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RESEARCH

Confocal Microscopy of Epithelial and Langerhans Cells of the Cornea in Patients Using Travoprost Drops Containing Two Different Preservatives

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Abstract The recently developed confocal cornea microscopy offers the opportunity to examine pathologies of the cornea and to gain insight into the activity of innate immunity. We aimed to investigate the corneal epithelial and Langerhans cell (LC) densities along with dry eye parameters in primary open-angle glaucoma (POAG) subjects, treated with either of two commercially available travoprost 0.004 % topical medications containing different preservatives. (1: benzalkonium chloride 0.015 % (TravBAK) and 2: polyquaternium-1 (PQ) 0.001 % (TravPQ). Consecutive case series of nineteen POAG patients on TravBAK (mean age: 64.8±13.6 years), nineteen POAG patients on TravPQ (mean age: 66.8±11.3 years) and nineteen age-matched healthy control subjects (63.8±8.2 years). Ocular surface disease index (OSDI), lid parallel conjunctival folds (LIPCOF), Schirmer test (ST) and tear break up time (TBUT) were assessed, and then corneal epithelial and LC densities were investigated with confocal microscopy. Tear production was significantly reduced in both glaucoma patient groups compared to healthy individuals ($p<0.05$). TBUT was significantly reduced and epithelial cell densities were significantly greater in patients treated with TravBAK compared to healthy individuals ($p<0.05$ for all). LC densities were greater in both glaucoma groups compared to control subjects ($p<0.05$ for all). Travoprost therapy may compromise ocular surface. The limited alertness of the corneal immune system found in patients with TravPQ can be considered as indicators of a less disturbed ocular surface and better controlled corneal homeostasis.

Keywords Benzalkonium chloride · Confocal microscopy · Langerhans cell · Polyquaternium

There was no proprietary interest involved in this study.

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Introduction

Benzalkonium chloride (BAK) is the principle preservative employed in topical therapy, used in ophthalmology. Although it is effective as an antimicrobial and antifungal agent, both in vitro and in vivo studies have demonstrated adverse effects on surface epithelial cell populations [1–6]. This is of particular concern with regard to topical medications used over long periods for chronic conditions, such as glaucoma. Recently a BAK-free formulation (travoprost 0.004 % with polyquaternium-1 0.001 % (Poliquad®) has been developed which has comparable safety, efficacy to travoprost preserved with BAK (0.015 %) [7]. Polyquaternium (PQ), like BAK is a quaternary ammonium compound with no detergent effect to the ocular surface. The molecule is more than 25 times greater than BAK and has been demonstrated to be less toxic to the corneo–conjunctival surface [6].

Changes in the human corneal microstructure can be examined with traditional histopathological and immunohistochemical methods, however, in vivo changes are not detectable by these approaches. The recently developed confocal cornea microscopy offers the opportunity to examine pathologies of the cornea in vivo and also of mucous membranes and superficial skin. The cornea is endowed with a heterogeneous population of immune cells [8]. Amongst them, the Langerhans cells (LCs) play a major role in corneal immune response by sensitising thymus dependent immune cells and they also participate in maintaining the corneal homeostasis. It has been demonstrated that BAK induces the IL-1 and TNF production in immortalised corneal and conjunctival epithelial cells [2]. Corneal LCs react to IL-1 and TNF-alpha with acceleration of their maturation and migration to the area concerned [8–10]. This observation drove our attention to investigate dry eye and subclinical microstructural alterations of the cornea by means of confocal microscopy in glaucoma patients treated with either BAK (TravBAK) or PQ preserved travoprost (TravPQ).

Methods

The study has been performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consent was obtained from all subjects, and the study protocol was reviewed and approved by an independent ethics committee of the institution. This was an observational study with analysis of dry eye parameters along with the epithelial and LC densities and Langerhans cell morphology (LCM) in patients with primary open angle glaucoma (POAG) and in age-matched controls. Nineteen eyes of 19 POAG patients with TravBAK (eight female and eleven male, mean age: 64.8±13.6 years) and nineteen eyes of 19 POAG patients with TravPQ (nine female and ten male, mean age: 66.8±11.3 years) and nineteen age-matched controls (ten female and nine male, mean age: 63.8±8.2 years) were enrolled into the study. Demographics are highlighted in Table 1. The inclusion criteria included: asymptomatic primary open angle glaucoma (POAG) patients on TravBAK (travoprost 0.004 % with preservative BAK 0.015 %, Travatan, S.A. Alcon-Couvreur N.V. Rijksweg B-2870, Puurs Belgium) and TravPQ (travoprost 0.004 % with preservative Poliquaternium 0.001 %, S.A. Alcon-Couvreur N.V. Rijksweg B-2870, Puurs Belgium) monotherapy used for at least one year prior to enrolment. Patients with previous eye surgery, diabetes, autoimmune disorders, ocular infections, trauma, allergy or contact lens wear were excluded. All examinations were carried out on the right eye only using the same room at each visit and a constant environment.

Examination of Dry Eye Related Parameters

The protocols for these procedures have been described in detail in our previous work [11]. In short and in investigational order: Ocular surface disease index (OSDI) questionnaire was used to describe subjective eye related discomfort. This 12-item scale was specially designed for the assessment of symptoms related to dry eye disease and their effect on vision. Lid parallel conjunctival folds (LIPCOF) were evaluated at the temporal aspect of the lower eyelid margin according to the protocol specified by Pult [12]. Schirmer test was carried out without anaesthesia with standardized Schirmer test strip (Haag-Streit, UK, Ref:4701001). The degree of wetting of the test strip represented the tear production and was considered as the Schirmer test result.

Table 1 Demographics of study participants

	Age (years)	Gender (F/M)	Therapy use (year)
Healthy individuals	63.8±8.2	10/9	0
Patients (TravBAK)	64.8±13.6	8/11	1.76
Patients (TravPQ)	66.8±11.3	9/10	1.69

In Vivo Confocal Corneal Microscopy

Image Acquisition

All subjects were examined with confocal laser-scanning microscope (HRT II RCM Heidelberg Engineering Inc., Heidelberg, Germany, Rostock Cornea Module), equipped with inbuilt Heidelberg Eye Explorer version 1.5.10.0 software. This confocal microscope uses a 670 nm wavelength laser source with a definition of 384×384 pixels over an area of 400 μm× 400 μm.

Before each examination, a drop of Oxybuprocaine-Humacain 0.4 % anesthetic (Human Pharmaceuticals, Gödöllő, Hungary) was instilled in the lower conjunctival fornix. In order to keep good control over the eye examined, a fixation light was used for the contralateral eye. To maintain a stable distance from the cornea to the microscope head, we used a disposable plastic cap (TomoCap®; Heidelberg). Carbomer gel (Vidisic; Dr. Mann Pharma, Berlin, Germany) served as a coupling medium. The positioning and constant contact of the eye relative to the plastic cap was monitored by an accessory digital camera set perpendicularly to the eye being examined. In contrast to the traditional histological cross section of the cornea, this method allowed en face images to be obtained.

Epithelial cells were examined at the corneal apex, while LCs densities were examined both at the corneal apex and at 6 o'clock in the periphery according to the examination scheme of Zhivov [13]. Images were taken at the depth of corneal epithelial cell layer (approx. 0–40 μm from the ocular surface) for the investigation of wing and basal epithelial cell densities and at the level of subbasal nerve plexus (approx. 40–60 μm from the ocular surface) to describe LC densities. The five best-focused images of each layer and location were considered for the analysis in a masked fashion.

Corneal Image Analysis

We used the inbuilt semi-automatic system of the device for calculating cell densities (cell number/mm^2). LCM was also evaluated and further classified according to our grading system described in our previous works [11, 14]. In short, for grading we chose the length of the LC processes to define maturation and to portray activation of LCs. LCM was evaluated on a 0–3 scale according to the size of the dendrites compared to the largest diameter of cell body. The longest visible dendrite of each LC was chosen to characterize maturation. Score 0 described the condition when cornea was devoid of LCs. Score 1 cells lacked processes. Score 2 (small processes) represented cells with processes not exceeding the longest diameter of the cell body. Score 3 (long processes) cells had longer processes then the largest diameter of the cell body. The average of LCM was calculated in each of the

figures selected and was used to describe the maturation of the LCs at both regions of the cornea.

Statistical Analysis

Comparisons between healthy individuals and glaucoma patients as well as between the two glaucoma patient groups were made with Mann–Whitney tests. P-values obtained from the tests with less than 0.05 were deemed statistically significant.

Results

Dry Eye Related Parameters

The results of functional assays can be seen in Table 2. Of note, both the OSDI and LIPCOF score were greater in patients treated with TravBAK and tear production was significantly reduced in both glaucoma patient groups compared to healthy individuals ($p<0.05$). TBUT was significantly reduced in patients treated with TravBAK compared to control.

In Vivo Confocal Corneal Microscopy

Epithelial cells and LC could be examined by in vivo confocal microscopy in all eyes. Results obtained by confocal microscopic examinations are listed in Table 3.

Of note, both the wing and basal cell number were significantly greater in patients treated with TravBAK compared to healthy subjects ($p<0.05$), Fig. 1. a–b). Basal cell number was significantly lower in patients with TravPQ therapy compared to TravBAK.

Table 2 Dry eye related results in healthy subjects and in glaucoma patients

Study group	OSDI	LIPCOF	Schirmer [mm/5 min]	TBUT [s]
Healthy individuals	11.7±6.6 (0–22.9)	1.3±0.5 (0–3)	12.4±2.8 (9–15)	12.9±3.6 (7–16)
All glaucoma pts.	20.2±15.7* (0–45)	1.7±0.7 (0–3)	7.8±4.1* (0–17)	10.3±4.5 (4–17)
Glaucoma patients according to the therapy used				
TravBAK	21.6±19.9* (0–45)	2.05±0.8* (1–3)	6.3±5.6* (0–17)	9.3±3.7* (4–17)
TravPQ	18.9±14.9 (0–50)	1.6±1.0 (0–3)	9.4±4.7* (0–17)	11.3±4.1 (5–17)

Data are expressed as mean ± SD with its minimum and maximum values. Comparisons between healthy individuals and glaucoma patients were made with Mann–Whitney tests

OSDI ocular surface disease index, *LIPCOF* Lid parallel conjunctival folds, *TBUT* tear break up time

*$p<0.05$ vs. healthy individuals

Central LC density was significantly greater in all glaucoma patient groups compared to control ($p<0.05$).

Central LC maturation score was significantly greater in glaucoma patient groups compared to control subjects ($p<0.05$) (Fig. 1/d,e).

Central LC density was significantly lower in patients with TravPQ compared to patients with TravBAK therapy ($p<0.05$) (Fig. 1/f).

LCM scores were significantly lower in patients on TravPQ compared to patients on TravBAK ($p<0.05$).

Discussion

Topical IOP lowering agents have been proven to induce ocular surface changes in patients treated for glaucoma [1–6, 15–17]. These changes can be attributed to the preservative BAK, while ocular hyperaemia could be caused by the prostaglandin analogue itself. BAK alone has significant in-vitro cytotoxicity to cultured ocular epithelial cells and has also been demonstrated to cause dose dependent reduction in viability of trabecular meshwork cells [15]. In vivo confocal microscopy is a powerful instrument to study the ocular surface and detect minute changes in certain conditions at a cellular level [18]. As the HRT II RCM uses laser light, the reliability of data collected greatly depends on the transparency of the organ examined, we therefore restricted our investigation on cornea to depict the cellular changes found in glaucoma patients on travoprost therapy. Pisella and associates demonstrated that dry eye symptoms are more prevalent in glaucoma patients using antihypertensive agents with preservatives employed [19]. The higher OSDI score found in our glaucoma patients coupled with the greater LIPCOF scores in patients with TravBAK underlines the detrimental effect of the preservative used and is consistent with Zhivovs' finding [20] and also are in line with Martones' results [1]. We found no difference between the two glaucoma groups in dry eye parameters examined, however the tear production and TBUT tended to be greater in patients with TravPQ which can be explained by the less toxic effect of polyquaternium-1 to the ocular surface.

The greater density of wing and basal epithelial cell found in patients with TravBAK could be considered as a consequence of proliferate stimuli from aponecrotic superficial cells and consistent with Martones' findings [1].

Epstein et al. have shown that even at low concentration BAK is capable of inducing markedly increased quantities of IL-1 and TNF-α both in the conjunctival and corneal epithelial cells in a dose dependent manner [2]. Multiple authors have demonstrated that these cytokines are important to regulate corneal LC migration and maturation [21, 22, 23]. LCs are antigen-presenting cells of the cornea and can be identified as bright, mostly oval or elongated particles with a diameter of

Table 3 Confocal microscopic results in healthy individuals and in glaucoma patients

Study group	Wing cells	Basal cells	LCC	LCP	c.LCM	p. LCM
Healthy individuals	5239.1±485 (4,450–6,249)	8663.4±490 (7,928–10,000)	25.9±26.6 (0–72)	66.7±34.4 (32–165)	0.77±0.71 (0–2)	2.22±0.54 (0–3)
All glaucoma pts.	5589.2±685 (4,427–7,294)	9262.4±790* (7,175–10,739)	54.1±33.6* (0–130)	122.1±74.4* (47–308)	1.23±0.65* (0–2)	2.42±0.64 (0–3)
Glaucoma patients according to the therapy used						
TravBAK	5,778±693* (4,660–7,294)	9,653±732* (7,842–10,739)	64.3±39.5* (0–130)	127±58.3* (68–308)	1.46±0.69* (0–2)	2.74±0.75 (0–3)
TravPQ	545.3±413 (4,427–6,126)	8755.3±828# (7,175–10,350)	48.4±32.7*# (0–104)	116.1±51.8* (47–226)	1.0±0.44*# (0–2)	2.23±0.53 (0–3)

Data are expressed as mean±SD with its minimum and maximum values. In cases of wing and basal cells, central and peripheral Langerhans cells (LCC and LCP respectively) the data represent the cell number/mm^2. c.LCM – LC morphology at the centre, p.LCM – LC morphology at the periphery. Comparisons between healthy individuals and glaucoma patients were made with Mann–Whitney tests

*$p<0.05$ vs. healthy individuals; # $p<0.05$ vs. TravBAK

up to 15 μm. They reside exclusively in the corneal epithelium in the vicinity of corneal subbasal nerves [24]. They are crucial for generation of immune responses and have a key role to modulate between immune defence and immune tolerance [25]. Zhivov et al. demonstrated significant elevation of central corneal LC densities in healthy volunteers treated with 0.01 % BAK solution just after six weeks and they experienced a quick drop and normalisation of these parameters after four weeks of cessation of drop administration [20]. We found the presence of LCs in the central cornea in eighteen out of 19 subjects (95 %) with TravBAK medication compared to control (9/19; 47 %). Furthermore we experienced that those LCs were in an activated stage. Opposite to that, we noted a lower density of basal epithelial cell number and also a lower LC number in patients with TravPQ. These alterations could be explained by the lack of the detrimental effect of BAK and are in concordance with Zhivov's findings [20]. The effect of the PQ to the ocular surface is controversial. Whitson et al. demonstrated little or no toxic effect of PQ to the human corneal epithelial cell line in their in vitro study [4]. Liang et al. showed that PQ did not induce obvious irritation, inflammatory infiltration or cell damage in their animal model [26]. On the contrary, Paimela et al. has brought to light that eye drops containing PQ evoke cytotoxicity and enhance the NF-κB driven inflammatory reaction in cultured HCE-2 cells [27]. The elevated densities of LCs in the cornea found in patients

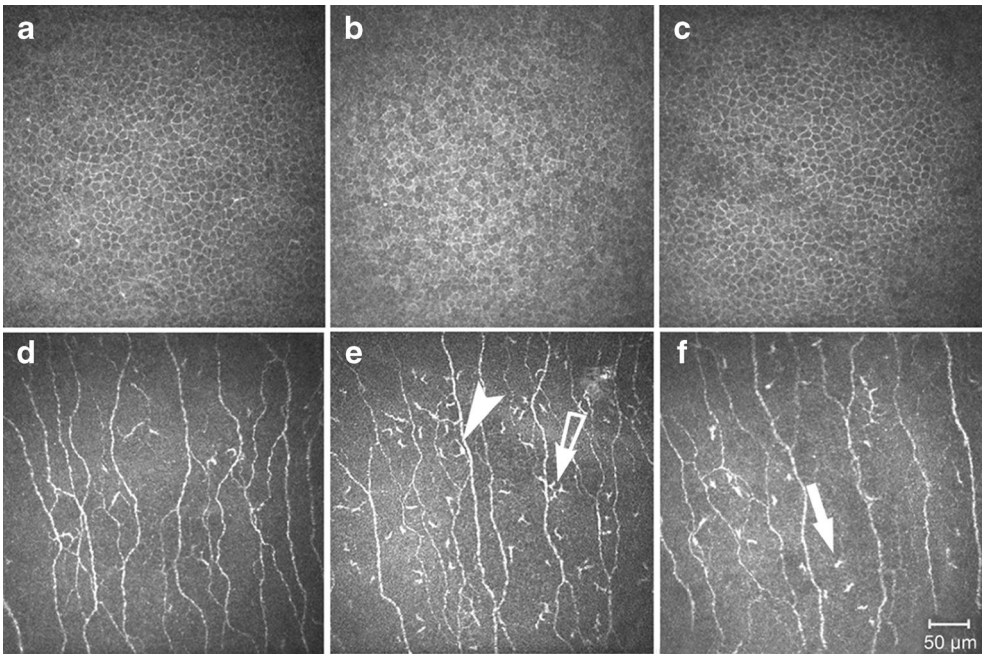

Fig. 1 Heidelberg Retina Tomograph (HRT) images of corneal basal cell layer and Langerhans cells (LC) at the corneal apex. *Top* row shows basal cell layer in control (**a**) and in patients with TravBAK (**b**) and TravPQ (**c**). *Bottom* row images are taken at the level of subbasal nerve plexus in control (**d**) and in patients with TravBAK (**e**) and TravPQ (**f**). White arrow indicates Langerhans cell morphology (LCM) grade 1, *Empty* arrow shows LCM 2, while arrowhead pointing at a LCM 3 LC. *Bar* represents 50 μm

both with TravBAK and TravPQ supports the immune modulating effect of chronic use of antihypertensive therapy and might reflect the ocular surface toxicity profile of the preservative concerned. Our study is the first to demonstrate corneal pathology at the cellular level in glaucoma patients treated with travoprost containing two different preservatives. Our findings call for attention that PQ might also interfere with the homeostasis of ocular surface.

The present study has certain limitations particularly confocal data on glaucoma patients without therapy. To date, there is no confocal investigation made on corneal epithelial and Langerhans cells of untreated glaucoma patients. We believe that no difference exists between healthy individuals and untreated glaucoma patients, which can be supported by Martone's findings, who could not find significant difference in epithelial cell densities between normal subjects and glaucoma patients treated with preservative-free hypotensive agent [1]. Furthermore, Ranno et al. found similar corneal confocal findings between normal subjects and untreated glaucoma patients with regards to nerve fibre tourtosity and reflectivity and concluded that their untreated glaucoma patients and controls had similar corneal parameters [28].

The higher LC number in the cornea in patients on TravPQ in relation to control subjects raises questions and needs to be further evaluated.

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Conflict of Interest None of the authors have financial, proprietary or other interest in any of the materials and instruments applied in the study. No financial support was received in this study.

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