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Ocular Surface Insight

Issue 16

Artificial Tear Medications for Evaporative Dry Eye Disease

Ocular Rosacea

The Effect of Anti-Inflammatory Topical Ophthalmic Treatments

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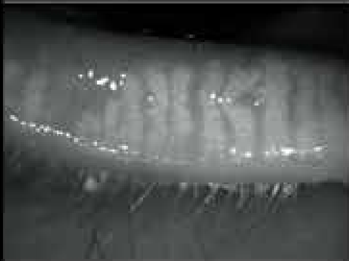
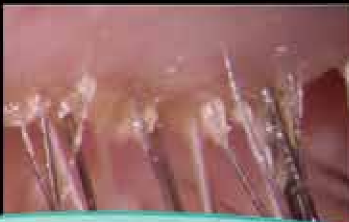


2 INFLAMMATION
Rosacea and blepharitis

3 DEMODEX



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Ocular Surface Insight

"The price of success is hard work, dedication to the job at hand, and the determination that whether we win or lose, we have applied the best of ourselves to the task at hand."

Vince Lombardi

Welcome to OSI Autumn issue!

It seems like only yesterday that many of us met at OSI Symposium & Dry Eye Masterclass, in London earlier this summer. I want to thank all our inspiring speakers, dedicated delegates and supporting industry colleagues who made the meeting such a great success.

Planning is already underway for our next OSI Symposium and Dry Eye Masterclass update, which will take place on the 24-25 March 2023 in Kensington, London. The key topics that will be covered are outlined on the back of this magazine, and more details will follow soon on our website. We set the bar high at this year's meeting, but we are committed to improve and expand to make this an unmissable event in the calendar for ophthalmologists and optometrists who share our passion for innovation, and best practice in ocular surface and dry eye disease.

In this issue we have a great article from Nikolina Budimlija detailing the different treatment and lifestyle adjustment options for ocular rosacea.

Prof. Ejaz Ansari and Hasan Naveed have produced an interesting review of the artificial tear options available for the treatment of evaporative dry eye.

Samer Hamada

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We stand with Ukraine!

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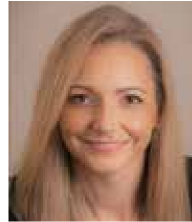

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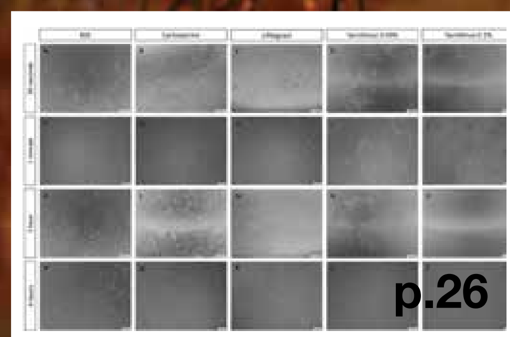

What's in the news?

The effects of breaks on digital eye strain, dry eye and binocular vision: Testing the 20-20-20 rule

The purpose of this study was to evaluate the benefits on the eyes of taking breaks based on the 20-20-20 rule.

Bespoke computer software using the laptop webcam to assess user breaks, eye gaze and blinking, and emitting personalized reminders of breaks based on the 20-20-20 rule, was downloaded onto the laptops of 29 symptomatic computer users. Digital eye strain (DES), binocular vision and dry eye were assessed before and after two weeks of using the reminders and one week after the discontinuation of the strategy. Binocular measurements included visual acuity, accommodative posture, stereopsis, fixation disparity, ocular alignment, accommodative facility, positive/negative vergences and near point of convergence. Symptoms were evaluated using the computer vision syndrome questionnaire, ocular surface disease index (OSDI), and symptom assessment in dry eye questionnaire (SANDE) versions one and two. Dry eye signs were assessed by measuring tear meniscus height, conjunctival redness, blink rate and incomplete blinking, lipid layer thickness, non-invasive keratograph break-up time, corneal and conjunctival staining and lid wiper epitheliopathy.

A decrease in the duration of computer work and the duration of breaks, along with an increase in the number of breaks taken per day was observed as a result of the 20-20-20 rule reminders ($p \le 0.015$). No changes on any binocular parameter were observed after the management period ($p \ge 0.051$), except for an increase in accommodative facility ($p = 0.010$). Dry eye symptoms and DES decreased with the rule reminders ($p \le 0.045$), although this improvement was

not maintained one week after discontinuation ($p > 0.05$). No changes on any ocular surface and tear film parameter were observed with the rule reminders ($p \ge 0.089$).

The authors concluded that the 20-20-20 rule is an effective strategy for reducing DES and dry eye symptoms, although 2 weeks was not enough to considerably improve binocular vision or dry eye signs.

Authors: Cristian Talens-Estarelles, Alejandro Cerviño , Santiago García-Lázaro, Andrej Fogelton , Amy Sheppard , James S Wolffsohn.
Publication: Cont Lens Anterior Eye. 2022 Aug 10;101744.doi: 10.1016/j.clae.2022.101744.

Short-term results of a pulsed therapy with hydrocortisone eye drops to treat moderate to severe dry eye in primary Sjögren syndrome patients

The authors investigated the safety and efficacy of short-term treatment with topical low-dose hydrocortisone sodium phosphate 0.335% (PFH) in patients with moderate to severe primary Sjögren syndrome (SS)-related dry eye disease (DED).

A retrospective single-centre interventional study. All patients received PFH for 6 days with a pulsed posology: three times daily for 2 days, twice daily for 2 days, and once daily for 2 days. This scheme was repeated for 3 consecutive months and then alternated for 3 months. Data were collected at baseline, 3 months, and 6 months of follow-up.

A total of 40 SS patients were enrolled. Conjunctival hyperaemia and corneal-conjunctival stain significantly improved ($p < 0.001$). Ocular Surface Disease Index score reduced significantly between baseline and 3 months and between baseline and 6 months ($p < 0.001$). The tear film osmolarity lowered significantly in each eye from baseline to 3 months and from baseline to 6 months ($p = 0.002$ and $p = 0.037$, respectively). Comparing results at 3 and 6 months, the Ocular Surface Disease Index score ($p = 1.000$), the frequency of lacrimal substitutes installation ($p = 0.632$), and tear film osmolarity (right eye $p = 0.518$, left eye $p = 1.000$)

did not change significantly. Intraocular pressure did not change during the study period.

The authors found that PFH eye drops with a pulsed posology improve signs and symptoms, not affecting the intraocular pressure in SS-related DED. Therefore, this pulsed treatment is safe and efficacious.

Authors: Thiago Gonçalves Dos Santos Martins, Paulo Schor, Luís Guilherme Arneiro Mendes, Andreas Anschütz, Rufino Silva.
Publication: Einstein (Sao Paulo). 2022 May 6;20:eAO6613. doi:10.31744/einstein_journal/2022AO6613.eCollection 2022.

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What’s in the news?

Dry Eye-Related Risk Factors for Digital Eye Strain

The objective of this survey was to evaluate dry eye-related lifestyle and demographic factors associated with digital eye strain (DES).

An anonymous online survey was conducted in 851 university students. Participants were classified into DES (computer vision syndrome questionnaire [CVS-Q]≥6) or non-DES (CVS-Q<6). Respondents completed three dry eye questionnaires (Ocular Surface Disease Index [OSDI]; 5-item Dry Eye Questionnaire [DEQ-5]; 8-item Contact Lens Dry Eye Questionnaire) and were surveyed on dry eye risk factors contemplated by the Tear Film and Ocular Surface Society Dry Eye Workshop II.

Six hundred twenty-eight participants were classified into the group with DES and 222 into the group without DES. Participants with DES slept fewer hours, spent more hours indoors with air conditioning, drank more caffeinated beverages, used the computer for longer periods, reported poorer health quality, and obtained a higher score in all questionnaires (P<0.025). A higher proportion of the participants were female, had several health disorders, and took several medications associated with dry eye (P<0.029). Multivariate logistic regression analysis revealed that stress (P=0.035), contact lens wear (P=0.011), hours of computer use per day (P=0.010), migraine headaches (P=0.013), and a higher OSDI (P<0.001) and DEQ-5 score (P<0.001) were associated with DES.

The conclusion from the results was that several dry eye-related risk factors and health conditions are associated with suffering from DES. Clinicians should acknowledge the relevance of triaging questions and dry eye disease risk factors when dealing with patients who view screens for extended periods.

Authors: Cristian Talens-Estarelles , José Vicente García-Marqués, Alejandro Cerviño, Santiago García-Lázaro.
Publication: Eye Contact Lens 2022 Oct 1;48(10):410-415.doi: 10.1097/ICL.0000000000000923.Epub 2022 Jul 8.

What’s in the news?

Omega-3 fatty acids in the management of dry eye disease-An updated systematic review and meta-analysis

The purpose of this study was to examine the effectiveness of omega-3 fatty acids in comparison to a placebo in the management of dry eye disease.

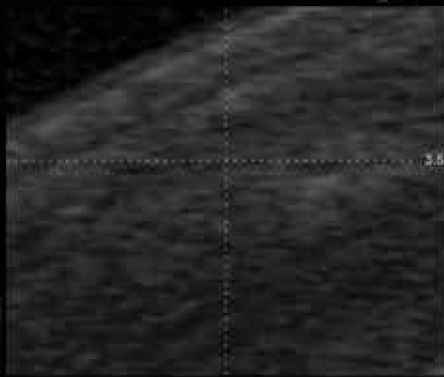
A systematic literature search was performed including randomised controlled trials (RCTs) comparing omega-3 versus placebo in the management of dry eye disease in human subjects. There were no language or time restrictions. Eligible trials were assessed for bias and assigned a risk-of-bias score. Data extraction was carried out using a standardised data extraction form, and meta-analysis was performed using a random effects model for continuous data. The outcome measures were Ocular Surface Disease Index (OSDI) scores, tear breakup time (TBUT) measurements, corneal staining and Schirmer’s score. Statistical heterogeneity was defined as substantial if the I2 test achieved a value >60%.

Eight parallel RCTs including 1107 subjects met eligibility criteria. None of the included studies achieved low risk of bias. Data synthesis demonstrated an improvement in the mean change in OSDI score from baseline to final assessment. Omega-3 supplementation conferred no evident improvement in corneal staining, TBUT or Schirmer’s score. There was considerable statistical heterogeneity in all four outcome measures.

This updated systematic review and meta-analysis indicates that omega-3 supplementation improves subjective symptoms in patients with dry eye disease.

Authors: Ciara O’Byrne, Michael O’Keeffe.
Publication: Acta Ophthalmol. 2022 Sep 22.doi: 10.1111/aos.15255.

Imaging beyond compare



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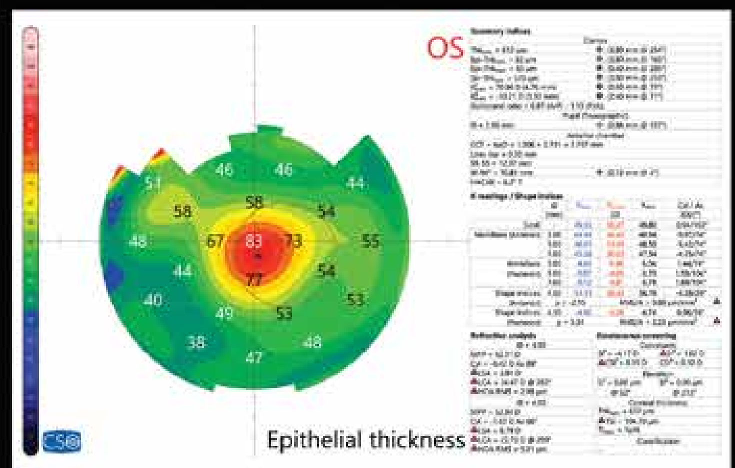

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Ocular rosacea

Rosacea is a chronic but treatable skin condition that primarily affects the central face, and is often characterized by flare-ups and remissions. Ocular rosacea is a form of rosacea that involves the eyelids and ocular surface and includes blepharitis, conjunctival hyperaemia, MGD and rosacea-associated keratitis.

Although rosacea can affect all segments of the population and all skin types, individuals with fair skin who tend to flush or blush easily are believed to be at greatest risk. The disorder is more frequently diagnosed in women, but tends to be more severe in men. It is uncommon in children and generally starts after the age of 30 years. There is also evidence that rosacea may tend to run in families, and may be especially prevalent in people of northern or eastern European descent. In Ireland it is know as “Course of the Celts”

What is the cause of ocular rosacea?

The exact cause of ocular rosacea is unknown. However, immunological factors, micro-organisms on the skin surface, and reactive blood vessels are involved.

- *Demodex folliculorum* mites, normal inhabitants of eyelash follicles, may stimulate inflammation in ocular rosacea and anterior blepharitis.
- Bacteria may play a role, as ocular rosacea improves with antibiotics. One theory is that bacterial lipases release toxic free fatty acids and glycerides from lipids secreted by meibomian glands.
- The pro-inflammatory cytokine interleukin 1-alpha (IL-1α) increases metallomatrix protein-9 (MMP-9) in the tear fluid. Upregulation of MMP-9 damages ocular tissues.

Other recent studies that have found associations between rosacea and increased risk for a growing number of potentially serious systemic diseases, suggesting that rosacea may be an outcome of systemic inflammation. Although causal relationships have not been determined, these have included cardiovascular disease, gastrointestinal disease, neurological and autoimmune diseases and certain cancers.

In some cases, rosacea symptoms can be triggered or made worse by exposure to ultraviolet light, high and low temperatures, stress, physical activity, alcohol, smoking, spicy food, and hot drinks.

Symptoms and clinical signs

Symptoms can include: itching, burning and soreness, epiphora, photophobia, foreign body sensation, dry eye, inflamed papules.

Clinical signs: Anterior and posterior blepharitis, keratitis, corneal ulcers and rarely scleritis and episcleritis.

Treatment of ocular rosacea

Conservative management

- Lid hygiene with wipes designed for blepharitis
- Warm compresses to closed eyelids for five to ten minutes daily can improve flow of meibomian gland secretions, chalazion and hordeolum.
- Artificial tears (ocular lubricants) reduce symptoms due to dry eye.
- Avoid wearing contact lenses if they irritate the in flamed eye.

Topical anti-inflammatory agents

- Topical steroids are used short-term to treat marked lid inflammation or rosacea keratitis.
- Topical ciclosporin is typically used in ocular rosacea that has not responded to topical steroids.

Systemic antibiotics

The following oral antibiotics are used for ocular rosacea:

- Tetracyclines such as doxycycline
- Macrolides such as erythromycin

They reduce bacteria, improve tear film stability and normalise Meibomian gland secretions.

Oral antibiotics are generally continued for 6–12 weeks, and then slowly tapered over the course of one to two months. Further courses of oral antibiotics can be used for disease flare-ups.

Intense pulse light (IPL) treatment

IPL therapy utilizes a high-intensity noncoherent light in the wavelength range of 500–1200 nm. It has been an effective treatment for many dermatological conditions as it demonstrates angiodestructive and anti-inflammatory properties that prove beneficial for erythema and swelling. It is thought that the superficial blood vessels in ocular rosacea are the primary site of IPL activity. The general understanding is that intense light is absorbed by chromophores in oxyhaemoglobin, causing abnormal blood vessels in inflamed areas to thrombose. Once closed, these abnormal blood vessels are no longer able to leak inflammatory mediators into the surrounding tissue. In addition, the temporary thermal effect of the light on meibomian glands is thought to decrease the viscosity of meibum, thus improving secretions and tear film quality.

Lifestyle Management

In addition to long-term medical therapy, rosacea patients can improve their chances of maintaining remission by identifying and avoiding lifestyle and environmental factors — often related to flushing — that may trigger flare-ups or aggravate their individual conditions. Identifying these factors is an individual process, however, because what causes a flare-up in one person may have no effect on another.

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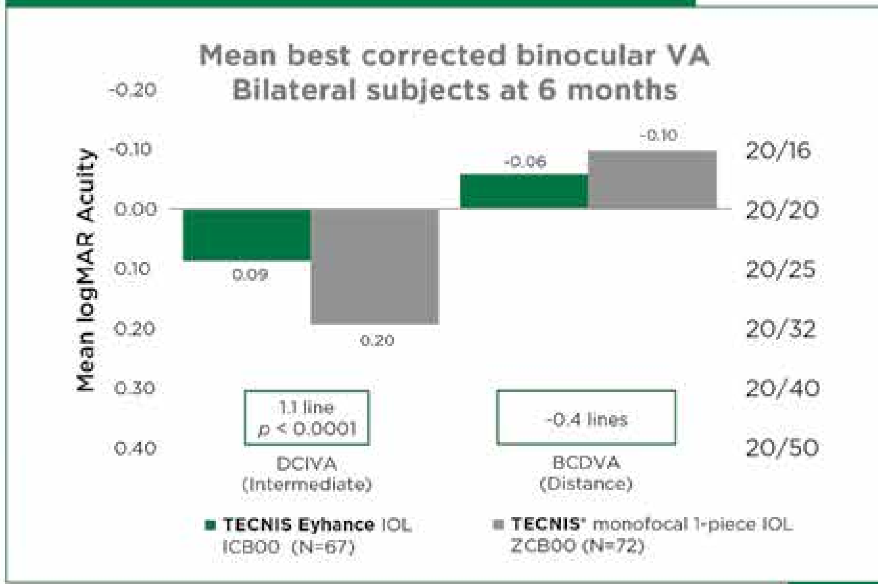

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Reference

1. Data on File, Johnson & Johnson Surgical Vision, Inc. Sep 2018. DOF2018CT4015.

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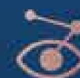

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Warnings and Precautions: May gradually change the iris colour; inform patients of this risk. Periorbital and eye lid skin darkening and eyelash changes have been reported. Periorbital and lid changes including deepening of the eyelid sulcus have been observed with prostaglandin analogues. Macular oedema has been reported during treatment with prostaglandin F2a analogues. Caution is recommended when in aphakic patients, pseudophakic patients with a torn posterior lens capsule or anterior chamber lenses, or in patients with known risk factors for cystoid macular oedema. Use with caution in patients with known predisposing risk factors for iritis/uveitis. Avoid contact with the skin and in patients who are pregnant or attempting to become pregnant. Patients must be instructed to remove contact lenses prior to application of VisuTRAX® and wait 15 minutes after instillation of the dose before reinsertion. Contains macrogolglycerolhydroxystearate which may cause severe allergic reactions.

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Legal category: POM.

Marketing Authorisation Number: PL 52223/0002.

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Date of Preparation: June 2022.

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1. VisuTRAX® Summary of Product Characteristics. **2.** European Glaucoma Society. Terminology and Guidelines for Glaucoma 5th Edition. October 2020. **3.** Quaranta L, et al. Safety and efficacy of travoprost solution for the treatment of elevated intraocular pressure. Clinical Ophthalmology 2015:9 633-643. **4.** Moussa WGEH, et al. Comparison of Efficacy and Ocular Surface Disease Index Score between Bimatoprost, Latanoprost, Travoprost, and Tafluprost in Glaucoma Patients. Journal of Ophthalmology. Volume 2018, Article ID 1319628, 7 pages. **5.** Parrish RK, Palmberg P, Sheu WP, XLT Study Group. A Comparison of Latanoprost, Bimatoprost, and Travoprost in Patients With Elevated Intraocular Pressure: a 12-week, Randomized, Masked-evaluator Multicenter Study. Am J Ophthalmol. 2003;135(5):688–703. **6.** Cai Z, et al. Analysis of the Responsiveness of Latanoprost, Travoprost, Bimatoprost, and Tafluprost in the Treatment of OAG/OHT Patients. Journal of Ophthalmology. Volume 2021, Article ID 5586719, 12 pages. **7.** Netland PA, Landry T, Sullivan EK, et al. Travoprost Compared With Latanoprost and Timolol in Patients With Open-angle Glaucoma or Ocular Hypertension. Am J Ophthalmol. 2001;132(4):472–484. **8.** Lewis R, et al. Travoprost 0.004% With and Without Benzalkonium Chloride: A Comparison of Safety and Efficacy. J Glaucoma 2007:16 98–103. **9.** Tatham AJ. The Use of Generic Medications for Glaucoma. Journal of Ophthalmology. Volume 2020, Article ID 1651265, 8 pages. **10.** VISUfarma Data on File. June 2022. **11.** MIMS www.MIMS.co.uk last accessed August 2022.

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Creating value in eye health

Benzalkonium Chloride, Even at Low Concentrations, Deteriorates Intracellular Metabolic Capacity in Human Conjunctival Fibroblasts

Yuri Tsugeno, Tatsuya Sato, Megumi Watanabe, Masato Furuhashi, Araya Umetsu,
Yosuke Ida, Fumihito Hikage, and Hiroshi Ohguro

By **Nadezhda S. Kudryasheva**, Academic Editor

Abstract

The objective of this study was to clarify the effects of benzalkonium chloride (BAC) on two-dimensional (2D) and three-dimensional (3D) cultures of human conjunctival fibroblast (HconF) cells, which are in vitro models replicating the epithelial barrier and the stromal supportive functions of the human conjunctiva. The cultured HconF cells were subjected to the following analyses in the absence and presence of 10–5% or 10–4% concentrations of BAC; (1) the barrier function of the 2D HconF monolayers, as determined by trans-endothelial electrical resistance (TEER) and FITC dextran permeability, (2) real-time metabolic analysis using an extracellular Seahorse flux analyzer, (3) the size and stiffness of 3D HconF spheroids, and (4) the mRNA expression of genes that encode for extracellular matrix (ECM) molecules including collagen (COL)1, 4 and 6, and fibronectin (FN), α-smooth muscle actin (α-SMA), ER stress related genes including the X-box binding protein-1 (XBP1), the spliced XBP1 (sXBP1) glucose regulator protein (GRP)78, GRP94, and the CCAAT/enhancer-binding protein homologous protein (CHOP), hypoxia inducible factor 1α (HIF1α), and Peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α). In the presence of BAC, even at low concentrations at 10–5% or 10–4%, the maximal respiratory capacity, mitochondrial respiratory reserve, and glycolytic reserve of HconF cells were significantly decreased, although the barrier functions of 2D HconF monolayers, the physical properties of the 3D HconF spheroids, and the mRNA expression of the corresponding genes were not affected. The findings reported herein highlight the fact that BAC, even such low concentrations, may induce unfavorable adverse effects on the cellular metabolic capacity of the human conjunctiva.

1. Introduction

It is well known that medical instillation therapy in the treatment of chronic ocular diseases including glaucoma, dry eye, and others can cause ocular surface adverse effects (OSAE) that can affect the eyelids, conjunctiva, and/or corneal epithelium [1,2,3,4], with the following symptoms often being associated with these conditions; redness, irritation, burning, fatigue, deteriorating visual acuity, infections, and others. As a possible causative factor, it has been suggested that for OSAE, toxicity by benzalkonium chloride (BAC), which is most commonly used in concentrations of 4~2 × 10–2% as a preservative in topical ophthalmic formulations [2,3,4,5,6], toward ocular tissue cells is the likely cause. The threshold concentration for inducing such toxic effects has been estimated to be approximately ~5 × 10–3% based upon several in vitro and in vivo studies showing that BAC exposure induced (1) a reduced survival of corneal cells [7,8,9,10,11,12], conjunctival cells [7,8,13,14], trabecular meshwork (TM) cells [15,16], and ciliary epithelial cells [9,13,15,17], loss of conjunctival goblet cells [13,14], (2) delayed corneal epithelial wound healing [18], (3) the induction of lymphocyte infiltration into conjunctival tissue [13,17], and (4) an increase in the levels of inflammatory cytokines in ocular tissues [9,10,12]. Alternatively, several clinical studies have reported that these BAC-induced adverse effects may partially be reversible upon withdrawal of the exposure to BAC [19,20,21,22,23,24]. However, in contrast, another previous study indicated that a short time exposure (30 min) to much lower concentrations of BAC ranging from 5 × 10–5% ~ or 10–3% toward immortalized human corneal epithelial cells (HCEs) caused DNA double-strand breaks (DSBs) and these breaks were concentration-dependent [25]. This information rationally suggests that BAC-induced risk may be evoked, even when much lower concentrations (5 × 10–5% ~ or 10–3%) of BAC are used as compared with the above estimated threshold levels. Therefore, to study the cytotoxic effects of BAC at much lower concentrations (10–5% and 10–4%) toward conjunctival tissues, we employed recently established in vitro models for the epithelial barrier and the stromal supportive functions of the human conjunctiva using two-dimension (2D) and three-dimension (3D) spheroid cultures of the human conjunctival fibroblasts (HconF) cells [26], and the following analyses were carried out: (1) barrier functions of 2D cultured HconF monolayers by trans-endothelial electron resistance (TEER) and FITC dextran permeability measurements, (2) measurements of real-time mitochondrial and glycolytic cellular function, (3) measurements of the size and hardness of the 3D HconF spheroids, and (4) quantitative PCR of major extracellular matrix (ECM) molecules, including collagen (COL)1, 4 and 6, and fibronectin, α-smooth muscle actin (α-SMA), ER stress related genes including the X-box binding protein-1 (XBP1), the spliced XBP1 (sXBP1) glucose regulator protein (GRP)78, GRP94, and the CCAAT/enhancer-binding protein homologous protein (CHOP), hypoxia inducible factor 1α (HIF1α), and Peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1α).

2. Materials and Methods

2.1. 2D Cell and 3D Spheroid Cultures Cultures of Human Conjunctival Fibroblasts (HconF)

BAC powder (CAS#8001-54-5, Nacalai Tesque, Kyoto, Japan) was dissolved in DMEM (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) adjusted to a concentration of 0.2%, and further diluted in 10% FBS (BioWest, Nuaillé, France). As a negative control, the same diluent was used. In the presence 10–5% or 10–4% BAC, or only diluent, HconF cells (ScienCell Research laboratories, CA U.S.A.) were 2D cultured and further maintained or subjected to 3D spheroid culturing for 6 days using hanging drop culture plates (# HDP1385, Sigma-Aldrich, St Louis, MO, USA), as described in our previous study [26]. Briefly, 2D cultured HconF cells in 150 mm 2D culture dishes at 37 °C in the Fibroblast Medium (FM, Cat. #2301, ScienCell Research laboratories, Carlsbad, CA, USA) [26] were maintained by changing the medium every other day. Alternatively, these 2D cultured HconF cells were washed with phosphate buffered saline (PBS), detached using 0.05% Trypsin/EDTA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and re-suspended in the Fibroblast Medium supplemented with 0.25% methylcellulose (Methocel® A4M, Sigma-Aldrich, St Louis, MO, USA). Then, approximately 20,000 HconF cells in 28 μL of the suspension were subjected to each well of the 3D hanging drop culture plate (# HDP1385, Sigma-Aldrich) (Day 0). Thereafter, on each following day until Day 6, half of the medium (14 μL) was exchanged by fresh medium.

2.2. Analysis of the Barrier Function of 2D HconF Cell Monolayers by TEER and FITC Dextran Permeability

In the absence and presence of 10–5% or 10–4% BAC, HconF cells were 2D cultured using a TEER plate (0.4 μm pore size and 12 mm diameter; Corning Transwell, Sigma-Aldrich) at 37 °C in the Fibroblast Medium as above. On Day 6, the TEER values between the 2D HconF monolayer were measured using an electrical resistance system (KANTO CHEMICAL CO. INC., Tokyo, Japan), and FITC-dextran permeability was estimated by measuring the fluorescence intensity that had permeated through the membrane from the basal compartment to the apical compartment during a period of 60 min as described in our previous study [27].

2.3. Seahorse Real-Time Bio-Cellular Metabolic Function Analysis of the 2D HconF Cells

As the bio-cellular function of 2D HconF cells, their oxygen consumption rate (OCR) and the extracellular acidification (ECAR) of 10–5% or 10–4% BAC-treated or un-treated 2D HconF cells were evaluated by a Seahorse XFe96 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) as described in the previous studies [28,29]. In brief, approximately 20,000 2D cultured HconF cells per well were set in an XFe96 Cell Culture Microplate (Agilent Technologies, #103794-100). The plate was centrifuged at 1600× g for 10 min, and the culture medium was replaced with 180 μL of assay buffer (Seahorse XF DMEM assay medium with 5.5 mM glucose, 2.0 mM glutamine, 1.0 mM sodium pyruvate (pH 7.4, Agilent Technologies, #103575-100)). Then, the assay plate was incubated in a CO2-free incubator at 37 °C for 1 h prior to the assay. OCR and ECAR were simultaneously measured on a Seahorse XFe96 Bioanalyzer under a 3-min-mixing and 3-min-measuring protocols using the following sequential injection of oligomycin (final concentration: 2.0 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, final concentration: 5.0 μM), rote-none/antimycin A mixture (final concentration: 1.0 μM), and 2-deoxyglucose (2-DG, final concentration: 10 mM). Valses of OCR and ECAR were normalized to the total protein per well after completion of assay.

2.4. Evaluation of the Size and Hardness of HconF Cell 3D Spheroids

The analyses of the physical properties, size, and hardness of the HconF 3D spheroids were performed as reported in our previous studies [30,31]. Briefly, the mean sizes of the 3D spheroids were measured using an inverted microscope (Nikon ECLIPSE TS2; Tokyo, Japan). Alternatively, for the hardness measurement, a single living 3D spheroid was placed on a 3-mm × 3-mm plate and compressed to achieve a 50% deformation during 20 seconds using a micro-compressor (MicroSquisher, CellScale, Waterloo, ON, Canada). The required force (μN) was measured, and force/displacement (μN/μm) was calculated.

2.5. Other Analytical Methods

Total RNA was extracted from the 2D or 3D cultured HconF cells and those were subjected to reverse transcription and real-time PCR as previously reported [32,33] using specific primers and probes (Supplemental Table S1).

As described in a recent report [32,33], all statistical analyses were performed using Graph Pad Prism 8 (GraphPad Software, San Diego, CA, USA). A significant difference at less than 0.05 between experimental groups by ANOVA followed by a Tukey's multiple comparison test was determined as statistically significant.

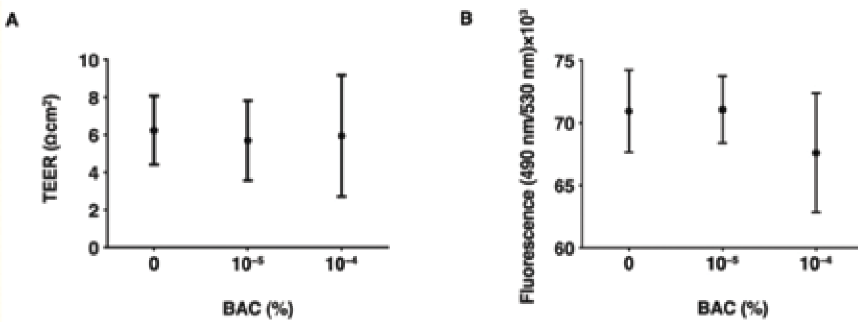
3. Results

Among ocular surfaces, the conjunctiva is well known to be involved in two different biological roles, namely, (1) a biological barrier by conjunctival epithelium [34] and (2) ocular tissue support, repair, and remodeling by conjunctival stroma [35,36]. In the current study, to investigate the effects of low concentrations of BAC at 10–5% or 10–4% on these conjunctival functions, under which no significant cytotoxicity was observed (Figure S1), we employed our recently established in vitro model using 2D and 3D cultures of HconF cells [26] which are thought to be related to the epithelial and stromal functions, respectively.

In a previous study we showed that, as the barrier function of the corneal epithelium, TEER values had significantly deteriorated even after a 20 min exposure to 10–3% of BAC [11]. Furthermore, 10–4% BAC also caused the significant up-regulation of IL-6 and IL-8 genes of 2D and the Matrigel®-assisted 3D spheroids of human trabecular meshwork (HTM) cells [37], although cytotoxic effects by BAC (2 × 10–2%) were not detected in the 3D corneal epithelial culture model [38]. However, in our established in vitro 3D spheroid model using HconF cells [26], HTM cells [31,39], human corneal stromal fibroblasts (HCSFs) [40] and human orbital fibroblast (HOFs) [41,42], several drugs including those treated with PGF2α, ROCK inhibitors, and others failed to induce cytotoxic effects but their physical properties were greatly modulated. These collective findings suggest that our established measurements of these physical properties of the 3D spheroids should be more sensitive in estimating cellular biological aspects, and therefore, it would be possible to use this methodology to evaluate effects of

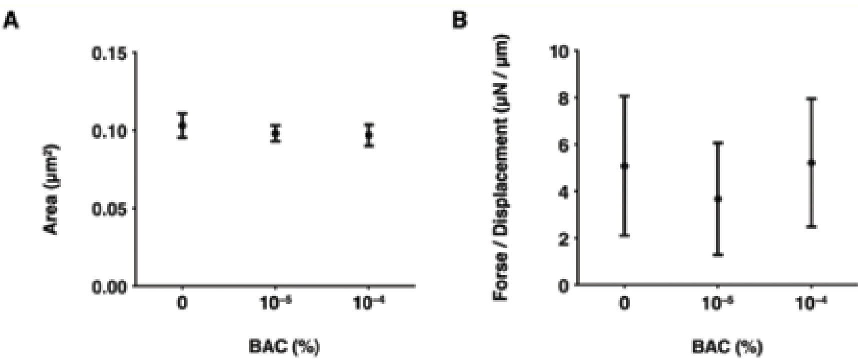
concentrations of BAC as low as 10−5% or 10−4%. Thus, initially, to study such low concentrations of BAC-induced effects on barrier function, TEER and FITC dextran permeability measurements of 2D HconF cell monolayers were conducted. As shown in Figure 1, both measurements were not affected at all by the presence of both 10−5% and 10−4% of BAC. Next, to estimate the influences of these low concentrations of BAC toward the tissue supportive functions of conjunctiva, the 3D HconF spheroid model was used. The result indicated that their physical properties, size, and stiffness were also not altered by these concentrations of BAC as similarly to the barrier functions of the 2D monolayer as above (Figure 2).

Figure 1



Effects of benzalkonium chloride (BAC) on barrier functions of HconF 2D monolayers. Barrier function based on TEER and FITC dextran permeability measurements were made on HconF cell 2D monolayers in the absence or presence of 10−5% or 10−4% BAC. Plots of the electric resistance (Ωcm2) by TEER and the absorbance of the amounts of permeated fluorescein are shown in (A) and (B), respectively. Experiments were repeated in triplicate (n = 5 each). All data are expressed; the mean ± the standard error of the mean (SEM). Statistical significance was evaluated by ANOVA followed by a Tukey's multiple comparison test.

Figure 2



Effects of BAC on the sizes and hardness of the HconF 3D spheroids. Analysis of the size and hardness of 3D HconF spheroids in the absence or presence of 10−5% or 10−4% BAC. The mean sizes (μm) and the force required to compress a single spheroid to the semidiameter (μN/μm) within 20 seconds are plotted in (A) and (B), respectively. Experiments were repeated in triplicate using fresh preparations (n = 16 spheroids each). All data are expressed; the mean ± the standard error of the mean (SEM). Statistical significance was evaluated by ANOVA followed by a Tukey's multiple comparison test.

To elucidate additional aspects related to the BAC-induced effects on the cellular physiology of the 2D HconF cells, mitochondria- and glycolysis-related functions were evaluated by a Seahorse real-time bio-cellular analyzer, since previous studies showed that upon BAC exposure, the OCR of rat liver mitochondria was substantially deteriorated [43], and glycolysis in bacteria was greatly modulated [44]. However, mitochondrial maximal respiration, mitochondrial respiratory reserve, and glycolytic reserve in HconF cells were significantly decreased by the presence of BAC, even at low concentrations, suggesting that BAC can adversely affect intracellular metabolic capacity (Figure 3A–D). The energy map visually indicated that metabolic response induced by FCCP-induced stress in HconF cells was impaired even with 10−4% BAC exposure, while BAC had no effect on baseline metabolism at either 10−5% and 10−4% concentrations (Figure 3E). These functional assays suggested that cellular mitochondrial and glycolytic functions were already affected even though the barrier functions by TEER and FITC dextran permeability of the 2D HconF monolayer and physical properties of the 3D HconF spheroid were not influenced in the presence of 10−5% and 10−4% BAC.

Figure 3 (On next page)

Effects of BAC on the mitochondrial and glycolytic functions in HconF 2D cells. A Seahorse real-time metabolic function analysis of HconF cells in the absence (blue dots, NT) or presence of BAC at concentrations of 10−5% (green dots) or 10−4% (brown dots) BAC. OCR (A) and ECAR (B) were measured at baseline and those with injections of sequential supplementation with a complex V inhibitor, oligomycin (Oligo), a protonphore, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), complex I/III inhibitors, rotenone/antimycin A (R/A), and a hexokinase inhibitor, 2-deoxyglucose (2DG). (C) Indicates the parameters of mitochondrial function. Basal OCR was calculated as the difference in OCR at the baseline and after the addition of R/A. ATP-linked respiration was calculated as the difference in OCR at the baseline and after the addition of Oligo. Proton leak was calculated as the difference between OCR after the addition of Oligo and OCR after the addition of R/A. Maximal respiration was calculated as the difference between OCR after the addition of FCCP and after the addition of R/A. Mitochondrial reserve capacity was calculated as the difference in OCR at baseline and after the addition of FCCP. (D) Indicates parameters of glycolytic function. Basal ECAR was calculated as the difference in ECAR at baseline and after the addition of 2DG. Glycolytic capacity was calculated as the difference between ECAR after the addition of Oligo and ECAR after the addition of 2DG. Glycolytic reserve was calculated as the difference in ECAR at baseline and after the addition of Oligo. (E) Energy map for cells in the absence or presence of 10−5% and 10−4% BAC. Experiments were performed using fresh preparations (n = 3). Data are expressed; the mean ± the standard error of the mean (SEM). * $p < 0.05$; ANOVA followed by a Tukey's multiple comparison test.

To study this issue further, the expressions of several genes including major ECM molecules including COL 1,4 and 6, FN and α-SMA, ER stress related factors, HIF1α, and PGC1α were analyzed by qPCR. As shown in Figure 4, Figure 5 and Figure 6, mRNA expression of these molecules was

not altered by these concentrations of BAC. Taken together with these gene expression analyses, Seahorse real time bio-metabolic measurements related to mitochondrial and glycolysis may be much more sensitive for evaluation of the physiological states of the living cells as compared with gene expressions of several related factors as well as physical analyses. Thus, these collective results suggest that some functional and morphological abnormality may be induced within human conjunctiva, even when such low concentrations of BAC are being used, especially in case with their long-term exposure.

Figure 3

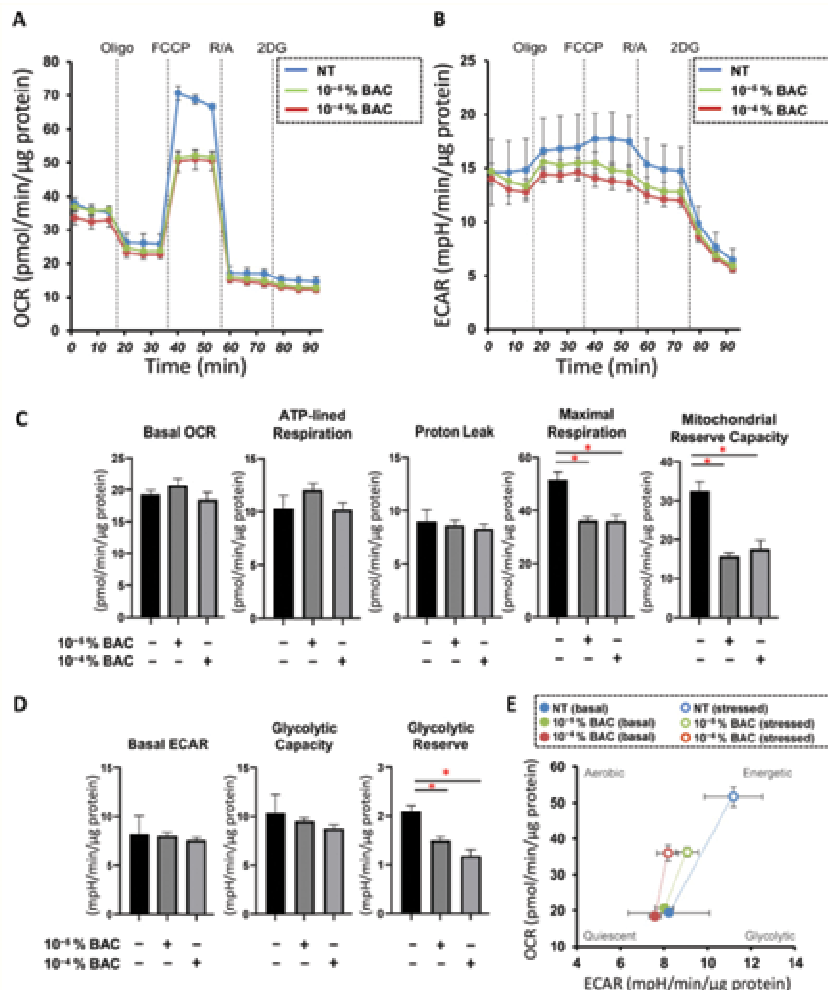


Figure 4

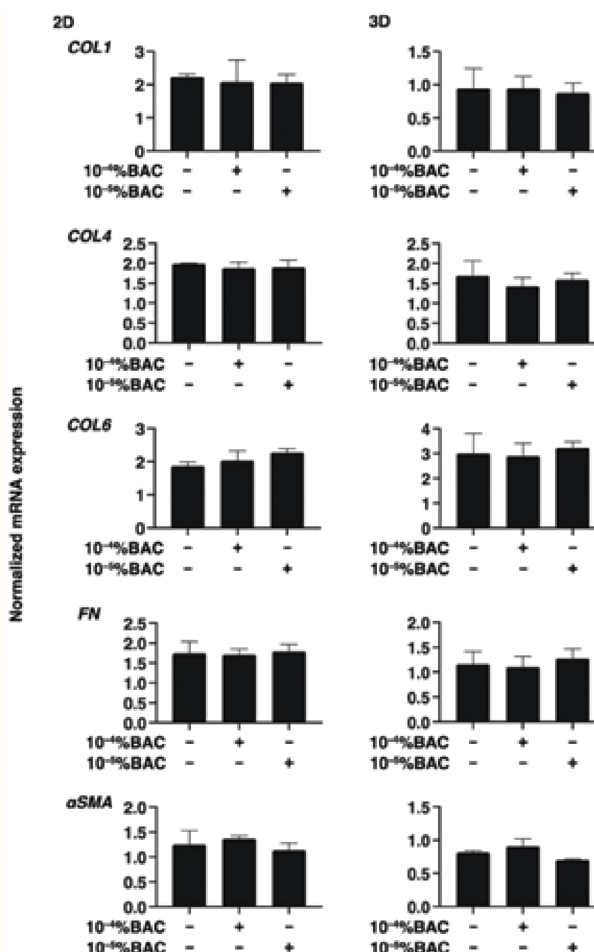
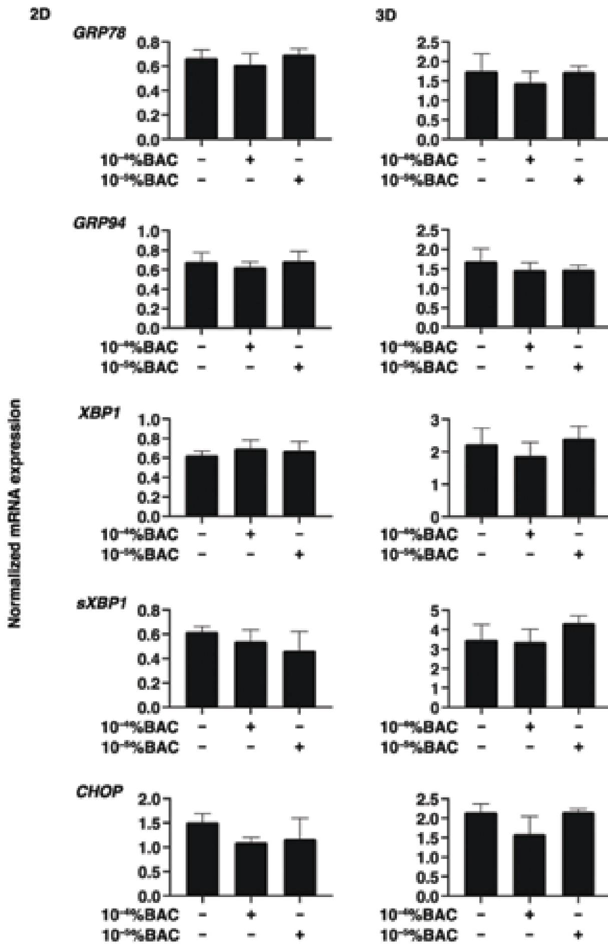


Figure 4
Effects of BAC on the gene expression of ECMs of HconF cells. 2D and 3D HconF cells were subjected to qPCR analysis for ECMs including COL1, COL4, COL6, FN, and aSMA in the absence or presence of 10–5% or 10–4% BAC. Experiments were repeated in triplicate using 3 different confluent 6-well dishes (2D) or 15 freshly prepared 3D HconF spheroids (3D) in each experimental condition. All data are expressed; the mean ± the standard error of the mean (SEM). Statistical significance was evaluated by ANOVA followed by a Tukey's multiple comparison test.

Figure 5



Effects of BAC on the gene expression of ER stress related factors of HconF cells. 2D and 3D HconF cells were subjected to qPCR analysis of ER stress-related genes in the absence or presence of 10–5% or 10–4% BAC; the glucose regulator protein (GRP)78, GRP94, the X-box binding protein-1 (XBP1), spliced XBP1 (sXBP1), and CCAAT/enhancer-binding protein homologous protein (CHOP). Experiments were repeated in triplicate using 3 different confluent 6-well dishes (2D) or 15 freshly prepared 3D HconF spheroids (3D) in each experimental condition. All data are expressed; the mean ± the standard error of the mean (SEM). Statistical significance was evaluated by ANOVA followed by a Tukey's multiple comparison test.

Figure 6

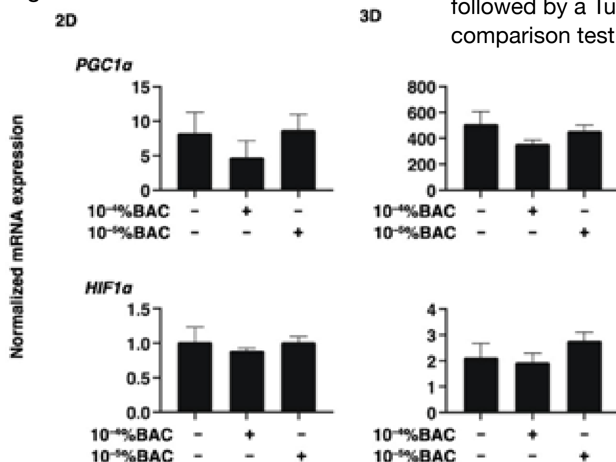


Figure 6

Effects of BAC on the gene expression of HIF1 and PGC1α of HconF cells. 2D and 3D HconF cells were subjected to qPCR analysis in HIF1α and PGC1α and 14 in the absence or presence of 10–5% or 10–4% BAC. Experiments were repeated in triplicate using 3 different confluent 6-well dishes (2D) or 15 freshly prepared 3D HconF spheroids (3D) in each experimental condition. All data are expressed; the mean ± the standard error of the mean (SEM). Statistical significance was evaluated by ANOVA followed by a Tukey's multiple comparison test.

4. Discussion

Since BAC, a polyquaternary ammonium detergent, was registered as a safe additive by the Environmental Protection Agency (EPA) in the United States in 1947 due to its broad-spectrum antimicrobial properties, BAC is now widely used in numerous agricultural, industrial, and clinical products [45,46,47]. Alternatively, it has been pointed out that toxicities could be induced in humans and other animals by oral uptake, by inhalation, or via an epidermal (including the eye) route [48]. Although BAC has not been reported to be carcinogenic, mutagenic, or genotoxic, an in vitro study demonstrated that the BAC concentrations should be controlled to as low as 1 mg/L to avoid possible risks for BAC induced genotoxic effects within plant and mammalian cells [49]. Furthermore, considerable cell toxicity toward human ocular and intranasal cells were observed in vitro on exposure to BAC concentrations as low as 10–3% [50] and 45 × 10–4% [51] respectively. In fact, the European Chemical Agency (ECHA) labels BAC as "causing severe skin burns and eye damage, is very toxic to aquatic life, is harmful if swallowed, and is harmful in contact with skin". Therefore, extensive in vivo as well as in vitro studies must be conducted in the case of using drugs containing BAC, including instillations.

Biochemically, it is known that BAC is capable of lysing cell membranes [52] and thus break cell–cell junctions within the corneal epithelium resulting in facilitating the penetration of the topically applied drugs into the anterior chamber [52,53]. It has been reported that BAC in topical ophthalmic formulations can penetrate through the ocular surface into the anterior chamber as well as the optic nerve [54,55]. Alternatively, it is also well known that BAC induces a number of adverse effects by stimulating inflammation in ocular surface tissues such as the conjunctiva [56,57,58,59]. In fact, a recent study reported that BAC exerts dose-dependent BAC toxic effects toward ocular surface epithelial cells using a mouse dry eye model [60]. Among several analyses using corneo-limbal epithelial cells (CLECs) in this study, a 24 h exposure of 10–2% BAC resulted in a significantly increased cytotoxicity, as evidenced by LDH assays as well as flow cytometry data on AnnV/PI stained cells as compared with non-treated controls. More interestingly, a significant reduction in both the colony forming

efficiency and the colony size of the CLEC cultures were observed when the exposure involved a 10−4% BAC solution. Such BAC-induced effects at lower concentrations were also detected by the mitochondrial analysis of human corneal epithelial cells [61], that is, BAC inhibited ATP production (IC50, 5.3 μM; 19 × 10−5%) and O2 consumption (IC50, 10.9 μM; 39 × 10−4%) and this inhibition was concentration-dependent. Taking the cationic property of BAC [52], a quaternary ammonium, into account, this observation is rationally supported by the fact that one of the major targets of BAC in the cell may be the only intracellular negatively charged mitochondria despite little information related to the influence of BAC on mitochondria.

Indeed, the findings presented in this study also demonstrated that significant reductions in mitochondrial and glycolytic reserve capacity are induced on exposure to BAC at concentrations of both 10−5% and 10−4% in 2D cultured HconF cells despite the fact that other analyses, including the barrier function of 2D monolayers, the physical properties of the 3D spheroids, and the mRNA expressions of several gene of ECM proteins, TIMPs, MMPs, and ER stress related factors were affected. Mitochondrial and glycolytic reserve refers to the ability of a cell to meet increased energy demands, and the retention of plasticity in these metabolic capacities has been reported to prevent cells from being driven into cellular senescence or cell death [62]. Therefore, the reduction of intracellular metabolic reserve induced by BAC exposure may potentially lead to an exacerbated cell dysfunction and cell death in HconF cells. However, as study limitations in the current investigation, additional information such as oxidative stress measurements, for instance, ROS production, or apoptosis will be required to confirm this speculation.

In conclusion, BAC, even at low concentrations, causes the deterioration of intracellular metabolic capacity in HconF cells. Since mitochondria play an indispensable role to maintain proper cellular functions, the effects of BAC noted herein raise great concerns for clinicians who are taking care of patients that are being administered formulations containing BAC, especially in cases of extended use.

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Artificial Tear Medications for Evaporative Dry Eye Disease

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Dry eye disease (DED) is a multifactorial condition of the ocular surface that exists on a continuum between aqueous deficiency and evaporative causes, underpinned by disruption of tear-film homeostasis.[1] It carries significant quality of life implications for patients, who can suffer from symptoms of ocular discomfort, reduced visual function, and develop progressive disease that can result in permanent injury to the ocular surface.[1,2]

Evaporative causes, with associated meibomian gland disease or ocular rosacea, are amongst the commonest causes of DED.[2] Treatment of the evaporative sub-type of DED is often challenging. Traditionally, treatments for evaporative DED include a combination of artificial tears, lid scrubbing and hygiene measures, warm compresses, and lid massage.

Refractory cases may also additionally benefit from intense pulsed light therapy and medications such as topical cyclosporin, topical azithromycin and oral doxycycline.[3] Thermal pulsation can also be considered for refractory cases or for patients who are poorly compliant with the long-term treatment schedules for evaporative DED[4].

In recent years there has been a tremendous drive from the ophthalmic pharmaceutical industry to develop novel therapies and artificial tear formulations that specifically target evaporative dry eye by enhancing the lipid layer of the tear film (Table 1). In 2021/22 a total of £171,060.00 was spent on lipid eye drops alone just in London and the South-East of England (pers comm Visufarma UK).
The rapid proliferation of these new therapies has outpaced the time required to understand their mechanisms, their position in the treatment regimen, and how to adapt them appropriately into clinical practice.

There are so many preparations on the market now, that the authors thought it would be opportune to provide a short synopsis of them. Therefore, this article aims to present a summary of the drops that are currently available for the treatment of evaporative DED. There are very few comparative studies between preparations (see table 1) and every distributor will wax lyrical about their own product(s), which can be very confusing for clinicians when deciding what to prescribe. However, regardless of which product is used, it is important to understand that the topical treatment is only one component of the multi-faceted approach to DED treatment (vide supra).

In order for treatment to be successful there are some very important general considerations to bear in mind. To aid compliance with therapy, clinicians must see the condition and treatment from the patient's perspective. At the outset, it is imperative to inform patients fully about the importance of daily eyelid care as well as eye drops, not to expect an immediate improvement (which can take several weeks), the protracted nature of therapy and to persevere with treatment to prevent relapses. This can be very demanding for patients, but encouragement and clear explanation in simple terms will help meet patients' expectations and develop trust in the doctor-patient relationship.

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Table 1: Common artificial tear formulations available for evaporative eye disease

Name of the Drop	Preserved/ Non-Preserved	Constituents/Concentrations	Evidence (Efficacy/Tolerability/ Comparative Studies)
Cationorm®	Preservative Free	Mineral oils, glycerol, tyloxapol, poloxamer 188, tris hydrochroloride, tromethamine, cetalkonium chloride and water [1]	Amrane et al. (2014) compared safety and efficacy of Cationorm® in patients with mild to moderate dry eye disease. Their study was a multicenter, open-label, comparative study which compared Cationorm® against a formulation of polyvinyl alcohol and povidone. The study found that Cationorm®, in addition to its moisturizing and lubricating properties, also helps stabilize the tear film due to its oily component. [2] Robert et al. (2016) further evaluated the efficacy and safety of Cationorm® in patients with moderate to severe dry eye disease. This study was designed as a multicenter, prospective, reference-controlled, parallel group, investigator-masked study. The results showed that Cationorm® was superior to hyaluronate sodium in improving symptoms for patients with moderate to severe dry eye disease. [3]
Systane® Balance (Novartis)	Preserved	propylene glycol, hydroxypropyl guar, mineral oil, dimyristoyl phosphatidylglycerol, polyoxyl 40 stearate, sorbitan tristearate, boric acid, sorbitol, edetate disodium and POLYQUAD® (polyquaternium-1) 0.001% preservative [4]	*No studies specifically on Systane Balance found on PubMed*
OPTIVE PLUS™ (Allergan)	Preserved	sodium carboxymethylcellulose 0.5%, glycerine 1.0%, castor oil 0.25%, polysorbate 80 0.5%, levocarnitine 0.25% and erythritol 0.25%, preserved with PURITE® 0.01% 5	Kaercher et al. (2014) conducted a prospective study of patients with dry eye disease and found Optive Plus® was well tolerated and effective in reducing the signs and symptoms of all types of dry eye but particularly recommended for lipid-deficient dry eye patients.[6]
EvoTears® (Scope Eyecare)	Preservative Free	100% Perfluorohexyloctane [7]	Mateo-Orbobia et at. (2020) have conducted a prospective multicentre study to ascertain the effects of perfluorohexyloctane on corneal surface and endothelium. They reported it to improve ocular surface measurable indicators.[8] Son et al. (2020) conducted a further study which looked at the efficacy and tolerability of the EvoTears® in evaporative dry eye disease patients who underwent cataract surgery. They found EvoTears improved tear film, ocular surface, and subjective impressions of patients with dry eye disease.[9]
VisuEVO® (VISUfarma)	Preservative Free	Liposomes from soya phospholipids, algae oil (DHA+EPA), Vitamin A palmitate, Vitamin D, disodium EDTA, PEG 400, Boric acid, Sodium tetraborate, buffered isotonic buffered with pH 7.20 [10]	Fogagnolo et al. (2020) conducted a multicenter, open-label, randomized, prospective study, prospective cohort study to assess VisuEvo® on dry eye disease (DED) in patients undergoing cataract surgery and found VisuEvo® to be efficacious from 2 weeks pre to 2 weeks post-operatively in such patients.[11] Fogagnolo et al. (2020) also conducted a multicenter, double-blind, 12-week crossover study to compare efficacy of VisuEvo® and Cationorm®. Both treatments were comparable in reducing the effects of evaporative and non-evaporative dry eye disease.[12]
Hylo® DUAL (Scope Eyecare)	Preservative Free	0.05% Sodium Hyaluronate and 2% Ectoin 13	*No studies specifically on Hylo DUAL found on PubMed*
Hycosan® Dual (Scope Eyecare)	Preservative Free	0.05% Sodium Hyaluronate and 2% Ectoin 14	*No studies specifically on Hycosan Dual found on PubMed*
Hycosan® Shield (Scope Eyecare)	Preservative Free	100% Perfluorohexyloctane 15	*No studies specifically on Hycosan Shield found on PubMed*
Viscotears Tri Action (Bausch & Lomb UK Ltd)	Preservative Free	Sacha inchi oil16 0.1% and glycerol, Trehalose 2%, Sodium hyaluronate 0.2%	*No studies specifically found on PubMed*

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The Effect of Anti-Inflammatory Topical Ophthalmic Treatments on In Vitro Corneal Epithelial Cells

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Abstract

Purpose

To compare the effect of three commonly prescribed anti-inflammatory eye drops on corneal epithelial cells in vitro.

Methods

Three different lines of human corneal epithelial cells were tested: primary cells cultured from donor tissue, commercially available primary cells, and immortalized cells. Cells were seeded on 96-well plates and treated with the following eye drops: cyclosporine 0.05%, lifitegrast 5%, and tacrolimus 0.03% or 0.1%. Exposure times tested were 30 seconds, 1 minute, 2 minutes, 1 hour, 2 hours, 4 hours, and 24 hours. Brightfield images and viability assays were analyzed 48 to 72 hours after the initiation of treatments. At least five replicates were tested per drug and time exposure.

Results

Commercially obtained primary cells showed reduced viability following 1 hour with tacrolimus 0.1% (8%; $P = 0.043$%) and 4 hours with tacrolimus 0.03% (17%; $P = 0.042$%). Lifitegrast exposure reduced primary cell viability after 4 hours (10%; $P = 0.042$). Cell viability in primary cells was not deleteriously affected following exposure to cyclosporine for up to 4 hours. A similar trend was observed in both primary cells cultured from donor tissue and immortalized human corneal epithelial cells, demonstrating greater decreases in cell viability in tacrolimus compared to lifitegrast and cyclosporine. Light microscopy imaging for analysis of cell morphology and confluence supported the results.

Conclusions

Tacrolimus showed the highest impact on corneal epithelium survival in vitro, and cyclosporine proved the most protective.

Translational Relevance

Comparing anti-inflammatory eye drops on corneal epithelial cells in vitro may inform eye drop selection and development for clinical purposes.

Keywords: dry eye disease, corneal epithelial cells, anti-inflammatory, cyclosporine, tacrolimus, lifitegrast

Introduction

One common condition associated with inflammation of the ocular surface is dry eye disease (DED). Dry eye–associated inflammation can lead to a variety of symptoms, including ocular discomfort, visual disturbances, and red eye.[1] The armamentarium of anti-inflammatory eye drops for the treatment of DED is wide and constantly evolving.[2,3] The principal commercially available anti-inflammatory eye drops for the treatment of DED in the United States, cyclosporine (CsA) ophthalmic emulsion 0.05% and lifitegrast ophthalmic solution 5.0%, may be interchangeably prescribed. Tacrolimus has been well established in ophthalmology for the treatment of many conditions, including uveitis, corneal transplant rejection, and keratoconjunctivitis.[4] For DED, treatment using tacrolimus initially started with off-label use of its skin ointment. Only more recently has its efficacy been explored in clinical trials and experimental studies and found to improve the status of the ocular surface in patients with Sjögren's syndrome–related DED.[5,6]

Studies comparing the effect of the above eye drops on corneal cells in vitro, however, are scarce.[7] Although these drops have clinically been found to be protective and reduce corneal damage in DED patients,[8–11] treatment-related discomfort such as instillation site burning and/or stinging sensation may limit patient adherence with treatment.[5–7,12–17] Clinically, the mechanism underlying these reported adverse effects is not well understood, as these symptoms are not typically accompanied by corresponding changes from baseline on slit-lamp examination.[18] Therefore, it is important to explore the impact of these drugs on corneal cells at a microscopic level to investigate potential causes such as epithelial cell toxicity. The epithelial cytotoxic effect of eye drops such as benzalkonium chloride–preserved anti-glaucoma medications[19,20] and natural tear substitutes[21] has been thoroughly investigated utilizing cultured human ocular surface cells, as well as in vivo confocal microscopy.[22,23] Studies comparatively analyzing the in vitro or in vivo effect of anti-inflammatory dry eye drops on human ocular surface cells, however, are limited. Our study aimed to evaluate and compare the effect of CsA, lifitegrast, and tacrolimus eye drops in an in vitro monolayer model of primary and immortalized human corneal epithelial (HCE) cells.

Materials and Methods

Donor Tissue

Human donor corneas, authorized for use for both clinical and research purposes by the decedents' families, were obtained from the San Diego Eye Bank (San Diego, CA). Unutilized corneoscleral rims were collected after trephination of full-thickness grafts for transplant surgery. Tissue was then preserved in OptiSol-GS (Bausch & Lomb, Rochester, NY) at 4°C for up to 72 hours until processing. Eleven corneas from 10 donors were used for this study. Donors' ages ranged from 48 to 78 years, with a median of 70.5 years.

Primary Cell Cultures

Primary HCE cells were either cultured from donor tissues or obtained from the American Type Culture Collection

(PCS-700-010; ATCC, Manassas, VA). For HCE cell cultures from donor tissues, the corneoscleral donor tissue rims collected as described previously were washed in phosphate-buffered saline (PBS, 21-040-CM; Corning, Inc., Corning, NY) and subsequently incubated at 37°C for 1 hour in 1-mg/mL Dispase II (D4693; MilliporeSigma, Burlington, MA) dissolved in HyClone Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, SH30023.01; Cytiva, Marlborough, MA). The epithelium was stripped off with gentle scraping using a surgical knife (ExactEtch; Cytosol Ophthalmics, Lenoir, NC) from the limbus to the center into PBS. The tissue was centrifuged at 1000 revolutions per minute for 5 minutes, and the cells were suspended in 1 mL corneal epithelial cell medium (PCS-700-030; ATCC) supplemented with corneal epithelial cell growth kit medium (PCS-700-040; ATCC), 33-µM Phenol red (P0290-100ML; MilliporeSigma), and 0.1× Antibiotic-Antimycotic (15240062; Thermo Fisher Scientific, Waltham, MA). Cells were plated on a 12-well plate and cultured at 37°C with 5% CO_2 in a humidified incubator until 80% to 100% confluent; the medium was changed three times a week. Then, cells were transferred to a six-well plate and finally passaged onto a 96-well plate for experiments. For passage, cells were digested by TrypLE (12604013; Thermo Fisher Scientific) for 5 minutes. All plates were coated with FNC Coating Mix (Athena Enzyme Systems, Baltimore, MD). Cells obtained from ATCC were treated according to the manufacturer's instructions with Corneal Epithelial Cell Basal Medium (PCS-700-030; ATACC) and Corneal Epithelial Cell Growth Kit (PCS-700-040; ATCC). Experiments were conducted on passage 2 in a 96-well plate.

Immortalized Cell Cultures

Immortalized human corneal epithelial (iHCE) cells were previously described by Shalom-Feuerstein et al.24 Cells were generously provided by the Ruby Shalom-Feuerstein laboratory (Technion, Israel Institute of Technology, Haifa, Israel). Cell medium was prepared using DMEM/F12 (01-170-1A; Biological Industries, Beit-Haemek, Israel) with 5% FBS (04-001-1B; Biological Industries), 5-µg/mL insulin (I9278; MilliporeSigma), 0.5% dimethyl sulfoxide (D2650; MilliporeSigma), 10-ng/mL EGF (PHG0311L; Rhenium Research Laboratory Equipment, Modi'in-Maccabim-Re'ut, Israel), and 1% penicillin–streptomycin (03-031-1B; Biological Industries). Cells were cultured at 37°C with 5% CO_2 in a humidified incubator.

In Vitro Treatments

The following anti-inflammatory drops were used for this study: CsA ophthalmic emulsion 0.05% (Restasis; Allergan, Irvine, CA), lifitegrast ophthalmic solution 5.0% (Xiidra; Shire, Lexington, MA), and tacrolimus 0.06% eye drops (Concept for Pharmacy, Kefar Saba, Israel) or 0.1% ophthalmic drops (San Diego Optimum Compounding Pharmacy, San Diego, CA). Tacrolimus 0.06% eye drops were further diluted in culture medium to prepare two testing drugs containing 0.03% and 0.1% tacrolimus, respectively.

When primary HCE cells obtained from ATCC and iHCE cells reached full confluence, cells were treated with 20% of the tested drug or 0.9% saline and 80% medium. The following drugs were tested: CsA 0.05%, lifitegrast 5.0%, and tacrolimus 0.03% or 0.1%. Treatment times tested for each drug were 30 seconds, 1 minute, 1 hour, and 4 hours. Following treatment, cells were rinsed twice with PBS and the medium was replaced. Brightfield images were captured in the center of each well using a 10× lens 72 hours after the initiation of treatments.

For validation and evaluation of time and concentration effect, the experiment was repeated with modifications on a separate line of primary cells cultured from human donor tissue as previously described. This time, cells were treated with 10% of the tested drug or balanced salt solution (BSS) and 90% medium. The treatments (CsA 0.05%, lifitegrast 5.0%, and tacrolimus 0.1%) were applied for 2 minutes, 1 hour, 2 hours, 4 hours, and 24 hours. The BSS group was compared to a no-treatment group.

Survival Assays

Cell survival was assessed using the XTT Cell proliferation kit (20-300-1000; Biological Industries) or CellTiter-Glo Luminescent Cell Viability Assay based on adenosine triphosphate quantification (G7570; Promega, Madison, WI). Assays were conducted 48 to 72 hours after applying the different treatments according to manufacturer's instructions. At least five replicates were performed for each tested drug or control at each time point.

Statistical Analysis

Statistical analysis was performed using JMP (SAS Institute, Cary, NC). At first, the non-parametric Kruskal–Wallis test was performed on a set of groups. Then, the Wilcoxon test with Steel adjustment was performed to obtain the statistical significance of individual groups compared to the control group. Comparisons between drugs were conducted using two-tailed Student's t-test statistical analysis (*$P < 0.05$, **$P < 0.01$).

Results

Concentration-Dependent Effect of Anti-Inflammatory Eye Drops on Primary HCE Cells

The cell viability analysis for primary HCE cells obtained from ATCC after treatment exposure is summarized in Figure 1. Cell survival, normalized to saline exposure, was significantly reduced after the 4-hour treatment with tacrolimus 0.03% (survival rate, 17%; SE = 3.59; n = 5; P = 0.042) (Fig. 1A) and after 1-hour treatment with tacrolimus 0.1% (8%; SE = 1.13; n = 5; P = 0.043) (Fig. 1B). Lifitegrast decreased cell viability after 4 hours (10%; SE = 2.21; n = 5; P = 0.042) (Fig. 1C). Cell viability on primary corneal epithelial cells was not altered following CsA treatment for up to 4 hours compared to saline (Fig. 1D).

Figure 1

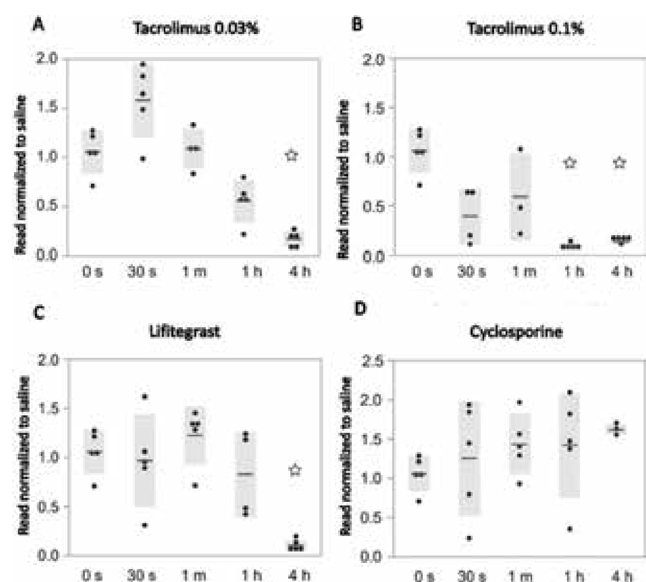
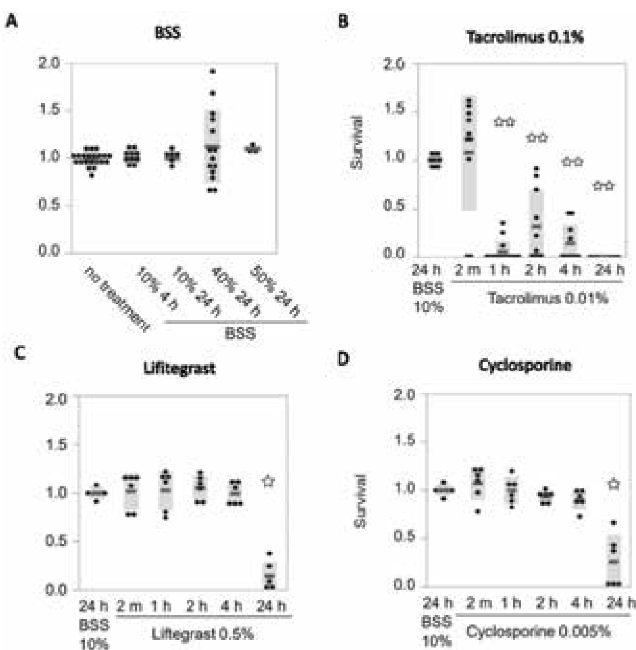


Figure 1.
Effect of dry eye drugs on commercially obtained primary epithelial cell cultures. Primary epithelial cell cultures obtained from ATCC treated with (A) tacrolimus 0.03%, (B) tacrolimus 0.1%, (C) lifitegrast, and (D) cyclosporine for 30 seconds, 1 minute, 1 hour, and 4 hours. Cell survival was analyzed using the XTT assay after 72 hours and normalized to treatments with saline.

To validate these results in a second line of primary cells and for individual variations, primary HCE cells derived from at least three different donors were tested, this time with longer time exposures (up to 24 hours) and a lower concentration: 10% of each drug in 90% of regular growth medium (Fig. 2). We noticed a significant decrease in cell survival only after 24 hours with CsA treatment (26%; SE = 10.6; n = 6; P = 0.022) (Fig. 2D) and lifitegrast (15%; SE = 5.63; n = 6; P = 0.022) (Fig. 2C). Tacrolimus 0.1% showed significant decreases after 1 hour (5.7%; SE = 2.95; n = 14; P = 0.0002) (Fig. 2B). BSS exposure did not have a deleterious effect on cell survival compared to the no-treatment group (Fig. 2A).

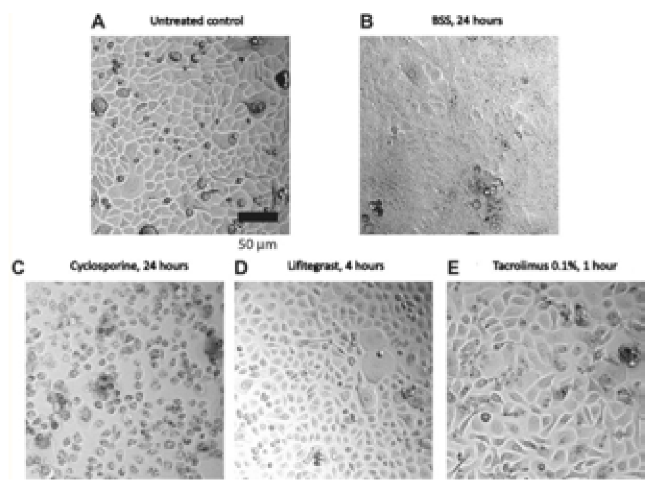
Figure 2.



Effect of dry eye drugs on primary epithelial cell cultures from corneal transplant donor tissue. Testing eye drops in a separate primary cell line for validation using a lower concentration and longer treatment time (compare with Fig. 1). Primary epithelial cell cultures were treated with (A) BSS as control, (B) tacrolimus 0.1%, (C) lifitegrast, and (D) cyclosporine for 2 minutes, 1 hour, 2 hours, 4 hours, and 24 hours. Cell survival was analyzed using the CellTiter-Glo Luminescent Cell Viability Assay after 48 hours.

Representative pictures of primary corneal epithelial cells derived from donors at critical time points after the start of drug application are shown in Figure 3. In conditions where cell survival was significantly decreased (Figs. 3C, 3E), cells showed various morphological changes such as interrupted connections, cellular elongation, and enlargement. At earlier and less toxic stages (Fig. 3D), cell shrinkage can be observed. As controls, XTT assays performed on both primary and immortalized cell cultures showed no significant differences in cell survival between cells treated with PBS and those treated with saline (Supplementary Fig. S1).

Figure 3.



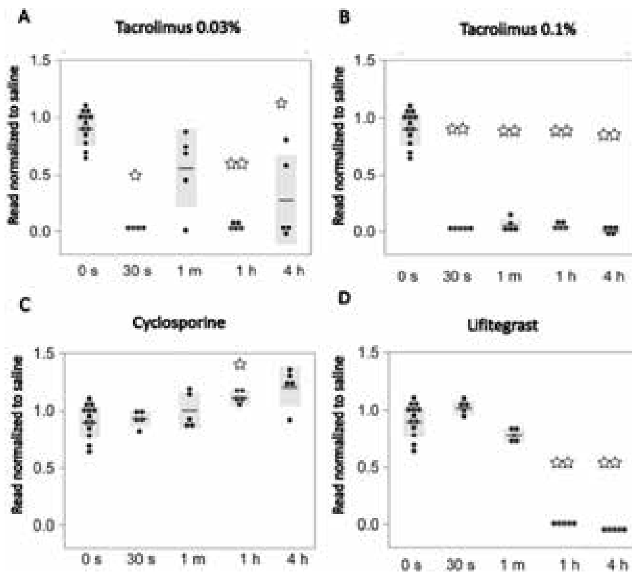
Representative pictures of cultured primary corneal epithelial cells at critical time points after the start of drug application. Phase contrast pictures of primary cells 24 hours after start of drug application with (A) untreated control, (B) BSS for 24 hours, (C) cyclosporine for 24 hours, (D) lifitegrast for 4 hours, and (E) tacrolimus for 1 hour. Note that BSS did not affect survival of cells but did alter transparency of the cells. Cells treated with 10% tested drug, 90% normal growth medium. Scale bar: 50 μm.

Effect of Anti-Inflammatory Eye Drops on iHCE Cells

Results were further tested on another in vitro model of corneal epithelial cells. Comparison of treatments on immortalized epithelial cell cultures is summarized in Figure 4. Cell viability was significantly impaired on immortalized cells following 30-second treatment with tacrolimus 0.03% (3%; SE = 0.19; n = 4; P = 0.015%) (Fig. 4A) and tacrolimus 0.1% (3%; SE = 0.44; n = 5; P = 0.006%) (Fig. 4B). Of note, although significant decreases in cell viability were observed after 30 seconds, 1 hour, and 4 hours, no significant difference in cell viability was observed in the 1-minute treatment of tacrolimus 0.03% (Fig. 4A). Lifitegrast decreased cell viability after 1-hour treatment on immortalized cells (1%; SE = 0.39; n = 5; P = 0.006) (Fig. 4D). Cell viability was not altered following CsA treatment compared to saline, and a significantly higher viability was noted on immortalized cells treated for 1 hour (111%; SE = 2.79; n = 5; P= 0.016) (Fig. 4C). Gross morphology in vitro changes following different treatments and time exposures in immortalized cells are demonstrated by brightfield microscopy images (Fig. 5). Imaging of immortalized epithelial cells supported quantitative data from survival assays and demonstrated altered morphology, elongation of cells, and disruption to intercellular connections with decreased cell survival.
A visualization and comparison of the overall results on cell survival in both primary and immortalized cell lines are shown in Figure 6.

Figure 4 (On next page).
Effect of dry eye drugs on immortalized epithelial cell cultures. Immortalized epithelial cell cultures treated with (A) tacrolimus 0.03%, (B) tacrolimus 0.1%, (C) lifitegrast, and (D) cyclosporine for 30 seconds, 1 minute, 1 hour, and 4 hours. Cell survival was analyzed using the XTT assay after 72 hours and normalized to treatments with saline.

Figure 4.

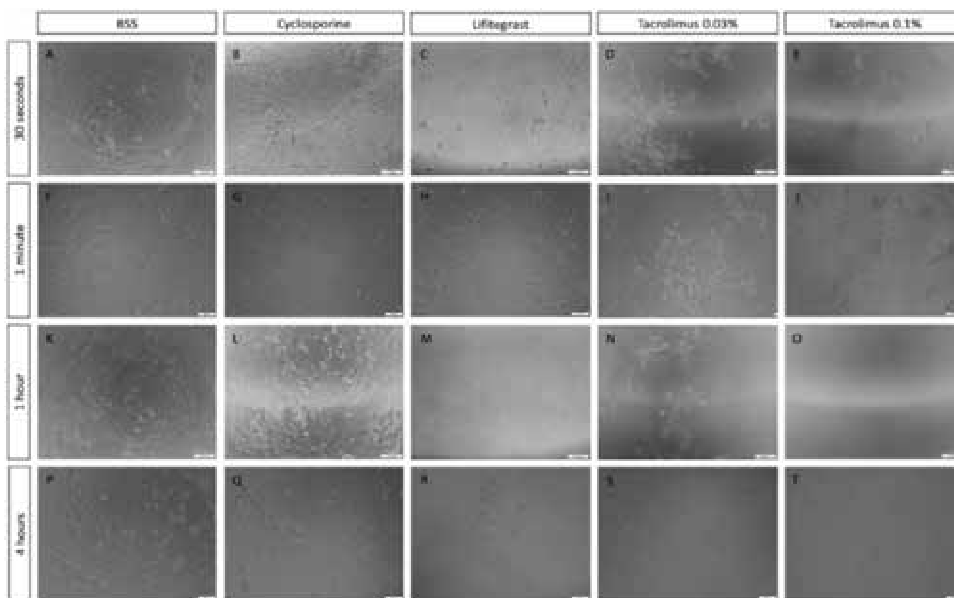


Comparison of eye drop effect on immortalized and commercially obtained primary epithelial cell cultures (a visualization of Figs. 1 and 4). Impact of cyclosporine, lifitegrast, and tacrolimus (0.03% and 0.1%) on the percentage of cell survival measured at 30 seconds, 1 minute, 1 hour, and 4 hours.

Discussion

Our study comparatively evaluated the in vitro effects of three common anti-inflammatory eye drops that are used in the treatment of DED. The results demonstrate a significant impact on cell viability of tacrolimus 0.1% and 0.03% in comparison to saline and BSS in models of both primary human corneal epithelial cells and immortalized human corneal epithelial cells. Due to tear dilution and rapid clearance of eye drops from the ocular surface within minutes,[25] effects at shorter periods of exposure and lower concentrations most likely have higher clinical relevance in mimicking physiologic conditions of a single eye drop instillation. Longer time durations may have some utility for chronic eye drop users.

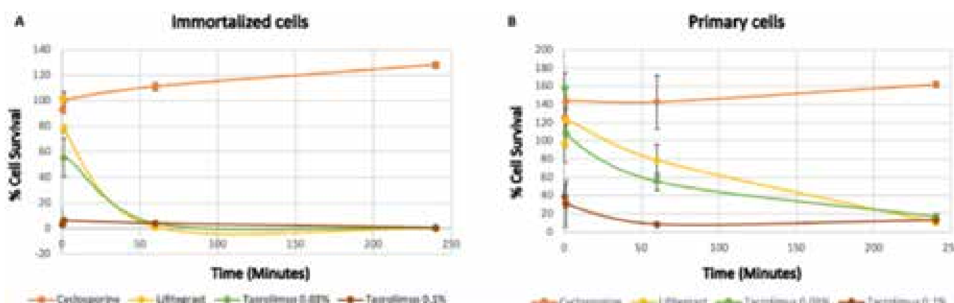
Figure 5.



Immortalized human corneal epithelial cells morphology following anti-inflammatory treatments. Brightfield images of iHCE cells 72 hours following treatments with saline (A, F, K, P), cyclosporine (B, G, L, Q), lifitegrast (C, H, M, R), tacrolimus 0.03% (D, I, N, S), and tacrolimus 0.1% (E, J, O, T). Treatment times: 30 seconds (A–E), 1 minute (F–J), 1 hour (K–O), and 4 hours (P–T). Cells treated with 20% tested drug, 80% normal growth medium. Scale bar: 200 µm.

There are several potential explanations for why tacrolimus decreased cell viability at significantly shorter time durations than CsA or lifitegrast. Tacrolimus is known to be a powerful immunosuppressive and anti-inflammatory drug and is estimated to be 10 to 100 times more potent than CsA,[26] despite having a similar molecular mechanism of action. The potency of tacrolimus for a variety of inflammatory diseases of the anterior segment could explain both its clinical efficacy and its increased toxicity. One theory is that transient toxicity may allow for better penetrance to the cornea, as a similar mechanism has been seen with other known corneal penetration-enhancer drugs such as ethylenediaminetetraacetic acid and preservatives such as benzalkonium chloride. These penetration enhancers are known to temporarily alter the corneal epithelial structure, which can cause mild irritation but has the advantage of allowing more drug to pass through the cornea.[27]

Figure 6.



More research is needed to reconcile the epithelial effect of tacrolimus with its clinical efficacy and potential. In a 2018 report, the application of 0.03% or 0.1% tacrolimus ointment to rat eyes significantly delayed epithelial healing and induced apoptosis; of note, when the authors treated primary corneal epithelial cells to concentrations of 0% to 0.003% for 24 hours, they

did not observe significantly decreased cell viability. These concentrations were significantly lower than those tested in our study (0.006%, 0.02%, and 0.01% after accounting for dilution). Thus, tacrolimus may be significantly more cytotoxic at high concentrations but more comparable to the other eye drops tested at lower concentrations. It is important to consider what concentrations would most accurately generalize to physiologic conditions in an in vitro study. Reports estimate that at most 5% of a drop penetrates the ocular structures, with the rest primarily draining through the nasolacrimal duct or being diluted by tears.[28,29] Another study showed that Restasis had limited delivery of CsA to the cornea compared to alternative cationic emulsions.[30] Our results do not negate the many studies supporting the efficacy and safety of tacrolimus as a treatment for DED but rather emphasize the importance of finding an optimum formulation that minimizes epithelial damage. Future research to evaluate the impact of concentration could test CsA and lifitegrast at different formulations and dilutions, in addition to tacrolimus. This could include 0.09% CsA drops (Cequa; Sun Pharmaceutical Industries, Princeton, NJ) or 2% compounded CsA drops.

The question remains whether our findings of the transient effect of tacrolimus versus CsA, and lifitegrast directly correlates to a clinical comparison of the three formulations. Our findings support the hypothesis that tacrolimus drops may lead to more frequent or severe complaints of adverse effects such as ocular stinging or burning. However, head-to-head comparisons of tacrolimus, lifitegrast, and CsA in clinical trials as treatments for DED are limited. A 2021 comparison of tacrolimus and CsA for the treatment of DED demonstrated that the two had comparable efficacy, but the authors did not comment on the incidence of adverse effects.5 Rates of adverse events vary among reports, with one clinical trial of dry eye patients treated with 0.03% tacrolimus drops showing that virtually all patients complained of moderate burning sensation for approximately 30 minutes after instillation of eye drops.[31] Hence, understanding epithelial toxicity and its relation to adverse effects has the potential to help improve patient adherence to these drugs.

Interestingly, in our study, CsA demonstrated a protective effect on the survival of immortalized epithelial cells in comparison to saline. This may be within expected lines, as ophthalmic emulsion of CsA has been previously been shown to induce a cytoprotective anti-apoptotic effect in vitro,10 and it corroborates well with the significant body of clinical evidence supporting the efficacy of CsA in patients with dry eye.[32,33]

There were several limitations to this study. First, we examined the effect of eye drops by measuring increases or decreases in cell viability. We intentionally quantified cell viability 3 days following treatments to test the end effect on cell survival, allowing the cells to either heal from any toxicity or execute the apoptotic program initiated by the drops. This experimental plan does not allow us to elucidate the molecular mechanisms leading to corneal epithelial cell death. Future experiments using gene expression profiling are needed to characterize the inflammatory markers and pathways responsible for these results. Furthermore, we applied the commercial eye drop emulsion to best model treatment in a real-life setting, but, as a result, we are unable to attribute the observed effects in this study to a single ingredient in the solution as marketed. Of note, as none of the eye drops used in this study contained preservatives, we did control for the presence of certain preservatives that are known toxins to the cornea.[34] Last, in this study, we applied eye drop emulsions to cultures of monolayer corneal epithelial cells. Prior studies have demonstrated that the stratified corneal epithelium model20 more closely mimics the layered human corneal epithelium in vivo, including the ability to test its barrier properties. However, in testing drug toxicity, the monolayer model has showed non-inferiority and enables high multi-well testing capacity.[35] Monolayer cells have furthermore been shown to be comparable in evaluation of cellular toxicity to both three-dimensional36 and in vivo models.[37]

Finally, some increased variability and unexpected findings were observed in our treatment groups. Lifitegrast and tacrolimus had more rapid-onset effects on cell viability in iHCE cells compared to primary cells. This may be due to differences in gene expression profile in iHCE cells that could lead to altered growth and heterogeneity.[35] Still, similar overall trends were observed, which turns this into an advantage, as performing our study in multiple cell lines allowed us to validate and control for possible variation between cell lines and laboratories.[38] Additionally, when immortalized cells were treated with tacrolimus 0.03% (Fig. 4), there was a significant decrease in cell survival at 30 seconds, 1 hour, and 4 hours of exposure but surprisingly not at 1 minute. This variability is most likely caused by the hydrophobic characteristics of tacrolimus, which does not make a homogeneous mixture as readily with the cell medium and thus could create different concentrations of the drug near the surface of the cells. This observation is relevant to the physiologic conditions of dry eye treatment, as surface irregularity may lead to altered concentration of the drug on different areas of the ocular surface.

Our findings on the effect of three anti-inflammatory eye drops on corneal epithelial cells in vitro have several translational implications. The sensitive detection of cytotoxicity and every effort to minimize it are of utmost importance, particularly because patients with more severe dry eye also simultaneously use eye drops more frequently, have reduced tear turnover rate, and have more susceptible corneal epithelium.[39,40] Patients with more susceptible corneal epithelium may benefit from eye drops that have more cytoprotective properties and less cytotoxic properties toward corneal epithelial cells both in vitro and in vivo.

Conclusions

The impact on corneal epithelial cell culture viability was most pronounced in tacrolimus-containing eye drops and observed in CsA and lifitegrast at longer time durations at the concentrations tested. These findings may serve as a useful resource in the selection and development of anti-inflammatory eye drops for clinical purposes and provide further insights into the study of inflammatory and cell death pathways.

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THE BODY DOCTOR.

What's in the news?

Hospitalizations for infections in primary Sjögren's syndrome patients: a nationwide incidence study

Primary Sjögren's syndrome (pSS) is an autoimmune disease with increased risk of infections. Here, the authors assessed whether pSS patients were at higher risk of hospitalization for community and opportunistic infections.

They selected newly hospitalized pSS patients between 2011 and 2018, through a nationwide population-based retrospective study using the French Health insurance database.
They compared the incidence of hospitalization for several types of infections (according to International Classification for Disease codes, ICD-10) between pSS patients and an age- and sex-matched (1:10) hospitalized control group. They calculated adjusted Hazard Ratios (aHR, 95% CI) adjusted on socio-economic status, past cardiovascular or lung diseases and blood malignancies factors.

Then they compared 25 661 pSS patients with 252 543 matched patients. The incidence of hospitalizations for a first community infection was increased in pSS patients [aHR of 1.29 (1.22-1.31), $p < .001$]. The incidence of hospitalization for bronchopulmonary infections was increased in pSS patients [aHR of 1.50 (1.34-1.69), $p < .001$, for pneumonia]. Hospitalizations for pyelonephritis and intestinal infections were increased [aHR of 1.55 (1.29-1.87), $p < .001$ and 1.18 (1.08-1.29), $p < .001$, respectively]. Among opportunistic infections, only zoster, and mycobacteria infections (tuberculosis and non-tuberculous) were at increased risk of hospitalization [aHR of 3.32 (1.78-6.18), $p < .001$; 4.35 (1.41-13.5), $p = .011$ and 2.54 (1.27-5.06), $p = .008$, respectively].

The authors concluded that pSS patients are at higher risk of hospitalisation for infections.
The increased risk of hospitalization for mycobacterial infections illustrates the potential bilateral relationship between the two conditions. Vaccination against respiratory pathogens and herpes zoster virus may help prevent some hospitalizations in pSS patients.
The key massage is that Primary Sjögren's syndrome (pSS) increases hospitalization risk for community infections: bronchopulmonary, skin, dental, ear-nose-throat, intestinal infections and pyelonephritis.
Hospitalisations for zoster and mycobacterial infections are also increased in this population. Dedicated preventive measures and vaccination campaigns could decrease the burden of infections in pSS patients.

Authors: Radjiv Goulabchand, Alain Makinson, Jacques Morel, Philine Witkowski-Durand-Viel, Nicolas Nagot, Paul Loubet , Camille Roubille, Danièle Noel, David Morquin, Kim Henry, Thibault Mura, Philippe Guilpain.
Publication: Ann Med. 2022 Dec;54(1):2672-2680.doi: 10.1080/07853890.2022.2126517.

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