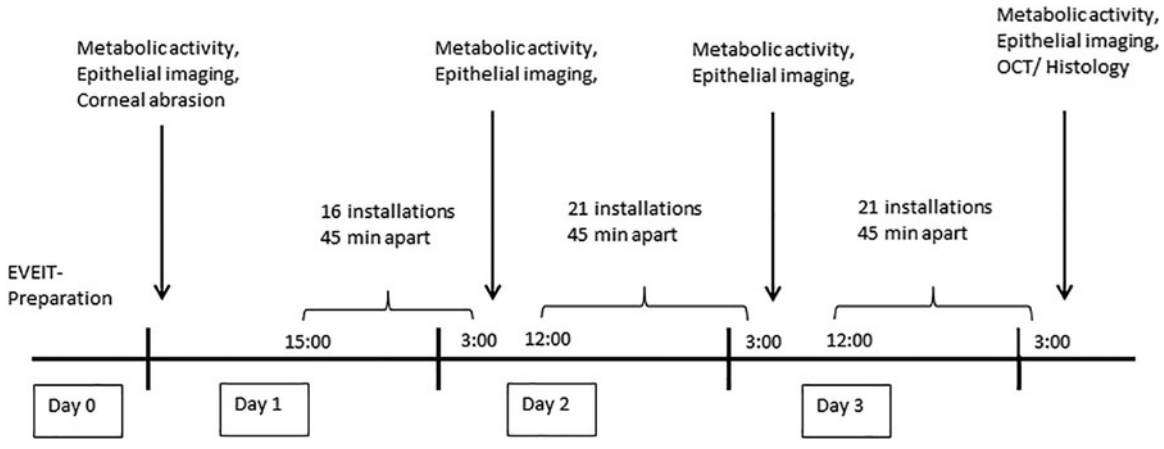

FIG. 1. Schema of experimental procedure and timeline.

any epithelial defects or opacity after this pre-incubation were excluded from further experiments.

During the experiments, the integrity of both the epithelial and endothelial sites was monitored daily for 3 days by using a phase-contrast microscope-integrated camera (KY-F1030U Camera, JVC, Bad Vilbel, Z16 APO Microscope, Wetzlar, Germany) connected to DISKUS software (Hilgers, Koenigswinter, Germany).

Before the corneal healing experiments started, 4 small epithelial abrasions measuring 2.01–3.05 mm^2 were induced by an abrasive corneal drill, which was placed on the cornea in a square pattern. Defect sizes were monitored by fluorescein sodium stains (0.17% aqueous solution), with yellow-green fluorescence indicating the areas of epithelial defects. To take daily measurements, the erosions were circumscribed by using a software tool of the microscope-integrated camera (KY-F1030U Camera, JVC, Bad Vilbel, Germany) mounted on a Z16 APO Microscope (Wetzlar, Germany) connected to DISKUS software (Hilgers, Koenigswinter, Germany). The erosion sizes (a sum of 4 erosions to each cornea) are given in square millimeters.

Metabolic activity

Before the experiments started, and daily thereafter, corneal metabolic activity was assessed. Therefore, the concentrations of glucose (GOD-PAP, Greiner Diagnostic GmbH, Bahlingen, Germany) and lactate (LOD-PAP, Greiner Diagnostic GmbH, Bahlingen, Germany) were quantified photometrically (EPOCH microplate reader, BioTek Instruments GmbH, Bad Friedrichshall, Germany) in the eluted medium of the anterior chamber after passing through the corneal endothelium.

Histology

Corneas embedded in paraformaldehyde 3.7% (w/w) were stained by using the conventional hematoxylin- and eosin-staining method. Sections (5 μm) were viewed and assessed with a digital camera (KY-F75U, JVC) mounted on a LEICA DM6000 B microscope [Leica Microsystems GmbH, Wetzlar, Germany]). A software tool of the microscope was used (DISKUS software, Bonn Bad Godesberg, Germany). For comparison to Cationorm and BAC-treated corneas, a control cornea was evaluated that underwent no treatment except for induction of 4 small epithelial abrasions before the healing experiments started.

Statistical analysis

Differences between corneal erosion sizes and glucose and lactate concentrations of artificial anterior chamber samples were determined by using the 2-tailed paired *t*-test.

Results

Corneal erosion

Before the drug application started, the induced corneal erosion size was $9.82 \pm 0.38\ mm^2$ for CAC and $10.13 \pm 0.65\ mm^2$ for the BAC 0.02% treatment group. There were no significant differences ($P > 0.05$) between treatment groups at the start. Under BAC 0.02% application, the erosion size continuously increased significantly to a final erosion size of $86.82 \pm 5.18\ mm^2$ ($P < 0.0001$, Fig. 2). For CAC, initial erosion decreased in size on day 1 ($P > 0.05$) and it increased up to day 3 ($29.51 \pm 16.87\ mm^2$, $P < 0.05$). Apart from 4 corneas that did not heal, 1 out of the 5 corneas had nearly healed by day 3 with a remaining erosion size of $0.89\ mm^2$. Representative photographs of fluorescein sodium dyed corneas of 1 cornea for each treatment group are shown in Fig. 3.

Corneal morphology

The histology of BAC 0.02% treated corneas reveals a loss of epithelium, stromal edema, and an undulating endotheial layer (Fig. 4). For CAC, epithelium is lost and the stroma shows minor edema compared with a regular microstructure of control cornea.

Corneal metabolism

As indicators of corneal metabolic activity, glucose and lactate concentrations were detected in artificial aqueous humor samples (Fig. 5).

For BAC, an increase of lactate concentrations became significant as early as day 2 (5.351 ± 0.341 mmol/L), when compared with baseline (3.702 ± 0.203 mmol/L, $P < 0.0001$).

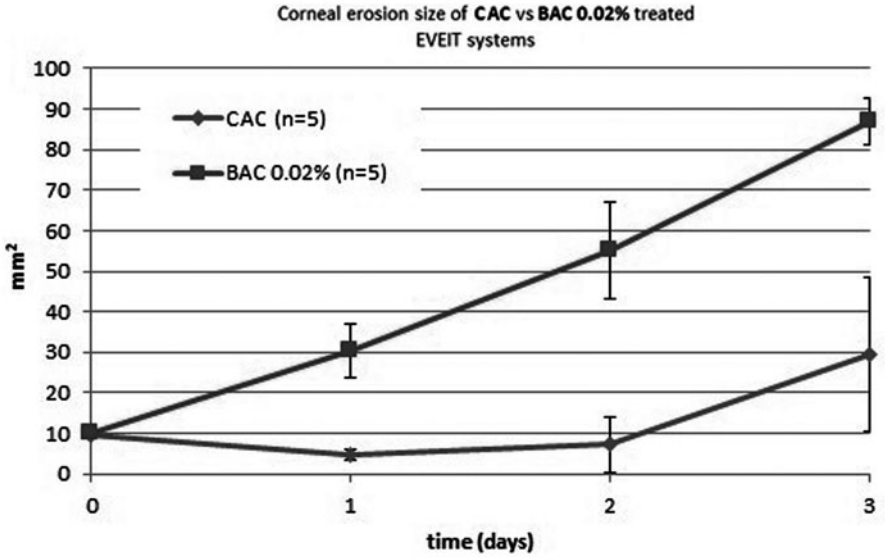

FIG. 2. Corneal erosion size (mm^2) of corneas within the EVEIT system treated with CAC versus BAC 0.02% (each $n = 5$) over 3 days. BAC, benzalkonium chloride; CAC, cetalkonium chloride; EVEIT, *ex vivo* eye irritation test.

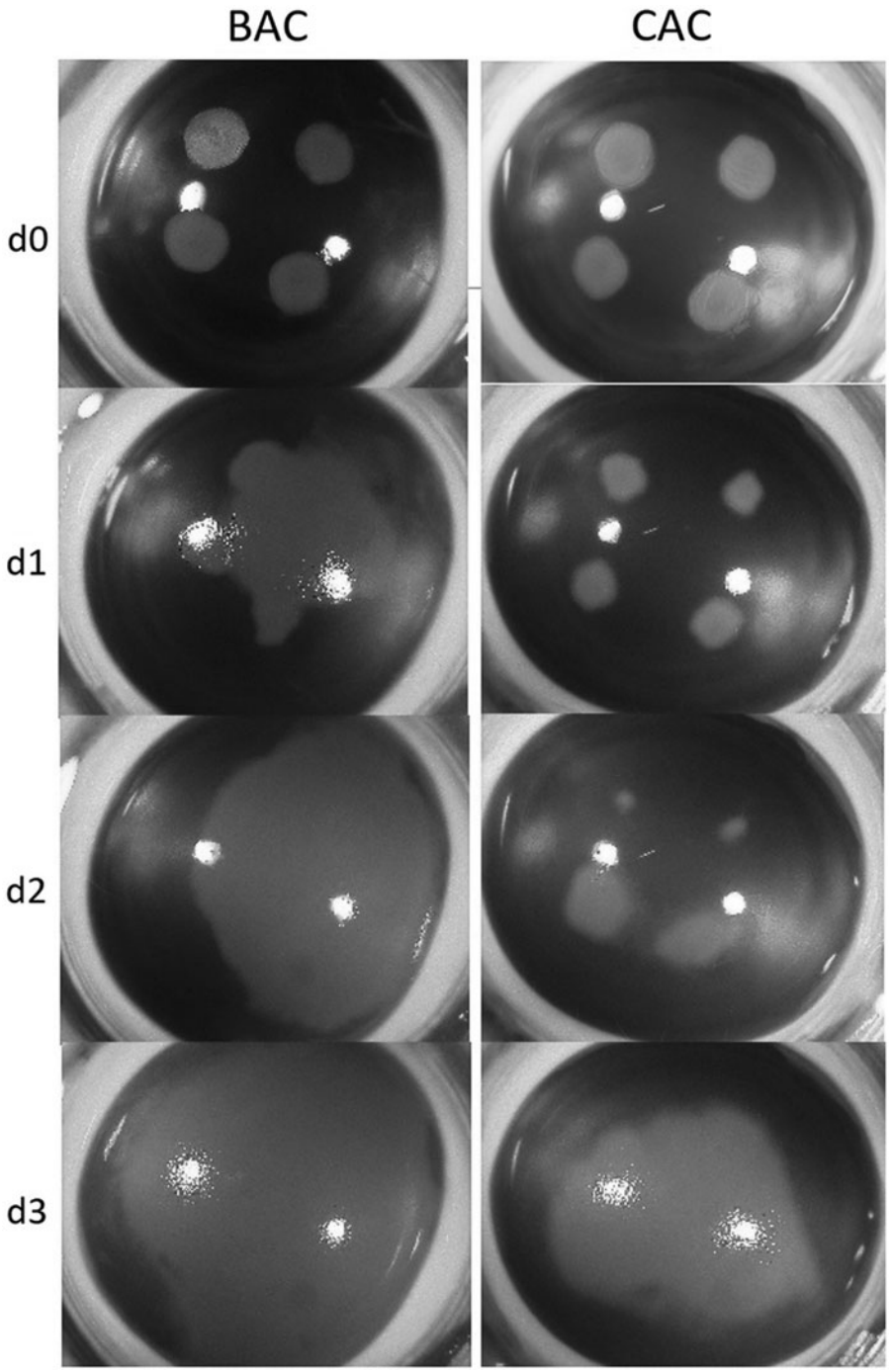

FIG. 3. Representative photographs of fluorescein sodium-stained corneas within the EVEIT system during application of CAC versus BAC 0.02% over 3 days.

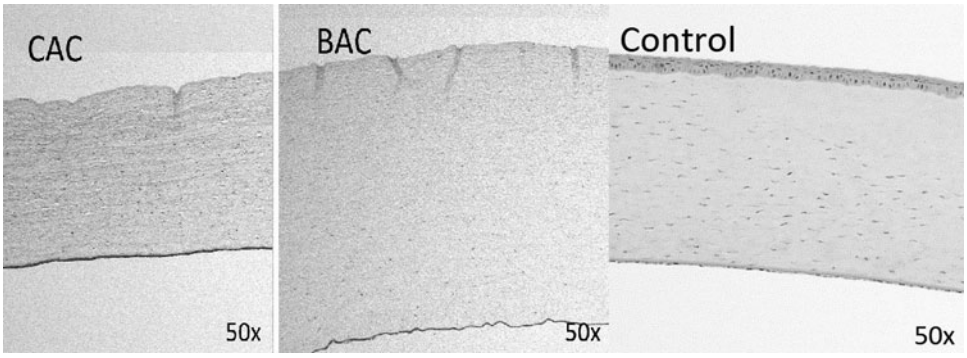

FIG. 4. H&E stained sections of corneas after 3 days of application of CAC and BAC 0.02%.

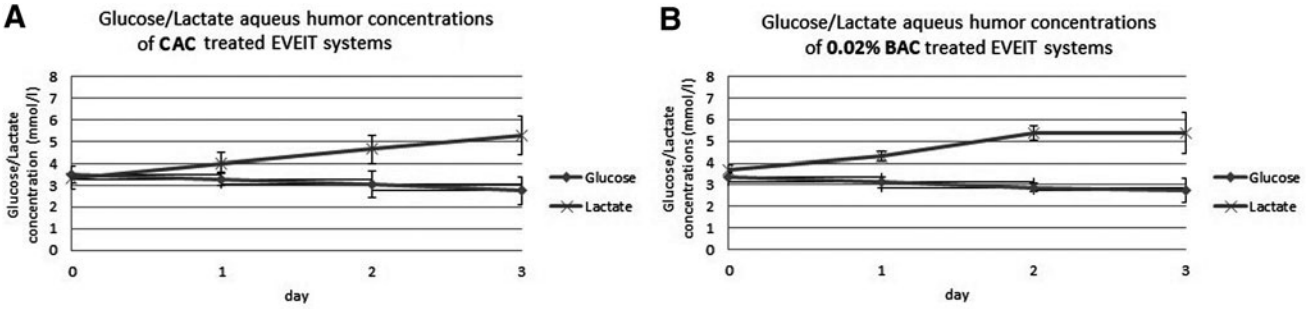

FIG. 5. Glucose and lactate concentrations within artificial aqueous humor sampled daily for CAC and BAC 0.02% treatment groups.

In the CAC corneas, lactate concentrations increased significantly from 3.353 ± 0.528 mmol/L on day 0 to 5.287 ± 0.886 mmol/L ($P < 0.005$) on day 3.

Glucose concentrations for BAC (day 0: 3.354 ± 0.207 mmol/L day 3: 2.751 ± 0.558 mmol/L) as well as CAC (day 0: 3.507 ± 0.338 mmol/L; day 3: 2.765 ± 0.64 mmol/L) showed a trend toward lower concentrations that did not become significant ($P > 0.05$).

Discussion

In the EVEIT system, we found epithelial corneal damage during intensive treatment with 2 common preservative formulations, BAC 0.02% and CAC. The CAC group showed much less damage in our *ex vivo* model (EVEIT). As a negative control for this study, we refer to several of our own previous experiments that presented perfect corneal healing within 2 days for hyaluronic acid-based unpreserved lubricant eye drops.[13,18,19]

The main discussion of this article is that the challenging work of Daull et al.[17] was reproduced exactly here, giving insight into the differences between living animal and *ex vivo* corneas. Daull's group is questioning the results of EVEIT compared with patients, showing that BAC and CAC are well tolerated by living rabbits, and thus that the EVEIT is over-predictive and gives false positive results in pharmaceutical evaluation.

Therefore, this poses the question: How valid is our *ex vivo* model?

In a first series of pre-validation experiments, we compared the EVEIT with the gold standard *in vivo* model, the Draize rabbit eye test (OECD Test Guideline 405).[20] When comparing the toxicity of 37 chemicals by GHS classification predictions, the classification of chemicals as irritating versus nonirritating resulted in 96% sensitivity, 91% specificity, and 95% accuracy. In this toxicological study, the EVEIT was over-predictive in 1 out of 37 chemicals. In another unpublished pre-validation experiment, corneas within the EVEIT were maintained vital for up to 20 days.

Looking at BAC toxicity, which was tested correctly according to GHS classification and former Draize test results, our results are supported by many studies.[7,8,10,11] A study on rats showed hampered corneal healing of BAC-preserved travoprost, compared with various other travoprost formulations when applied once daily.[21] Clinically, a benefit of switching from BAC-preserved to BAC-free ophthalmic solutions (1/daily) did not occur within 4 weeks. Instead, it took at least 3 months according to a study on glaucoma patients.[22] In a similar study on patients already showing superficial punctate keratopathy under BAC 0.02% preserved latanoprost (Xalatan®, Pfizer Ltd.) nightly treatment, patients saw improvement as early as 2 weeks after switching to polyquaternum-1 preserved travoprost (sofZia®, Alcon Laboratories).[23] The overall majority of studies indicate long-term ocular surface toxicity of BAC.[24–27] In those studies, BAC concentration ranged from 0.001% to 0.02% and was applied once daily.

When defining the BAC results of anti-glaucomatous drugs, little imagination is needed to find a dose-dependent effect for preserved lubricant eye drops that are applied at least 3 times daily.

Looking at CAC, our study indicated corneal toxicity, but to a lesser extent compared with BAC. Regarding CAC, only a small number of studies have addressed its toxicity. Liang et al. saw CAC toxicity when applied for a short term in the Draize test. This toxicity was evident for CAC in solution but not for CAC as an emulsion.[28] The CAC within Cationorm is such an emulsion, and more specifically a cationic nanoemulsion. In another study, Daull et al. treated wounded rabbit corneas with Cationorm, which did not affect corneal wound healing compared with various lubricant eye drops.[29] In this study, low-dose exposure of twice daily applications over 5 days did not even show epitheliopathy for BAC 0.02%.

To understand the differences between the EVEIT results of our study presented here and the living animals result of Daull et al.,[17] we must focus on the differences in the circumstances of living animals and isolated exposed corneas. In living animals, we have continuous production of tears, especially with a higher tear clearance in wounded eyes.[30] There is evidence that lipids are secreted by harderian and lacrimal glands, and that tear film is essential to maintain the corneal integrity. In experiments involving the removal of harderian and lacrimal glands in living rabbits,[31] there is evidence that the surface integrity without any chemical intervention becomes unstable after 56 days of experiments (group A of citation 31). Thus, we know and show explicitly that the reaction of the cornea in an isolated system is comparable to the unprotected cornea of a living rabbit missing tear flow and lipid protection in the EVEIT system. Thus, the application of nanoemulsions containing lipids and CAC is tolerated in living animals without any disease of the tear and lipid production system even when corneal wounds are applied. There is a tendency of corneal wound healing during 24 h in the EVEIT System too, but after this period the decontamination of CAC by the isolated cornea becomes expired, and the toxic effects of this low concentrated antibacterial and membrane active ingredient lead to increased corneal erosions.

Thus, the EVEIT model indicates drug toxicity that otherwise would most likely only be detected by long-term use.

Formal discussion to publication;[17]

The authors Daull and Garrigue declare that there is no financial interest. There is evidence that both are employees of enterprises distributing the CAC-containing compound Cationorm and Ikervis®. Moreover, Daull and Garrigue are co-inventors of the drugs[32,33] and thus as European employees share income from the marketing. There is a considerable conflict of interest due to the published paper of our group[13] and this publication, which interferes with the commercial aims of the authors.

Our results suppose that there is a limitation in the use of these CAC-containing drugs compared with unpreserved artificial tears, which we tested several times to promote corneal healing and stable epithelium in similar approaches.[13–15,19]

In contrast to the authors' interest in their product, our group has no commercial interest in the data. We are convinced that animal-free testing is reasonable and predictive. The limitations in comparison to experiments using living animals are clearly visible in the missing tear film and clearance of the ocular surface and thereby earlier identification of ocular alterations. This is obvious within the comparison of[17] and this publication. The advantage of the systematic approach of the EVEIT is as evident by this comparison. We put the EVEIT without patent protection to support the use of animal experiment-free evaluation of chemicals and ocular drugs without limitation.

The data presented here underline that the EVEIT is constructed as a toxicity test, showing time-compressed side effects that occur over the course of months in patients, as previously demonstrated for BAC, phosphate buffer, and other substances.[13–15,34] On repeated application, the EVEIT, as an *ex vivo* model, is very well suited for the worst-case prediction of tolerability and toxic effects of eye drops in particular, and many other chemicals in general.

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Author Disclosure Statement

No competing financial interests exist.

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