Annals of Anatomy 222 (2019) 129–138

Contents lists available at ScienceDirect

Annals of Anatomy

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

RESEARCH ARTICLE

In vitro effects of benzalkonium chloride and prostaglandins on human meibomian gland epithelial cells☆

Anca Rath[a], Michael Eichhorn[a], Katharina Träger[b], Friedrich Paulsen[a], Ulrike Hampel[a,b,*]

[a] Department of Anatomy II, Friedrich Alexander University of Erlangen-Nürnberg, Universitätsstraße 19, Erlangen, Germany
[b] Department of Ophthalmology, University Medical Center of the Johannes Gutenberg University Mainz, Langenbeckstr. 1, Mainz, Germany

ARTICLE INFO

Article history:
Received 17 October 2018
Received in revised form 4 December 2018
Accepted 11 December 2018

Keywords:
Meibomian gland
Benzalkonium chloride
Prostaglandins

ABSTRACT

Purpose: Benzalkonium chloride is the most widely used preservative in ophthalmic topical solutions. The aim of this study was to investigate the influence of BAC as a single substance or as a component of several commercially available ophthalmic solutions on meibomian gland epithelial cells *in vitro*.
Materials and methods: An immortalized human meibomian gland epithelial cell line (HMGEC) was used and cells were cultured in the absence or presence of fetal bovine serum to assess cell morphology, cell proliferation, cell viability (MTS assay) and impedance sensing (ECIS) after stimulation with BAC. Further, the viability of HMGECs stimulated with BAC-containing and BAC-free bimatoprost, travoprost and latanoprost was evaluated using the MTS assay. Real-time PCR analysis for hyperkeratinization associated genes (cornulin, involucrin) was performed.
Results: In the absence of serum, the proliferation rate of HMGECs decreased starting with 0.1 μg/ml BAC. At concentrations of 50 μg/ml BAC and higher, cell viability was reduced after 10 min exposure with a corresponding change in cell morphology. Toxicity of BAC-containing ophthalmic solutions was greater than that of BAC alone, whereas BAC-free alternative products did not significantly influence cell viability. Confluence, cell-cell contacts and serum-containing medium appeared to facilitate HMGECs survival. Expression rate of involucrin and cornulin declined after exposure to preserved bimatoprost and BAC.
Conclusions: BAC showed cytotoxic effects on HMGECs starting with a concentration of 0.1 μg/ml. The combination of BAC and prostaglandin-analogs might have a synergistic effect which results in higher toxicity than BAC alone. Unpreserved eye drops and eye drops preserved with Polyquaternium-1 are less damaging to HMGECs.

© 2019 Elsevier GmbH. All rights reserved.

1. Introduction

Dry eye disease (DED) is a disease of the ocular surface that manifests in eye discomfort, visual disturbance and often as ocular surface damage (Craig et al., 2017). This condition affects 3-45% of the European and US-population aged 50 years and older and has a major impact on the quality of life. (Stapleton et al., 2017) Investigations revealed that long-term glaucoma therapy with preserved eye drops is an important risk factor for corneal alteration and DED (Jaenen et al., 2007; Baudouin et al., 2010; Pisella et al., 2002).

However, the most common preservative is at the same time the one with profound toxicity: benzalkonium chloride (BAC) (Martínez-Soroa et al., 2016; Nelson et al., 2017; Noecker, 2001). BAC is a quaternary ammonium salt with antiseptic properties which interferes with membrane permeability. Even a short-term exposure to BAC appears to be toxic if it occurs repeatedly (e.g. topical antiglaucoma treatment). Friedlaender et al. (2006) analyzed the BAC clearance from tear fluid after instillation of 35 μl gatifloxacin ophthalmic solution 0.3% preserved with 50 μg/ml BAC (hence 1.75 μg BAC per instillation). They conclude that the concentration sank to 6.4 μg/ml within 30 sec, and below the detection limit in under 20 min after instillation. The rapid elimination of BAC from the tear film might creates the illusion that its toxic effect

☆ The present work was performed in fulfilment of the requirements for obtaining the degree "Dr. med."

* Corresponding author at: Department of Ophthalmology, Mainz University Hospital, Langenbeckstr. 1, 55131 Mainz, Germany.
E-mail addresses: anca_gavrilut@yahoo.ca (A. Rath), michael.eichhorn@fau.de (M. Eichhorn), katharina.traeger@unimedizin-mainz.de (K. Träger), friedrich.paulsen@fau.de (F. Paulsen), ulrike.hampel@unimedizin-mainz.de (U. Hampel).

https://doi.org/10.1016/j.aanat.2018.12.003
0940-9602/© 2019 Elsevier GmbH. All rights reserved.

could be of little significance. On the contrary, experimental data has shown that exposure to BAC causes significant damage in stromal, (Mietz et al., 1994) and epithelial tissue (conjunctival, corneal, and goblet cells), (Herreras et al., 1992; Burnstein, 1985; Chen et al., 2011) and causes functional and morphological changes such as disruption of tight junctions, (Chen et al., 2011) cell growth arrest, and eventually cell death at concentrations as little as 0.0001%. (De Saint Jean et al., 1999; Ciancaglini et al., 2008) During repeated exposure the damage cumulates and extends even to the goblet cells of the conjunctival fornix (Barabino et al., 2014; Lin et al., 2011). It is unclear whether this is due to a mere addition of new damage or to accumulation of BAC in the adjacent tissue. However, BAC has been shown to induce dry eye-like alterations including decreased tear break-up time, increased corneal inflammation, epithelial apoptosis, and epithelial desquamation in animal models of mouse (Xiao et al., 2012), rat (Pauly et al., 2007), and rabbit (Ichijima et al., 1992). The toxicity of BAC is not limited to the superficial layers of the eye, but it has rather been found to be able to penetrate to the lens capsule, (Desbenoit et al., 2013) the trabecular meshwork, and retinal tissue (Brignole-Baudouin et al., 2012) after repeated application of BAC-containing solutions. Whether ophthalmic solutions used in glaucoma treatment which combine prostaglandin-like substances and BAC affect cells to a lesser extent, such as has been described in other cell lines, is uncertain.

Until recently the damage BAC causes to meibomian glands remained poorly investigated. These sebaceous glands that lay inside the tarsal plate and open at the rim of the eyelids produce an oily secretion and proteins which appear to play the main role in the formation of the very thin lipid layer that prevents the aqueous part of the tear film from evaporation or collapsibility (Knop et al., 2011). Clinical studies indicate that a long-term use of antiglaucoma eye drops alters the function and morphology of meibomian glands leading to meibomian gland dysfunction (MGD) (Arita et al., 2012a; Arita et al., 2012b). The damage is due to hyperkeratinization which ascends from the orifice side, leading to structural changes of the canaliculi and atrophy of the acini. This process is considered to be a main factor in the pathogenesis of DED. (Knop et al., 2011) To our knowledge, it has not yet been investigated whether BAC additionally accelerates this process by interfering with the acini. Given the ability of BAC to penetrate deeper layers which we mentioned above, (Desbenoit et al., 2013; Brignole-Baudouin et al., 2012) and the findings of oncology research that a tumor with a size of $\leq$1 mm (hence about the distance between conjunctival tissue, and the meibomian tissue) survives without vascularization due to sufficient penetration of nutrients, it appears of great importance to tackle the role it plays in this process.

We employed an immortalized human meibomian gland epithelial cell line (HMGEC) to study the effect of different BAC concentrations on proliferation, viability, and cell barrier function (Liu et al., 2010). For our experiments we chose both proliferating cells and cells differentiated with fetal bovine serum (FBS). The suitability of HMGECs for such investigations is supported by prior results of our working group and by results of other groups. These show that proliferating cells resemble the basal cell layer of meibomian glands. If, however, FBS is added to the culture media the cells resemble those of mature meibomian tissue (eg. accumulation of lipid droplets, formation of desmosomes and cytokeratin filaments), (Hampel et al., 2015; Hampel and Garreis, 2017; Xie et al., 2018). Secondly, we assessed whether BAC induces hyperkeratinization-like changes in HMGECs. As surrogate parameter, we chose to measure the gene expression rate of involucrin and cornulin, two proteins often detected in squamous epithelia and markers of keratinization.

Furthermore, we compared the *in vitro* effects of several commercially available topical ophthalmic solutions often used in the clinical setting to treat glaucoma. We chose products containing prostaglandin-like substances recommended as first choice interventions in the therapy of glaucoma by the European Glaucoma Society (2017). Subsequently, we compared BAC-containing solutions to their BAC-free correspondents and to BAC alone.

2. Materials and Methods

2.1. Cell Culture

SV40 immortalized human meibomian gland epithelial cells (HMGEC) were cultured as previously reported (Liu et al., 2010). HMGECs were cultured in keratinocyte serum-free medium (SFM) to study "basal", proliferating cells or in serum-containing (SCM) medium to induce differentiation as it is found in "mature" cells (Liu et al., 2010). The SFM contained 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (BPE), Penicillin and Streptomycin at 37 °C and 5% CO_2 (Thermo fisher, Life Technologies, Darmstadt, Germany, # 17005042). Furthermore, HMGEC were placed in SCM (10% FBS (Biochrom, Berlin, Germany, # S0415) in equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 (Biochrom, Berlin, Germany, # FG4815) with 10 ng/mL EGF (Sigma-Aldrich, Taufkirchen, Germany, #E4127)) for 24 h after reaching 70% to 90% confluence to induce differentiation. Details of the differentiation and stimulation procedures are depicted in Fig. 1.

2.2. Cell Morphology

Morphological changes in HMGEC due to BAC were monitored by seeding on sterile slides placed in 6 well cell culture plates (CELLSTAR/Greiner Bio-One, Frickenhausen, Germany) in SFM. At 90% confluence, the cells were divided into two groups and stimulated according to Fig. 1. Both cells cultured with and without serum were stimulated with BAC (0.1, 1, 10, 100, 1000 μg/ml), in corresponding culture medium for 24 h. Cells were washed twice then fixed with 4% paraformaldehyde in PBS and stained with Sudan III as described previously (Hampel et al., 2015). Control HMGECs were stimulated with corresponding culture medium. Three independent experiments with duplicates were performed.

2.3. Cell Proliferation Assay

The proliferative activity of HMGEC while stimulated with BAC was tested for 7 days, in order to assess cumulative toxicity of BAC at concentrations which appeared uncritical during the first 24 h. 5000 HMGECs were seeded in 6-well cell culture plates (CELLSTAR/Greiner Bio-One, Frickenhausen, Germany; n = 6 per concentration and counting point) and cultured for 24 h in SFM. Then the cells were stimulated for 7 days with BAC (0.01, 0.1, 1 μg/ml) in SFM. Control HMGECs were cultured in SFM and analysed at the same time points. After 3, 5 and 7 days the cells were collected by trypsinization. Cell number was calculated with a hemocytometer (Brand, Wertheim, Germany).

2.4. Metabolic Cell Activity Assay (MTS Assay)

The metabolic activity of HMGEC stimulated with BAC was assessed by the CellTiter 96 AQueous MTS Assay System (Promega, Mannheim, Germany; product contains the reagent 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS). 50% confluent HMGECs cultured in SFM or SCM according to the flow chart in Fig. 1 were used for the viability testing. The cells were stimulated for 24 h with BAC (0.01, 0.1, 1 μg/ml) in corresponding media. When stimulated with higher concentrations of BAC (25, 50, 100, 200 μg/ml) and commercially available antiglaucoma ophthalmic

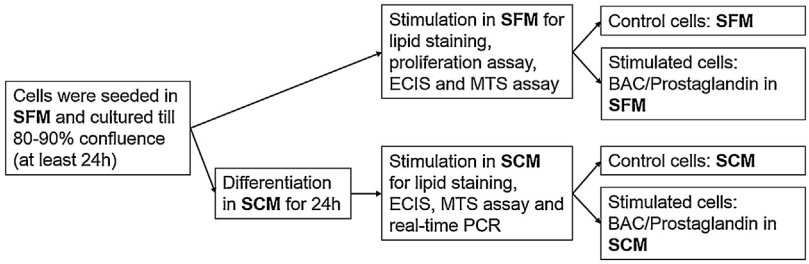

Fig. 1. Stimulation scheme. HMGECs were cultured in serum free medium (SFM) and/or serum containing medium (SCM) throughout the study.

Table 1
Topical antiglaucoma products used for MTS assay.

Product	Active substance	BAC concentration (μg/ml)
Lumigan® 0.1%	Bimatoprost 100 μg/ml	200
Lumigan® 0.3%	Bimatoprost 300 μg/ml	50
Lumigan® UD 0.3%	Bimatoprost 300 μg/ml	None
Taflotan®	Tafluprost 15 μg/ml	100
Taflotan® Sine	Tafluprost 15 μg/ml	None
Travatan®	Travoprost 40 μg/ml	None (Polyquad®)
Xalatan®	Latanoprost 50 μg/ml	200
Monoprost®	Latanoprost 50 μg/ml	None

Polyquad® (0.001%Polyquaternium-1).

solutions (Table 1), the stimulation was conducted with BAC in SCM for 10 min. Metabolic activity was measured photometrically according to manufacturer's protocol at the indicated time points in a plate reader (MWG-Biotech, Ebersberg, Germany) at 490 nm. Control cells were cultured in corresponding medium (SFM or SCM) and analysed at the same time points. Three independent experiments with duplicates were performed for Fig. 5A + B, five independent experiments with duplicates were performed for Fig. 5C + D.

2.5. Electric cell-substrate impedance sensing (ECIS)

The electric cell-substrate impedance sensing method (ECIS, Applied BioPhysics, München, Germany) was used as valuable tool for real time monitoring of both cell behaviour such as attachment, growth, barrier function, and cell toxicity during stimulation with BAC. Pre-coated and stabilized ECIS 8W10E+ arrays were incubated with 6×10^4 cells (per well) suspended in SFM. The array station was placed in a standard 37 °C incubator with 5% CO_2 allowing the cells to attach for 24 h. The stimulation started by replacing the culture medium with 400 μl BAC (0.01, 0.1, 1.0 μg/ml) in either SFM or SCM. Control cells were cultured in corresponding BAC-free culture medium. 300 μl of the stimulating solution were exchanged every 48 h while pausing the experiment. Data were collected real-time throughout the experiment for 6 days and analysed using ECIS Software and were run as multi-frequency time scan. The data is presented as the mean ± SEM from three independent experiments with a total of 10 wells per concentration.

2.6. RNA Preparation and Complementary DNA (cDNA) Synthesis

Crude RNA was purified with isopropanol and repeated ethanol precipitation and contaminating DNA was destroyed by digestion with RNase-free DNase I (30 minutes, 37 °C; Boehringer, Mannheim, Germany). The enzyme DNase was heat-inactivated for 10 minutes at 65 °C. Reverse transcription of all RNA samples to first-strand cDNA was performed by RevertAidTM H Minus M-MuL V Reverse transcriptase Kit (Fermentas, St. Leon-Rot, Germany) according to manufacturer's protocol. For each reaction, 2 μg total RNA and 10 pmol oligo 18 primer (Fermentas, St. Leon-Rot, Germany) were used.

2.7. Real-time PCR

Quantitative real-time PCR with SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) as a double-stranded DNA–specific fluorescent dye was performed using a LightCycler® 480 II system (Roche, Penzberg, Germany). Each reaction contained 2 μl cDNA and 18 μl LightCycler480 5 x probe mastermix consisting of 10 μl SYBR Green, 0.25 μl forward primer, 0.25 μl reverse primer and 7.5 μl nuclease-free water. Primer sequences were as follows: cornulin (CRNN) 5′- CAA CTG CAC AGC GCT CAC, reverse 5′-TCC ACA GTT GCT GGA TCG T; involucrin (IVL) forward 5′-AGC TCG ACA GGC ACC TTC T, reverse 5′-ACC CAT CAG GAG CAA ATG AA; 18S ribosomal RNA (rRNA) forward GGT GCA TGG CCG TTC TTA, reverse TGC CAG AGT CTC GTT CGT TA. 18S rRNA was used because its expression was not influenced by any of the mediators investigated. PCR was initiated for 2 minutes at 50 °C, followed by 1.5 minutes at 94 °C. The program continued with 55 cycles of 20 seconds at 94 °C and 60 seconds at 62 °C. Each assay included duplicates of each cDNA sample and a "no-template" control with nuclease-free water. Relative amounts of expression of messenger RNA (mRNA) for involucrin, cornulin and 18S rRNA were calculated using the ΔΔCt method (Pfaffl, 2001). The expression of 18S rRNA was used to normalize samples for the amount of cDNA used per reaction.

2.8. Statistics

Flow cytometric results and viability were calculated as arithmetic mean ± SEM, and significance was determined using One-Way ANOVA with $p < 0.05$ regarded as significant. The comparison of stimulation groups was done with Bonferroni post test. Results of the ECIS assays and real-time-PCR were calculated as arithmetic mean ± SEM, and significance values were calculated by means of the two-way ANOVA with $p < 0.05$ regarded as significant. The comparison of stimulation groups was performed with the help of Dunns comparison.

3. Results

3.1. Evaluation of cell morphology and cytotoxicity induced by BAC

Cells stimulated with BAC showed morphological changes and cell detachment when exposed to ≥10 μg/ml BAC. Fig. 2 depicts the occurring modifications and the capacity of HMGECs to store fatty vesicles of cells exposed to BAC and stained with Sudan III.

In the control group and the group stimulated with 1 μg/ml BAC, both HMGECs stimulated in SFM or SCM remained confluent and maintained a normal shape. At 10 μg/ml BAC, cells cultured in SFM changed their shape to round with marginal position of the nucleus.

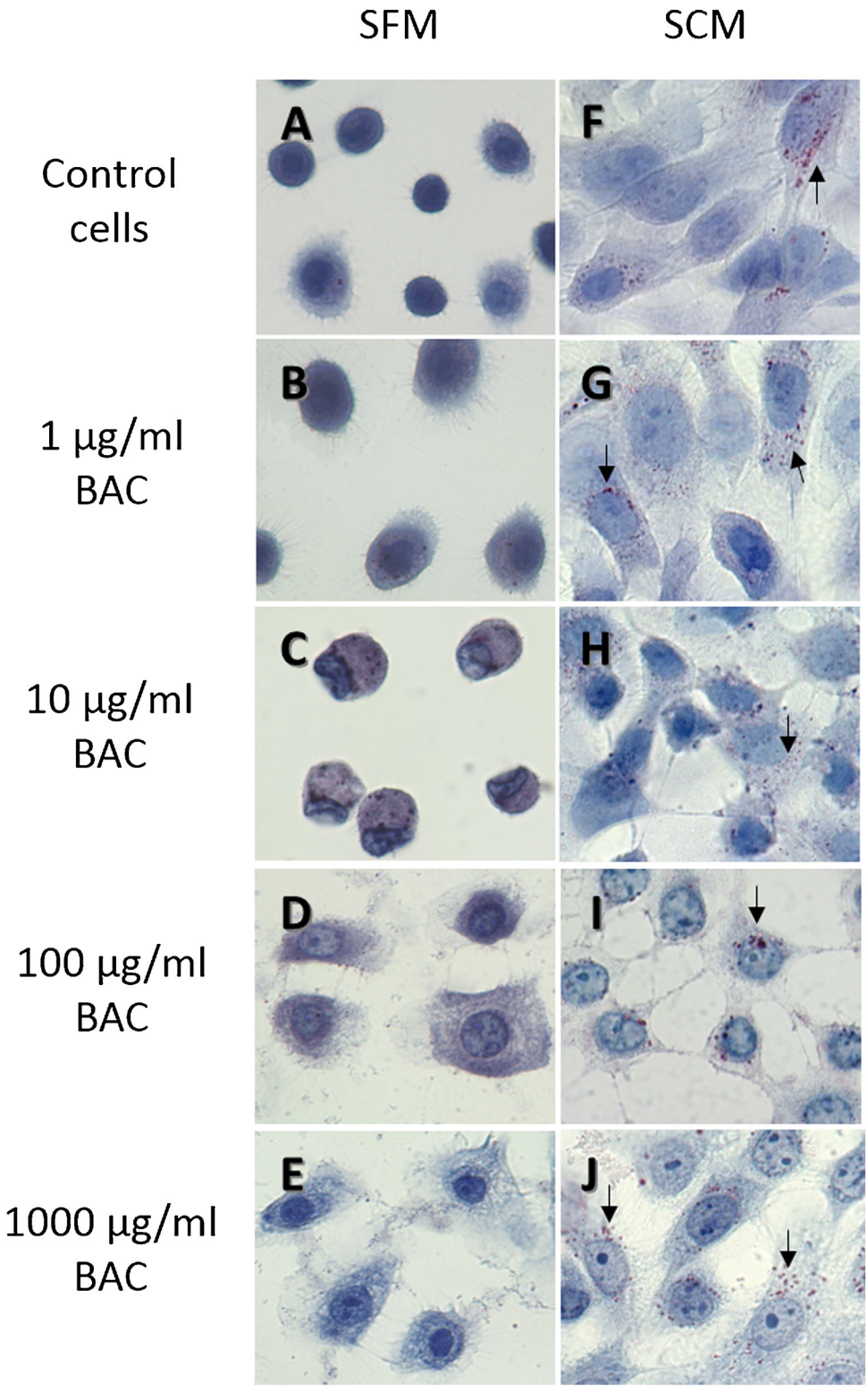

Fig. 2. Sudan III staining of HMGECs after stimulation with BAC in SFM (A-E) and SCM (F-J) for 24 h. Cells stimulated with BAC in SFM showed changes in morphology at 10 µg/ml BAC or more. When cultured with SCM, changes occurred starting from 100 µg/ml BAC. Unlike cells in SFM, cells in SCM built cell connections and stored lipid droplets (red). The arrows show stored lipid droplets. (A + F) control HMGECs; (B + G) 1 µg/ml BAC; (C + H) 10 µg/ml BAC; (D + I) 100 µg/ml BAC; (E + J) 1000 µg/ml BAC. Three independent experiments with duplicates were performed. Original magnification 60x.

Additionally, a large proportion of the HMGECs detached from the surface of the culture plate. Cells cultured in SCM revealed no modification in shape when exposed to 10 µg/ml BAC, but cytoplasmic storage of fatty vesicles near the nuclei. When the concentration was raised to 100 µg/ml BAC, HMGECs underwent a modification in morphology in the presence of SCM. These cells shrunk, and the cytoplasm-nucleus-ratio altered in favour of the nuclei. However, the nuclei and the cell junctions seemed to remain intact. The evaluation of the changes in cells incubated with 1000 µg/ml BAC was difficult, because after only a few seconds, the stimulation solution

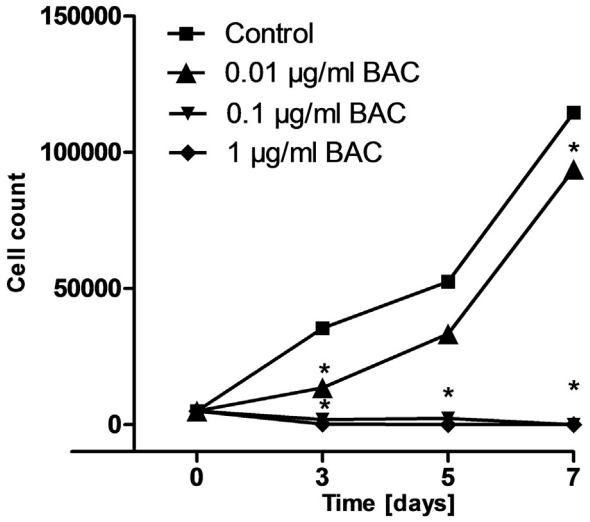

Fig. 3. Effects of BAC on proliferation of HMGECs. HMGECs were cultivated in sterile 6-well culture plates at 5000 cells/well and stimulated with BAC (0.01, 0.1, 1.0 μg/ml) in SFM for up to 7 days. Control cells were incubated with SFM. Cells were counted after 3, 5 and 7 days. BAC (0.01; 0.1 and 1.0 μg/ml) inhibited proliferation of HMGEG compared to control HMGECs from day 3 of stimulation onwards. (mean ± SEM; n = 6, *p<0.05, Two Way ANOVA).

turned cloudy and precipitated on the cells to a hardly washable layer. This phenomenon occurred regardless of the culture medium type (SFM or SCM). The cytoplasm of HMGECs stimulated with this mixture had a granulated aspect whereas the cell shape and the amount of cell junctions remained similar to those of control cells. The same modification occurred in HMGECs stimulated with 100 μg/ml BAC in SFM.

3.2. Proliferation under BAC treatment

We investigated the proliferation of HMGECs at different BAC concentrations. We incubated HMGECs with BAC (0.01, 0.1, 1 μg/ml) in SFM for 7 days to evaluate their proliferative activity under these conditions and performed cell counts after 3, 5 and 7 days. All concentrations of BAC reduced the proliferation rate of HMGECs; but day 5 & 7 gave the greatest discriminatory power: the cells incubated with 0.01 μg/ml BAC partially recovered and proliferated at a similar rate as the control cells. On the contrary 0.1 and 1.0 μg/ml BAC led to a permanent decrease of the cell number. While 0.1 μg/ml BAC allowed cell survival and even a slight proliferative activity up to 3 days, 1 μg/ml BAC induced a drastic decrease in cell number ($p < 0.05$). However, on day 5 cell numbers for both 0.1 and 1.0 μg/ml BAC declined to a similar amount and did not recover until the end of the experiment ($p < 0.05$).

We used the ECIS system to explore the further cytotoxic reaction of HMGECs to BAC. Additionally to cell survival, this method reflects modifications in cell barrier function, respectively to the integrity of the cellular network. Inter- and trans-cellular leakage is proportional to the change in impedance measured over the electrodes. Impedance values are normalized to impedance at t = 0 min (24 h after seeding). HMGECs seeded on ECIS electrode-containing dishes (8W10E+) at 10^4 cells/well were stimulated with BAC continuously for 168 h. Independently of the culture medium (SFM or SCM) used during stimulation, at concentrations of 0.01 and 0.1 μg/ml BAC the HMGECs impedance showed no significant difference to control cultures (Fig. 4).

At 1.0 μg/ml, however, cells showed different responses to BAC depending on the culture medium being used. The cells stimulated with 1.0 μg/ml BAC in SCM revealed no difference in impedance compared to other stimulation groups and control cells (Fig. 5B). Cells stimulated with 1.0 μg/ml BAC in SFM (Fig. 5A) demonstrated a decrease of impedance occurring after 24 h of stimulation. No recovery could be detected throughout the experiment. Furthermore, the impedance continuously dropped until the end of the experiment.

3.3. Viability of HMGECs after exposure to BAC, preserved and unpreserved ophthalmic prostaglandins

We further studied the effect of BAC on cell viability by MTS assay. As shown in Fig. 4A and B, stimulation of HMGECs for 24 h with 0.01, 0.1 or 1.0 μg/ml BAC did not lead to significant differences in viability between stimulation groups and control HMGECs cultured in SFM or SCM. Subsequently, we applied the concentrations which were usually used in medical products (50, 100 or 200 μg/ml BAC) for 10 min. We allowed HMGECs to recover in BAC-free medium for 24 h before we measured their viability.

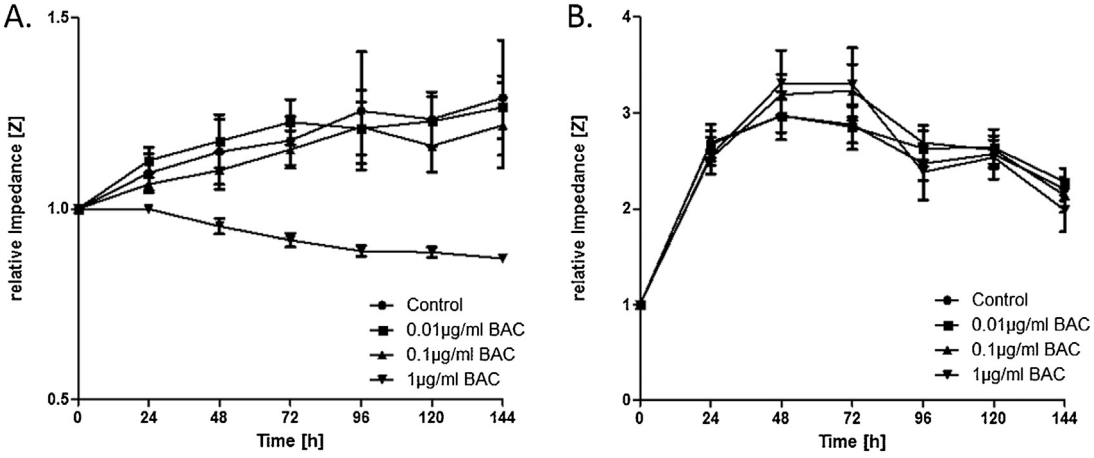

Fig. 4. Effect of BAC on cell barrier properties of HMGECs measured by ECIS. HMGECs were seeded in ECIS electrode-containing dishes (8W10E+) at 10^4 cells/well and stimulated with BAC in SFM or SCM (0.01, 0.1, 1 μg/ml) continuously for 168 h. Control HMGECs were cultured in corresponding culture medium. Cellular impedance was measured continuously at a single frequency of 4000 Hz. Increase in resistance corresponds to increasing cell barrier function and number of adherent cells. Impedance values were normalized to impedance at t = 0 min. The data is presented as the mean ± SEM from three independent experiments with a total of 8wells per concentration. (A) Comparison of the effect of HMGECs stimulated with BAC in SFM and control cells. Cells stimulated with 1 μg/ml BAC showed a significant decrease of impedance ($p < 0.01$). Stimulation with 0.1 and 0.01 μg/ml BAC did not show significant difference in impedance compared to control cells. (B) Comparison of the effect of HMGECs stimulated with BAC in SCM and control cells. Data show no significant difference between stimulated and control HMGECs. (One Way ANOVA).

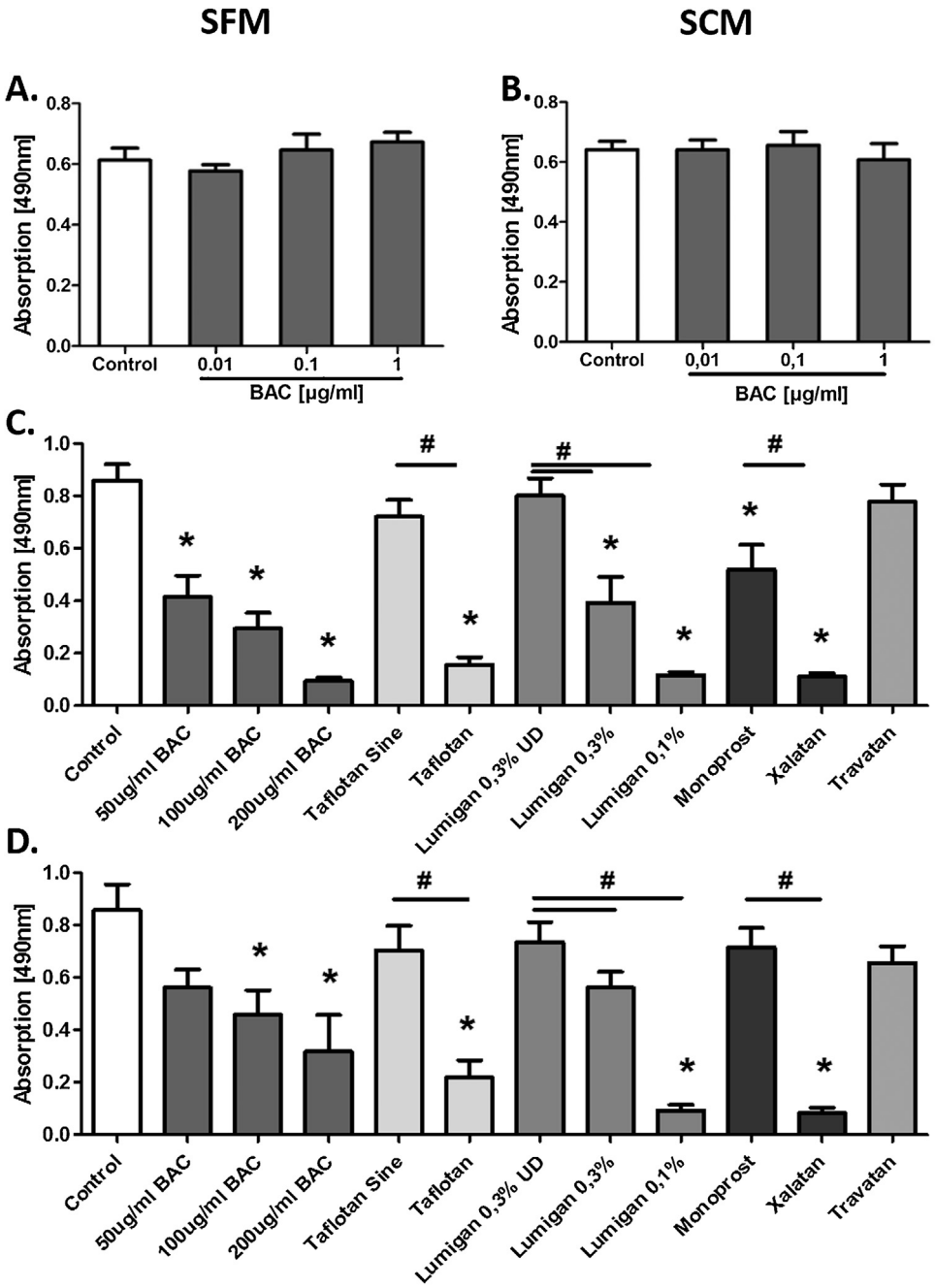

Fig. 5. Effects of BAC and antiglaucoma ophthalmic solutions on viability of HMGECs using MTS assay. Viability was determined in HMGECs cultured in SFM (A + C) and SCM (B + D) after stimulation with BAC. (A + B) HMGECs were stimulated for 24 h with BAC (0.01, 0.1, 1.0 μg/ml). Viability of stimulated cells did not differ from control cells. (C) HMGECs were stimulated with BAC (50, 100, 200 μg/ml), Taflotan®, Taflotan® Sine, Lumigan® 0.3%, Lumigan® 0.1%, Lumigan® 0.3% UD, Monoprost®, Xalatan® or Travatan® for 10 min and incubated for further 24 h in BAC-free culture medium. (C) Cells incubated with BAC in SFM showed significant loss in viability in all groups compared to control cells. No significant reduction in viability was detected in cells stimulated with BAC-free Lumigan®, Taflotan® and Travatan®. BAC-containing eye drops lead to significant reduction in cell viability compared to both control cells and the BAC-free correspondent. Monoprost® also led to significant reduction in cell viability. (D) Cells incubated with 100 and 200 μg/ml BAC in SCM showed significant loss in viability compared to control cells, while 50 μg/ml did not influence cell viability significantly. No significant reduction in viability was detected in cells stimulated with either BAC-free solution. BAC-containing Taflotan®, Lumigan® 0.1% and Xalatan® led to significant reduction in cell viability compared to both control cells and the BAC-free correspondent. Lumigan® 0.3% did not lead to significant decrease in viability. Data are presented as the mean ± SEM. Three independent experiments with duplicates were performed for Fig. 5A + B, five independent experiments with duplicates were performed for Fig. 5C + D. (- *p<0.05 compared to control cells, # p<0.05 compared to reference column, One Way ANOVA).

The metabolic activity of HMGECs cultured in SFM (Fig. 4C) diminished ($p < 0.05$) by 52%, 76% and 89% compared to control cells. Such high decrease ($p < 0.05$) in cell viability was only measured in HMGECs stimulated with 200 μg/ml BAC in SCM, measuring 67%. Even though on first sight it appears that toxicity amplifies in a dose dependent manner, this difference was only significant between 50 μg/ml and 200 μg/ml in cells cultured in SFM. Moreover, the absorption did not differ significantly between either of these groups in cells cultured in SCM.

Whether exposure to the combination of BAC and prostaglandin-like substances impacts the viability of HMGECs in a different manner than BAC alone or the BAC-free alternative products was then investigated using the MTS assay. For this purpose, cells underwent stimulation with several antiglaucoma

eye drops. As depicted in Fig. 5C and D, BAC-free Lumigan® 0.3% UD, Taflotan® Sine and Travatan® did not lead to significantly lower cell viability compared to control cells in SFM or SCM.

The viability of cells in SFM cultured with all BAC-containing eye drops decreased significantly whereas cells cultured in SCM tolerate BAC and BAC-containing solutions slightly better. No significant difference between control cells, 50 μg/ml BAC and Lumigan® 0.3% (contained 50 μg/ml BAC) were measurable in the SCM group (Fig. 5D).

In cells previously cultured in SCM, Lumigan® 0.1% (200 μg/ml BAC) reduced the viability significantly more than Lumigan® 0.3% (50 g/ml BAC). Furthermore, the alteration due to BAC (50 μg/ml and 200 μg/ml) did not differ significantly from that of Lumigan® 0.3%.

However, viability of cells treated with Taflotan®, Lumigan® 0.1% and Xalatan® corresponded to that of cells incubated with 200 μg/ml in both SFM and SCM cultured cells and was significantly lower than control cells.

When comparing effects of latanoprost-containing products on cells in SCM there was no significant difference between BAC-containing Xalatan® and 200 μg/ml BAC and between Monoprost® and control cells. The same observation was made with BAC-preserved and unpreserved tafluprost-containing products.

3.4. Expression of involucrin and cornulin under BAC exposure

We have evaluated BAC-dependent induction of gene expression for involucrin und cornulin in HMGECs cultured in SCM. No significant difference was measurable in the expression rate of involucrin and cornulin in control and low-dose stimulated (0.01, 0.1, 1.0 μg/ml BAC) HMGECs (Fig. 6A + B). In contrast, gene expression rate was significantly lower ($p < 0.05$) than in control cells when cells were stimulated with Lumigan® 0.3% undiluted (Fig. 5C + D) for both involucrin and cornulin. Interestingly, HMGECs stimulated with 25 μg/ml BAC revealed the lowest expression rate for both involucrin and cornulin, while 50 μg/ml did not display a significant modification in expression of neither gene ($p > 0.05$).

4. Discussion

Meibomian glands are an essential element in maintaining the moisturization of the ocular surface (Millar and Schuett, 2015). These holocrine glands produce an oily secretion, the meibum, which has qualities unique in the human body (Millar and Schuett, 2015). If the glands are dysfunctional as in MGD, it leads to changes in volume and functionality of the gland as well as to an increase in meibum viscosity and subsequently to symptoms of DED (Knop et al., 2011). Patients who undergo long-term treatment with antiglaucoma eye drops are more likely to be affected than the normal population (Baudouin et al., 2010; Arita et al., 2012a; Arita et al., 2012b). The addition of preservatives in commercial eye drops is usually cited as the causative agents of cytotoxicity. As BAC is the most often used preservative in this type of medication (Sullivan et al., 2014), we decided to investigate how harmful BAC is to meibomian gland cells *in vitro*.

Our *in vitro* results demonstrate that the cytotoxicity of BAC is apparent at concentrations well below the ones used in eye drops (commercially used concentrations: 50, 100 or 200 μg/ml BAC). Our studies show that cytotoxic effects of BAC start at 0.1 μg/ml, 1000 x less than in commercial eye drop products. We chose to test such low concentrations of BAC (0.01, 0.1 and 1 μg/ml BAC) on account of the results presented in Fig. 2, which show that cell death occurs at concentrations ≥10 μg/ml within less than 24 h and due to data collected from other cell lines, which show these as critical concentrations of BAC. (Iwasawa et al., 2013) The absence of cytotoxicity is reflected in the lack of morphological changes compared to control HMGECs (Fig. 2) and to insignificant or partly reversible influence on cell functionality (Figs. 3–5), especially looking at highly confluent cells.

The rise in concentration, cell confluence and exposure period are of great importance when tackling further results as we can see when comparing Figs. 3 and 4. While 0.01 μg/ml BAC was tested safe for at least 6 days during our experiments, in a similar experiment Chen et al. showed that even a 5fold gain in concentration induces a significant decrease in cell number within 5 days. (Chen et al., 2018) The toxicity is increased if the concentration is further raised by the factor 10 in both our results (Fig. 3) and the data published by Chen et al. (2018). On the contrary, cells seeded at a higher density tolerate 0.1 μg/ml BAC for a longer period as seen in Fig. 4. We limited the stimulation period to 7 days because of a steady development of the cell survival for all tested concentrations (Figs. 3 snd 5). While it was not verified for HMGECs, in some cell lines it has been shown that high cell density enables them to be more resistant to external influences (Grayson et al., 2008; Heng et al., 2011; Wang et al., 2009). We consider that his could be the reason for the discrepancy between these results.

Cell morphology and viability remained comparable to control cells even at 1.0 μg/ml BAC in SFM if exposure was limited to 24 h (Figs. 2 and 5 A). If we prolonged the stimulation, BAC was toxic to HMGECs after less than 48 h when cultured in serum-free medium (Figs. 3 and 4 A). After three days of stimulation, microscopic assessment revealed profound toxicity and loss of cell viability of HMGECs. When FBS was added to the media the cells appeared to be more resistant to BAC. FBS is rich in nutrients and cytokines which appear to influence cell growth, morphology and metabolism, and induced the development of characteristic similar to mature meibomian cells. (Bettger and McKeehan, 1986; Hampel et al., 2015) The rise in impedance in Fig. 4B indicates that cells cultured in SCM develop properties (for example increase in cell surface and cell contacts) which help them survive despite exposure to an otherwise toxic amount of BAC.

Fig. 2 demonstrates that 10 μg/ml BAC in SFM was the critical concentration regarding survival of HMGECs during the first 24 h. In our model cultured HMGECs reacted with detachment from the culture plate surface and changed their morphology to round appearance with a marginal nucleus while metabolic activity was significantly lower after 10-minute stimulation (Fig. 5C). Regardless, no cell modification was detectable with light microscopy at 10 μg/ml BAC in SCM. Interestingly, Chen et al. found that a 30-minute incubation with 5 μg/ml BAC in both SFM and SCM significantly reduces cell survival rates and the phosphorylation of the AKT-signalling (Chen et al., 2018), a pathway involved in cell survival and proliferation. When applied to our data, it appears that disruption of this pathway is critical for cells in SFM, while cells in SCM appear to compensate this alteration for some time. At a careful lecture, we noticed that Chen et al. do not state whether the stimulation of HMGECs with BAC and SCM were started simultaneously or whether the cells were first differentiated with SCM without BAC. If the cells were not differentiated beforehand, this would explain the discrepancy between Chen et al.'s (2018) and our results and makes them not comparable.

At a concentration of 100 μg/ml, relevant morphological changes started to occur in HMGECs in the presence of serum as well. The volume ratio of the cytoplasm diminished in favour of the nucleus, a phenomenon often described in cells at the beginning of the apoptotic process. (Lang, 2007; Orlov and Novikov, 1996) Moreover, high BAC concentrations appear to cause necrosis in another cell line. (Debbasch et al., 2001) Accordingly, cell viability reduced significantly after exposure to 100 μg/ml BAC for 10 min. In addition, as demonstrated in Fig. 5D we found a poor dose depen-

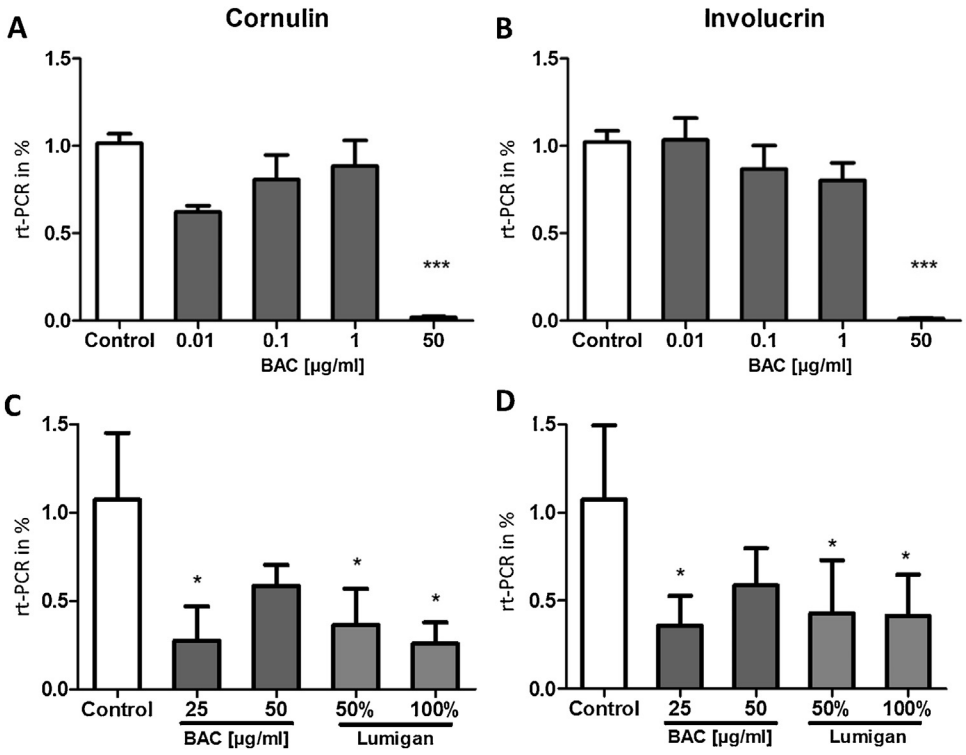

Fig. 6. Effects of stimulation with BAC on the expression rate of involucrin and cornulin genes in HMGEC. RNA was isolated, reverse transcribed to cDNA, and then amplified by a real-time PCR detection system to measure mRNA levels of involucrin and cornulin in HMGECs cultured in SCM after stimulation with BAC and Lumigan® diluted in SCM. Target genes were normalized to 18S RNA. **(A + B)** Gene expression rate of cornulin and involucrin did not differ significantly after stimulation with 0.01, 0.1 and 1.0 μg/ml BAC compared to control cells, while stimulation with 50 μg/ml BAC led to a highly significant decrease compared to control HMGECs. (C + D) Stimulation with 25 μg/ml BAC and 100% Lumigan® concentrations showed significant differences in the gene expression compared to control HMGECs. The data are presented as the mean ± SEM from three independent experiments. (* $p<0.05$, One Way ANOVA).

dent decrease in viability of HMGECs cultured in SCM that became apparent between 50 and 200 μg/ml BAC.

What strikes is that HMGECs cultured at high BAC concentrations in serum-free medium (100 and 1000 μg/ml BAC) and cells cultured at 1000 μg/ml BAC in SCM maintained a shape similar to untreated cells. The cytoplasm, however, had granulated inclusions and indicated that high concentrations of BAC affected HMGECs in a different manner than 10 μg/ml or lower concentrations. A thorough evaluation of this observation rendered difficult due to a durable precipitate that developed when BAC was mixed with culture medium in concentrations as high as 100 or 1000 μg/ml. Hence, it is uncertain if these changes occurred because of BAC alone or if they were rather associated with the precipitate that covered the cells. We consider that more studies are required to investigate this phenomenon.

Even though BAC has been suspected to be the main cytotoxic component of ophthalmic topical solution, other studies show that this might not be the only factor (Gasset et al., 1974; Burstein, 1980). Kam et al. (2016) observed that bimatoprost also induces a significantly lower rate of phosphorylation of the protein kinase B in the AKT-signal pathway. This results in negative influence on proliferation and survival of HMGECs which is consistent with our results regarding the viability of HMGECs in SCM. The viability differs significantly between cells stimulated with 50 μg/ml and 200 μg/ml BAC while *in vitro* the effects of Lumigan® 0.3% (contains 50 μg/ml BAC) and Lumigan® 0.1% (contains 200 μg/ml BAC) do not differ significantly from corresponding BAC concentrations in SCM. This contradicts the findings of Barabino et al. who conclude that bimatoprost has some protective role against the cytotoxicity of BAC (Barabino et al., 2014). Hence, the content of BAC is important when referring to cytotoxicity. However, there is no significant modification in viability when the BAC-free product is applied to HMGECs. As it is known that BAC facilitates the penetration of other substances into tissues (van der Bijl et al., 2001; van der Bijl et al., 2002), it is possible that the combination of a prostaglandin-like substance and BAC or other substances contained in these eye drops reaches deeper tissue levels and creates damage to an even greater extent than BAC alone, favouring the development of DED. Our study does not bring sufficient evidence for longer antiglaucoma treatment that usually is needed in patients with glaucoma. Nevertheless, Arita et al. (Arita et al., 2012a; Arita et al., 2012b) described alteration of the meibomian glands in patients who underwent antiglaucoma treatment. They found that there is no correlation between the meiboscore and the duration of topical therapy (Arita et al., 2012a). Although we could show that all BAC-containing products had severe influence on the viability of HMGEC compared to their BAC-free correspondent, we could only show this correlation for bimatoprost and BAC. This does not necessarily mean that bimatoprost has more disadvantages than other prostaglandin-like substances. Due to the lack of products that contain latanoprost, tafluprost, and BAC in various concentrations, such a comparison regarding other active substances is not possible at the moment.

It appears that only eye drops with BAC affect the viability of HMGECs but not eye drops with another preservative Polyquad®. There is no significant difference between the viability of control cells and cells treated with Travatan® and no significant difference between cells treated with Travatan® and cells treated with other preservative-free antiglaucoma eye drops.

When looking at cells in SFM Monoprost® stands out from the group of BAC-free ophthalmic solutions used in this study. The viability of HMGECs in SFM decreases significantly compared to control cells but was still significantly higher than its BAC-containing correspondent Xalatan®. This suggests on one hand

that there is a synergistic cytotoxic effect of BAC and other prostaglandin-like substances. On the other hand, it is possible that prostaglandin-like substances have negative effects on HMGECs to a certain extent as well. Smedowski et al. (2014) have demonstrated that BAC-free latanoprost is toxic to corneal epithelial cells *in vitro*. Our findings are therefore not only inconsistent with a previously described protective effect of prostaglandins but they rather suggest that this combination increases toxicity compared to BAC alone and therefore increases the risk for MGD and DED (Chang et al., 2015). Zhang et al. also showed that the negative effects on proliferation and survival of HMGECs are not limited on prostaglandin-like substances but are also present under the influence of other medication used for the treatment of glaucoma, pilocarpine (acetylcholine-analogue) and timolol (ß-blocker) (Zhang et al., 2017).

Parameters like drug diffusion and interaction with other tissue types represent an important disadvantage of our model and make a direct extrapolation to *in vivo* data impossible. Xiong et al. (2008) reported significant decreases in Schirmer test results and in goblet cell density, increase in fluorescein scores and damage in cornea and conjunctiva of rabbits after a 7-day treatment with 0.1% BAC twice daily (0.1% BAC corresponds to 100 μg/ml). Lin et al. (2011) noted similar results in a mouse model treated with 0.2% BAC for 7 days. The changes described in both animal models showed similar signs as DED but did not include information about the meibomian gland. Yet iatrogenic caused DED is often associated with MGD. The hyperkeratinisation of meibomian gland ducts and the high viscosity of meibum that occur in MGD, favour the obstruction of the secretion orifice and subsequently induces mechanical damage to the acini (Knop and Knop, 2009; Paulsen et al., 2015). If BAC can be isolated from the lens capsule (Desbenoit et al., 2013), it appears plausible for it to penetrate 1 mm deep into the tarsal tissue and affect the deeper layers. The consequence of this is hyporegeneration, and thus gland atrophy. More studies are necessary to quantify what depth BAC can reach in the tarsal tissue which would enable us to better estimate the effects of a long-term treatment.

As mentioned above, squamous metaplasia and hyperkeratinisation, especially of the duct epithelium, have been suggested to be important factors in MGD (Gutgesell et al., 1982; Lee and Tseng, 1997; Jester et al., 1981). We found that expression of cornulin and involucrin, two proteins who are found at higher levels in differentiated keratinocytes (Contzler et al., 2005; Dowdall et al., 2015), did not differ significantly from that of control cells when cultured with 0.01, 0.1 and 1.0 μg/ml BAC for 24 h (Fig. 6). Furthermore, we identified a significant reduction of cornulin and involucrin mRNA after a short treatment (10 min) with undiluted and 50% diluted Lumigan® 0.3% and the corresponding BAC amounts in SCM. Lumigan® 0.3% was considered suitable because it has the lowest BAC concentration (50 μg/ml BAC) and 50% diluted Lumigan® 0.3% to further ensure some cellular metabolic activity, which is obligate when proteins are quantified.

5. Conclusion

The presence of FBS, cell density, cell-cell-interactions and cell connections play a protective role in HMGECs and result in a higher tolerance against BAC. Nevertheless, BAC exerts its toxicity at concentrations equal to or lower than those which we meet in antiglaucoma eye drops, regardless of the type of media being used. If BAC is used in serum-free medium, damage is noticeable even at a concentration factor 1000fold less than what is contained in commercial eye drops. This effect seems to be even more pronounced during the use of BAC-preserved commercially available eye drops, whereas unpreserved eye drops, and eye drops preserved with Polyquaternium-1 are less damaging to HMGECs. Lee et al. cites that 1986 Champeau and Edelhauser could demonstrate accumulation of BAC in eye tissue (Lee et al., 2017). Therefore, it appears likely for BAC to accumulate in the tarsal tissue and maybe reach a toxic concentration on the level of the meibomian glands. If this truly is the case, then BAC could be a more important factor in the pathogenesis of iatrogenic DED then previously assumed.

Acknowledgements

This work was supported in part by the German Research Foundation (DFG, Program Grant PA738/9-2, FP, and HA6344/2-1, UH) and Sicca Forschungsförderung of the Association of German Ophthalmologists.

References

Arita, R., Itoh, K., Maeda, S., Maeda, K., Furuta, A., Tomidokoro, A., Aihara, M., Amano, S., 2012a. Comparison of the long-term effects of various topical antiglaucoma medications on meibomian glands. Cornea 31 (11), 1229–1234.

Arita, R., Itoh, K., Maeda, S., Maeda, K., Furuta, A., Tomidokoro, A., Aihara, M., Amano, S., 2012b. Effects of long-term topical anti-glaucoma medications on meibomian glands. Graefes Arch. Clin. Exp. Ophthalmol. 250 (8), 1181–1185.

Barabino, S., Antonelli, S., Cimbolini, N., Mauro, V., Bouzin, M., 2014. The Effect of preservatives and antiglaucoma treatments on the ocular surface of mice with dry eye. Invest. Ophthalmol. Vis. Sci. 55 (10), 6499.

Baudouin, C., Labbé, A., Liang, H., Pauly, A., Brignole-Baudouin, F., 2010. Preservatives in eyedrops: the good, the bad and the ugly. Prog. Retin. Eye Res. 29 (4), 312–334.

Bettger, W.J., McKeehan, W.L., 1986. Mechanisms of cellular nutrition. Physiol. Rev. 66 (1), 1–35.

Brignole-Baudouin, F., Desbenoit, N., Hamm, G., Liang, H., Both, J.P., Brunelle, A., Fournier, I., Guerineau, V., Legouffe, R., Stauber, J., Touboul, D., Wisztorski, M., Salzet, M., Laprevote, O., Baudouin, C., 2012. A new safety concern for glaucoma treatment demonstrated by mass spectrometry imaging of benzalkonium chloride distribution in the eye, an experimental study in rabbits. PLoS One 7 (11).

Burstein, N.L., 1980. Preservative cytotoxic threshold for benzalkonium chloride and chlorhexidine digluconate in cat and rabbit corneas. Invest. Ophthalmol. Vis. Sci. 19 (3), 308–313.

Burnstein, N.L., 1985. The effects of topical drugs and preservatives on the tears and corneal epithelium in dry eye. Trans. Ophthalmol. Soc. U. K. 104 (Pt 4), 402–409.

Chang, C., Zhang, A.Q., Kagan, D.B., Liu, H., Hutnik, C.M., 2015. Mechanisms of benzalkonium chloride toxicity in a human trabecular meshwork cell line and the protective role of preservative-free tafluprost. Clin. Exp. Opthalmol. 43 (2), 164–172.

Chen, W., Li, Z., Hu, J., Zhang, Z., Chen, L., Chen, Y., Liu, Z., 2011. Corneal alternations induced by topical application of benzalkonium chloride in rabbit. PLoS One 6 (10).

Chen, X., Sullivan, D.A., Sullivan, A.G., Kam, W.R., Liu, Y., 2018. Toxicity of cosmetic preservatives on human ocular surface and adnexal cells. Exp. Eye Res. 170, 188–197.

Ciancaglini, M., Carpineto, P., Agnifili, L., Nubile, M., Fasanella, V., Lanzini, M., Calienno, R., Mastropasqua, L., 2008. An *in vivo* confocal microscopy and impression cytology analysis of preserved and unpreserved levobunolol-induced conjunctival changes. Eur. J. Ophthalmol. 18 (3), 400–407.

Contzler, R., Favre, B., Huber, M., Hohl, D., 2005. Cornulin, a new member of the "fused gene" family, is expressed during epidermal differentiation. J. Invest. Dermatol. 124 (5), 990–997.

Craig, J.P., Nichols, K.K., Akpek, E.K., Caffery, B., Dua, H.S., Joo, C.K., Liu, Z., Nelson, J.D., Nichols, J.J., Tsubota, K., Stapleton, F., 2017. TFOS DEWS II Definition and Classification Report. Ocul. Surf. 15 (3), 276–283.

De Saint Jean, M., Brignole, F., Bringuier, A.F., Bauchet, A., Feldmann, G., Baudouin, C., 1999. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. Invest. Ophthalmol. Vis. Sci. 40 (3), 619–630.

Debbasch, C., Brignole, F., Pisella, P.J., Warnet, J.M., Rat, P., Baudouin, C., 2001. Quaternary ammoniums and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. Invest. Ophthalmol. Vis. Sci. 42 (3), 642–652.

Desbenoit, N., Schmitz-Afonso, I., Baudouin, C., Laprévote, O., Touboul, D., Brignole-Baudouin, F., Brunelle, A., 2013. Localisation and quantification of benzalkonium chloride in eye tissue by TOF-SIMS imaging and liquid chromatography mass spectrometry. Anal. Bioanal. Chem. 405 (12), 4039–4044.

Dowdall, J.R., Sadow, P.M., Hartnick, C., Vinarsky, V., Mou, H., Zhao, R., Song, P.C., Franco, R.A., Rajagopal, J., 2015. Identification of distinct layers within the stratified squamous epithelium of the adult human true vocal fold. Laryngoscope 125 (9), E313–E319.

European Glaucoma Society Terminology and Guidelines for Glaucoma, 4th Edition – Chapter 3: Treatment principles and options. Supported by the EGS Foundation. Br. J. Opthalmol. 2017. 101(6), 130-195.

Friedlaender, M.H., Breshears, D., Amoozgar, B., Sheardown, H., Senchyna, M., 2006. The dilution of benzalkonium chloride (BAK) in the tear film. Adv. Ther. 23 (6), 835–841.

Gasset, A.R., Ishii, Y., Kaufman, H.E., Miller, T., 1974. Cytotoxicity of ophthalmic preservatives. Am. J. Ophthalmol. 78 (1), 98–105.
Grayson, W.L., Bhumiratana, S., Cannizzaro, C., Chao, P.H., Lennon, D.P., Caplan, A.I., Vunjak-Novakovic, G., 2008. Effects of initial seeding density and fluid perfusion rate on formation of tissue-engineered bone. Tissue Eng. A 14 (11), 1809–1820.
Gutgesell, V.J., Stern, G.A., Hood, C.I., 1982. Histopathology of meibomian gland dysfunction. Am. J. Ophthalmol. 94 (3), 383–387.
Hampel, U., Garreis, F., 2017. The human meibomian gland epithelial cell line as a model to study meibomian gland dysfunction. Exp. Eye Res. 163, 46–52.
Hampel, U., Schröder, A., Mitchell, T., Brown, S., Snikeris, P., Garreis, F., Kunnen, C., Willcox, M., Paulsen, F., 2015. Serum-induced keratinization processes in an immortalized human meibomian gland epithelial cell line. PLoS One 10 (6).
Heng, B.C., Zhao, X., Xiong, S., Ng, K.W., Boey, F.Y., Loo, J.S., 2011. Cytotoxicity of zinc oxide (ZnO) nanoparticles is influenced by cell density and culture format. Arch. Toxicol. 85 (6), 695–704.
Herreras, J.M., Pastor, J.C., Calonge, M., Asensio, V.M., 1992. Ocular surface alteration after long-term treatment with an antiglaucomatous drug. Ophthalmology 99 (7), 1082–1088.
Ichijima, H., Petroll, M.W., Jester, J.V., Cavanagh, D.H., 1992. Confocal Microscopic Studies of living rabbit cornea treated with benzalkonium chloride. Cornea 11 (3), 221–225.
Iwasawa, A., Ayaki, M., Niwano, Y., 2013. Cell viability score (CVS) as a good indicator of critical concentration of benzalkonium chloride for toxicity in cultured ocular surface cell lines. Regul. Toxicol. Pharm. 66 (2), 177–183.
Jaenen, N., Baudouin, C., Pouliquen, P., Manni, G., Figueiredo, A., Zeyen, T., 2007. Ocular symptoms and signs with preserved and preservative-free glaucoma medications. Eur. J. Ophthalmol. 17 (3), 341–349.
Jester, J.V., Nicolaides, N., Smith, R.E., 1981. Meibomian gland studies: histologic and ultrastructural investigations. Invest. Ophthalmol. Vis. Sci. 20 (4), 537–547.
Kam, W.R., Liu, Y., Ding, J., Sullivan, D.A., 2016. Do cyclosporine A, an IL-1 receptor antagonist, uridine triphosphate, rebamipide, and/or bimatoprost regulate human meibomian gland epithelial cells? Invest. Ophthalmol. Vis. Sci. 57 (10), 4287.
Knop, E., Knop, N., 2009. Meibomian glands: part IV. Functional interactions in the pathogenesis of meibomian gland dysfunction (MGD). Ophthalmologe 106 (11), 980–987.
Knop, E., Knop, N., Millar, T., Obata, H., Sullivan, D.A., 2011. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. Invest. Ophthalmol. Vis. Sci. 52 (4), 1938–1978.
Lang, F., 2007. Mechanisms and significance of cell volume regulation. J. Am. Coll. Nutr. 26 (5 Suppl), 613S–623S.
Lee, S.H., Tseng, S.C., 1997. Rose bengal staining and cytologic characteristics associated with lipid tear deficiency. Am. J. Ophthalmol. 124 (6), 736–750.
Lee, J.H., Lee, J.S., Kim, S., Lee, J.E., 2017. Comparison of cytotoxicities and wound healing effects of diquafosol tetrasodium and hyaluronic acid on human corneal epithelial cells. J. Physiol. Pharmacol. 21 (2), 189.
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, S., Hatton, M.P., Khandelwal, P., Sullivan, D.A., 2010. Culture, immortalization, and characterization of human meibomian gland epithelial cells. Invest. Opthalmol. Vis. Sci. 51 (8), 3993.
Martínez-Soroa, I., de Frutos-Lezaun, M., Ostra Beldarrain, M., Egía Zurutuza, A., Irastorza Larburu, M., Bachiller Cacho, M., 2016. Determination of phosphate concentration in glaucoma eye drops commercially available in Spain. Arch. Soc. Esp. Oftalmol. 91 (8), 363–371.
Mietz, H., Niesen, U., Krieglstein, G.K., 1994. The effect of preservatives and antiglaucomatous medication on the histopathology of the conjunctiva. Graefes Arch. Clin. Exp. Ophthalmo. 232 (9), 561–565.
Millar, T.J., Schuett, B.S., 2015. The real reason for having a meibomian lipid layer covering the outer surface of the tear film – a review. Exp. Eye Res. 137, 125–138.
Nelson, J.D., Craig, J.P., Akpek, E.K., Azar, D.T., Belmonte, C., Bron, A.J., Clayton, J.A., Dogru, M.D., Dua, H.S., Foulks, G.N., Gomes, J.A.P., Hammitt, K.M., Holopainen, J., Jones, L., Joo, C.K., Liu, Z., Nichols, J.J., Nichols, K.K., Novack, G.D., Sangwan, V., Stapleton, F., Tomlinson, A., Tsubota, K., Willcox, M.D.P., Wolffsohn, J.S., Sullivan, D.A., 2017. TFOS DEWS II introduction. Ocul. Surf. 15 (3), 269–275.
Noecker, R., 2001. Effects of common ophthalmic preservatives on ocular health. Adv. Ther. 18 (5), 205–211.
Orlov, S.N., Novikov, K.N., 1996. The regulation of cell volume: the mechanisms, coupled cellular reactions and pathophysiological significance. Fiziologicheskii zhurnal imeni IM Sechenova 82 (8–9), 1–15.
Paulsen, F., Garreis, F., Hampel, U., 2015. Pathophysiologie in Meibom-Drüsen-Dysfunktion – Aktuelle Diagnose und Therapieoptionen. UNI-MED, Bremen.
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. Invest. Ophthalmol. Vis. Sci. 48 (12), 5473.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29 (9), e45.
Pisella, P.J., Pouliquen, P., Baudouin, C., 2002. Prevalence of ocular symptoms and signs with preserved and preservative free glaucoma medication. Br. J. Ophthalmol. 86 (4), 418–423.
Smedowski, A., Paterno, J.J., Toropainen, E., Sinha, D., Wylegala, E., Kaarniranta, K., 2014. Excipients of preservative-free latanoprost induced inflammatory response and cytotoxicity in immortalized human HCE-2 corneal epithelial cells. J. Biochem. Pharmacol. Res. 2 (4), 175–184.
Stapleton, F., Alves, M., Bunya, V.Y., Jalbert, I., Lekhanont, K., Malet, F., Na, K.S., Schaumberg, D., Uchino, M., Vehof, J., Viso, E., Vitale, S., Jones, L., 2017. TFOS DEWS II epidemiology report. Ocul. Surf. 15 (3), 334–365.
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. Invest. Opthalmol. Vis. Sci. 55 (6), 3866.
van der Bijl, P., van Eyk, A.D., Gareis, A.A., Thompson, I.O., 2002. Enhancement of transmucosal permeation of cyclosporine by benzalkonium chloride. Oral Dis. 8 (3), 168–172.
van der Bijl, P., van Eyk, A.D., Meyer, D., 2001. Effects of three penetration enhancers on transcorneal permeation of cyclosporine. Cornea 20 (5), 505–508.
Wang, L., Seshareddy, K., Weiss, M.L., Detamore, M.S., 2009. Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering. Tissue Eng. A 15 (5), 1009–1017.
Xiao, X., He, H., Lin, Z., Luo, P., He, H., Zhou, T., Zhou, Y., Liu, Z., 2012. Therapeutic effects of epidermal growth factor on benzalkonium chloride–induced dry eye in a mouse model. Invest. Ophthalmol. Vis. Sci. 53 (1), 191.
Xie, H.T., Sullivan, D.A., Chen, D., Hatton, M.P., Kam, W.R., Liu, Y., 2018. Biomarkers for progenitor and differentiated epithelial cells in the human meibomian gland. Stem Cells Transl. Med. 9999, 1–6.
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. Invest. Opthalmol. Vis. Sci. 49 (5), 1850.
Zhang, Y., Kam, W.R., Liu, Y., Chen, X., Sullivan, D.A., 2017. Influence of pilocarpine and timolol on human meibomian gland epithelial cells. Cornea 36 (6), 719–724.