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RESEARCH ARTICLE

Acute corneal toxicity of latanoprost with different preservatives

Masafumi Uematsu[1], Yasser Helmy Mohamed[1,2], Naoko Onizuka[1], Ryotaro Ueki[1], Daisuke Inoue[1], Azusa Fujikawa[1], Hitoshi Sasaki[3], and Takashi Kitaoka[1]

[1]*Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan,* [2]*Department of Ophthalmology, EL-Minia University Hospital, EL-Minia, Egypt, and* [3]*Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan*

Abstract

Purpose: To investigate the corneal toxicity of Xalatan and three latanoprost generics using transepithelial electrical resistance (TER) and scanning electron microscopy (SEM).
Methods: Corneal TER changes after a 60-s exposure to Xalatan (latanoprost 0.005% preserved with 0.02% BAC), and latanoprost generics (Latanoprost PF BAC free, Latanoprost Nitten SB containing sodium benzoate and Latanoprost Towa containing 0.01% BAC with sodium chloride polysorbate 80 as additive) were measured in living rabbits. Corneal damage was also examined by SEM. Hank's balanced salt solution (HBSS) was used as a control.
Results: There was a significant decrease in the corneal TER after exposure of the cornea to Xalatan ($p < 0.01$) and all latanoprost generics ($p < 0.01$: Latanoprost PF, $p < 0.05$: Latanoprost Nitten SB, Latanoprost Towa) as compared to HBSS. All latanoprost generics showed less TER decrease in the corneal TER as compared to Xalatan ($p < 0.01$). SEM revealed that superficial cells of Xalatan-treated corneas were damaged and exhibited degenerated microvilli. Conversely, the superficial cells of corneas exposed to HBSS or all latanoprost generics appeared normal and had normal microvilli under SEM examinations.
Conclusion: The corneal toxicity of Xalatan is greater than that of latanoprost generics. Xalatan contains 0.02% BAC, which may be responsible for the corneal toxicity.

Keywords

Benzalkonium chloride, cornea, generics, latanoprost, transepithelial electrical resistance, Xalatan

History

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Introduction

Glaucoma is a severe, sight-threatening disease that occurs in a large population of patients. In Japan, prevalence of all glaucoma and glaucoma suspect were estimated to be 5.0 and 7.5% respectively[1]. Anti-glaucomatous therapy aims to prevent progressive optic nerve damage, usually by lowering intraocular pressure. Prostaglandin (PG) analogs have become first line treatments among glaucoma medications. Topical PG analogs are generally well tolerated and their ocular adverse effect profiles are quite similar. These PGs are superior to beta-adrenoceptor antagonists in terms of lowering IOP, and they induce no severe side effects during long-term clinical use. Among these PGs, latanoprost presents a highly advantageous balance in terms of IOP lowering efficacy and tolerance and is still the mostly frequently used PG analog worldwide[2]. However, a commercial solution of latanoprost is proposed in a preserved formulation, which raises a number of issues, especially in patients with an abnormal or sensitive ocular surface.

Address for correspondence: Masafumi Uematsu, Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8501, Japan. Tel: +81-95-819-7345. Fax: +81-95-819-7347. E-mail: uematsu1124@outlook.jp

The most widely used preservative is benzalkonium chloride (BAC) in ophthalmic drugs. The harmful effects of BAC on conjunctival and corneal epithelial cell layers have been shown both in numerous clinical and experimental studies[3]. BAC caused damage to the tear film, corneal and conjunctival surfaces of those patients receiving long-term treatment for glaucoma with eye drops preserved with BAC in usual concentrations[4]. Several *in vivo* animal studies and *ex vivo* studies using human tissue-derived cell lines have demonstrated detrimental effects of BAC[4,5]. The ocular surface side effects of antiglaucoma medications should not be neglected because they may deeply impact patients' quality of life, compliance or later surgical outcome. Reducing the exposure to preservatives may lessen the adverse events, which could lead to better tolerability, lower treatment discontinuation and higher adherence in patients treated with antiglaucoma medications. This in turn would improve outcomes for these patients, both in terms of glaucoma management and their quality of life, which may also contribute to a lower cost of long-term glaucoma complications[6].

Measurement of corneal transepithelial electrical resistance (TER) is a suitable method for evaluating corneal permeability and irritancy quantitatively and continuously. TER reflects the barrier function of the epithelium. Lower corneal TER means more electrical current penetrates through the damaged superficial cells and tight junctions between

them. In addition, it is reported to be a very sensitive test for measuring electrical properties of the cornea[7]. We developed a new method of measuring the TER of live rabbit cornea and demonstrated that BAC concentrations between 0.005 and 0.02% immediately caused acute corneal barrier dysfunction[8–10].

Recently, lots of generic formulations for the commercially available latanoprost were approved and launched in Japan, but many of them include BAC as a preservative. The purpose of this study was to evaluate and compare the toxicological profile of many generic drugs for latanoprost in Japan with a commercially available Xalatan containing 0.02% BAC as a preservative (Pfizer, New York, NY) using our TER measuring method.

Materials and methods

Chemicals

Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS) was obtained from Invitrogen Corp. (Carlsbad, CA). Commercially available anti-glaucoma eye drops, such as Xalatan containing 0.02% BAC (Pfizer, New York, NY), Latanoprost PF BAC free (Nitten, Nagoya, Aichi, Japan), Latanoprost Nitten SB containing sodium benzoate (Nitten, Nagoya, Aichi, Japan) and Latanoprost Towa containing 0.01% BAC with sodium chloride polysorbate 80 as additive (Towa, Kadoma, Osaka, Japan) were used in this study. The eye drop ingredients are listed in Table 1.

Experimental animals

Male white Japanese rabbits (KBT Oriental, Tosu, Japan) weighing 2.5–3.0 kg were individually housed in cages under a controlled temperature (21 °C) and humidity (50 ± 5%) and a 12:12 h light/dark cycle at the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. Initiation of the study occurred once the rabbits reached weights of 3.0–4.0 kg, as this was the point where the corneal diameters were of suitable size for experimentation. The rabbits were treated in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal TER measurement *in vivo*

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine (Ketalar, Sankyo, Tokyo, Japan) and 5 mg/kg xylazine (Celactal, Bayer HealthCare, Osaka, Japan). After a small incision was made with an 18-gauge sharp needle (Terumo, Tokyo, Japan) in the peripheral cornea, a 1.0-mm diameter custom-made Ag/AgCl electrode (Physiotech, Tokyo, Japan) was inserted into the anterior chamber. A 6.0 mm internal diameter (0.28 cm^2 inner area) nitrile rubber O-ring (Union Packing, SAN-EI, Osaka, Japan) was fixed on the cornea using biomedical adhesive (Alon-Alpha A, Sankyo, Tokyo, Japan). Subsequently, 80 μL of HBSS was placed inside the ring, with the second electrode then placed in HBSS on the cornea. The TER was measured in real time using a volt-ohm meter (EVOMX, World Precision Instruments, Sarasota, FL) that generates a ±20 μA AC square wave current at 12.5 Hz. In a period of just a few seconds, 1 mL of the test solutions was gently poured into the ring, with all the overflow aspirated. After an exposure period of 60 s, the rings were washed out using 1 mL of HBSS. After obtaining the TER of the cornea before and after the exposure, results were then calculated as a percentage of the pre-exposure TER value (100%). This specific methodology and photographs of the *in vivo* corneal TER measurement system have been previously published[8–10].

In this study, the influences of commercially available anti-glaucoma eye drops, such as Xalatan (Pfizer, New York, NY), Latanoprost PF BAC free (Nitten, Nagoya, Aichi, Japan), Latanoprost Nitten SB containing sodium benzoate (Nitten, Nagoya, Aichi, Japan) and Latanoprost Towa containing 0.01% BAC with sodium chloride polysorbate 80 as additive (Towa, Kadoma, Osaka, Japan) on corneal TER changes were determined. Three corneas are used for the corneal TER measurement in each group. We chose this number as we found this to be sufficient for our statistical analyses in our previous TER studies[8–10].

Scanning electron microscopy (SEM) observation

The rabbits were anesthetized with an intramuscular injection of 30 mg/kg ketamine and 5 mg/kg xylazine. Corneas were evenly soaked in the test solution for 60 s. After washing the cornea, the rabbits were immediately sacrificed using a lethal dose of intravenous sodium pentobarbital (Nembutal, Dainippon Pharmaceutical, Osaka, Japan). The corneas were carefully excised, fixed in 4% glutaraldehyde in 0.05 M cacodylate buffer for 1 h and then post-fixed in 1% osmium tetroxide in veronal acetate buffer containing 0.22 M sucrose. The fixed materials were dehydrated through a series of ethanol washes. Corneas were placed in *t*-butyl alcohol, treated in a freeze-drying apparatus (EIKO ID-2, EIKO, Tokyo, Japan), and then sputter-coated with gold using an auto fine coater (JEOL JFC-1600, JEOL, Tokyo, Japan). After processing, the surface of the corneal epithelium was observed by a scanning electron microscope (Hitachi S2360, Hitachi, Ibaragi, Japan).

Table 1. The ingredients included in the latanoprost eye drops.

Xalatan	Latanoprost PF	Latanoprost SB	Latanoprost Towa
BAC (0.02%)	Boric acid	Sodium benzoate	BAC (0.01%)
pH regulator	Trometamol	Boric acid	Sodium chloride
Tonicity adjusting agents	Polyoxyethylene castor oil	Trometamol	Polysorbate 80
	EDTA	Polyoxyethylene castor oil	pH regulator
	pH modifier	EDTA	
		pH modifier	

Statistical analysis

All results were expressed as the mean ± standard error of at least three experiments. In our preliminary study, we confirmed that the %TER values in the control corneas followed a normal distribution. Therefore, statistical comparisons were performed using an analysis of variance followed by a Tukey test for the TER measurements. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Corneal exposure to Xalatan and some generics

The mean corneal TER for the live rabbits used in this study was $875.6 \pm 156.9\,\Omega\,cm^2$. Figure 1 shows the TER changes that occurred after corneal exposure to HBSS, Xalatan and some generics. Relative TER value after exposure to HBSS solution was $95.3 \pm 3.1\%$. There was a significant decrease in the corneal TER after exposure of the cornea to Xalatan (Relative TER value $= 16.8 \pm 1.2\%$; $p < 0.01$), Latanoprost PF BAC free (Relative TER value $= 63.4 \pm 4.8\%$; $p < 0.01$), Latanoprost Nitten SB (Relative TER value $= 75.3 \pm 4.4\%$; $p < 0.05$) and Latanoprost Towa (Relative TER value $= 76.1 \pm 2.1\%$; $p < 0.05$) compared to HBSS. Xalatan showed significantly lower TER compared to that of all latanoprost generics ($p < 0.01$).

Scanning electron microscopy observation

The superficial cells of the cornea of control eyes exposed to HBSS solution were normal in appearance with normal microvilli revealed with SEM (Figure 2A). In contrast, the superficial cells were damaged and exhibited degenerated microvilli in Xalatan solution revealed with SEM (Figure 2B). As regards the superficial cells of the cornea exposed to Latanoprost PF BAC free, Latanoprost Nitten SB and Latanoprost Towa appeared normal in appearance with normal microvilli with SEM examinations (Figure 2C–E).

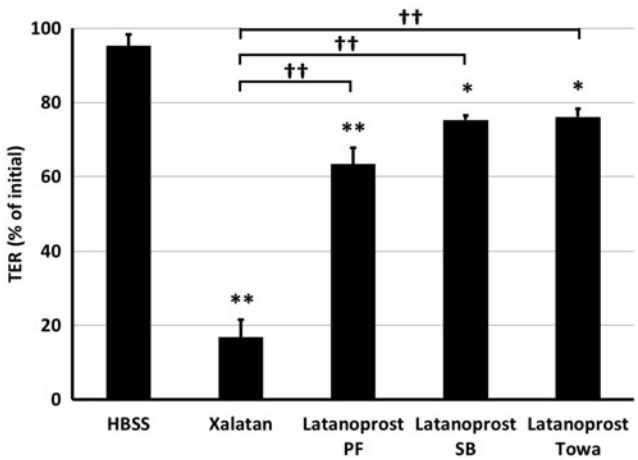

Figure 1. Corneal TER changes after exposure to HBSS, Xalatan and latanoprost generics (Latanoprost PF BAC free, Latanoprost Nitten SB and Latanoprost Towa) for 60 s. Data represent the percentage compared to the pre-exposure value. Each value is the mean ± SE ($n = 3$). *$p < 0.05$, **$p < 0.01$ as compared with HBSS. ††$p < 0.01$ as compared with Xalatan (Tukey test).

Discussion

Among the PG analogs, latanoprost is a first line prostaglandin analog formulation for patients with glaucoma to control IOP, and exhibits potent pharmacological activity with one daily application[11]. However, the first commercial solution of latanoprost (Xalatan) is proposed in a preserved formulation (BAC 0.02%), which raises a number of issues, especially in patients with an abnormal or sensitive ocular surface. Multiple studies have indicated that the toxicity of antiglaucoma eye drops is largely due to their preservative, BAC. BAC is one of the most commonly used preservatives because of its higher antimicrobial efficiency, stability and low cost[3,12]. Over more than two decades, a huge number of animal and *in vitro* studies, using a considerable variety of models, cells and tissues, have demonstrated that BAC may cause or enhance harmful consequences on the eye structures of the anterior segment, including the tear film, cornea, conjunctiva and even trabecular meshwork. Despite these consistent and solid data, and warnings coming from observational surveys and individual case series, BAC is still used as the main preservative in eye drops and very few alternatives have been developed[13].

Many methods have been used to evaluate corneal irritation and permeability induced by ophthalmic drugs. Ocular irritability is conventionally tested according to the modified procedure of Draize by scoring the degree of damage to rabbit eyes[14,15]. Alternative methods include evaluation of toxicity in cultured ocular cells, direct confocal microscopic analysis[16] and various other approaches using isolated animal corneas[17]. Corneal drug permeability has been evaluated by diffusion experiment *in vitro*[18]. The epithelial barrier function in humans has been examined by measuring the permeability of fluorescence[19]. Drug toxicity must be rapidly evaluated because topically instilled drugs become rapidly diluted with tears. However, ocular surface changes are difficult to be elicited within a short period using the previous described methods. We previously described a method of assessing acute corneal change after drug instillation by measuring TER *in vivo* within seconds[8]. In general, TER reflects the barrier function of epithelium, with lower corneal TER values indicative of the penetration of greater amounts of electrical current through the damaged superficial cells and tight junctions existing in the epithelium. Thus, TER is a sensitive, reliable and versatile test of corneal epithelial barrier function and useful indicator of corneal toxicity[8–10,20]. Our developed corneal TER measurement system *in vivo* using custom-designed thin stick electrodes and a volt-ohm meter can measure the barrier function of the intact cornea in rabbits. This design more accurately reflects the clinical instillation of ophthalmic drugs and gives us relevant data about the acute corneal toxicity of some eye drops[8–10].

There have been some concerns about the measurement of the corneal TER, as the electrical profile may be altered by the status of the Meibomian lipid layer[21], mucin layer[22] and polysorbate 80[23]. To reduce the effect of the lipid layer of the tear film, we conducted TER measurements after washing the ocular surface three times with 1 ml of HBSS. Mucins are highly *O*-glycosylated glycoproteins that have been implicated in the barrier function of the corneal epithelium.

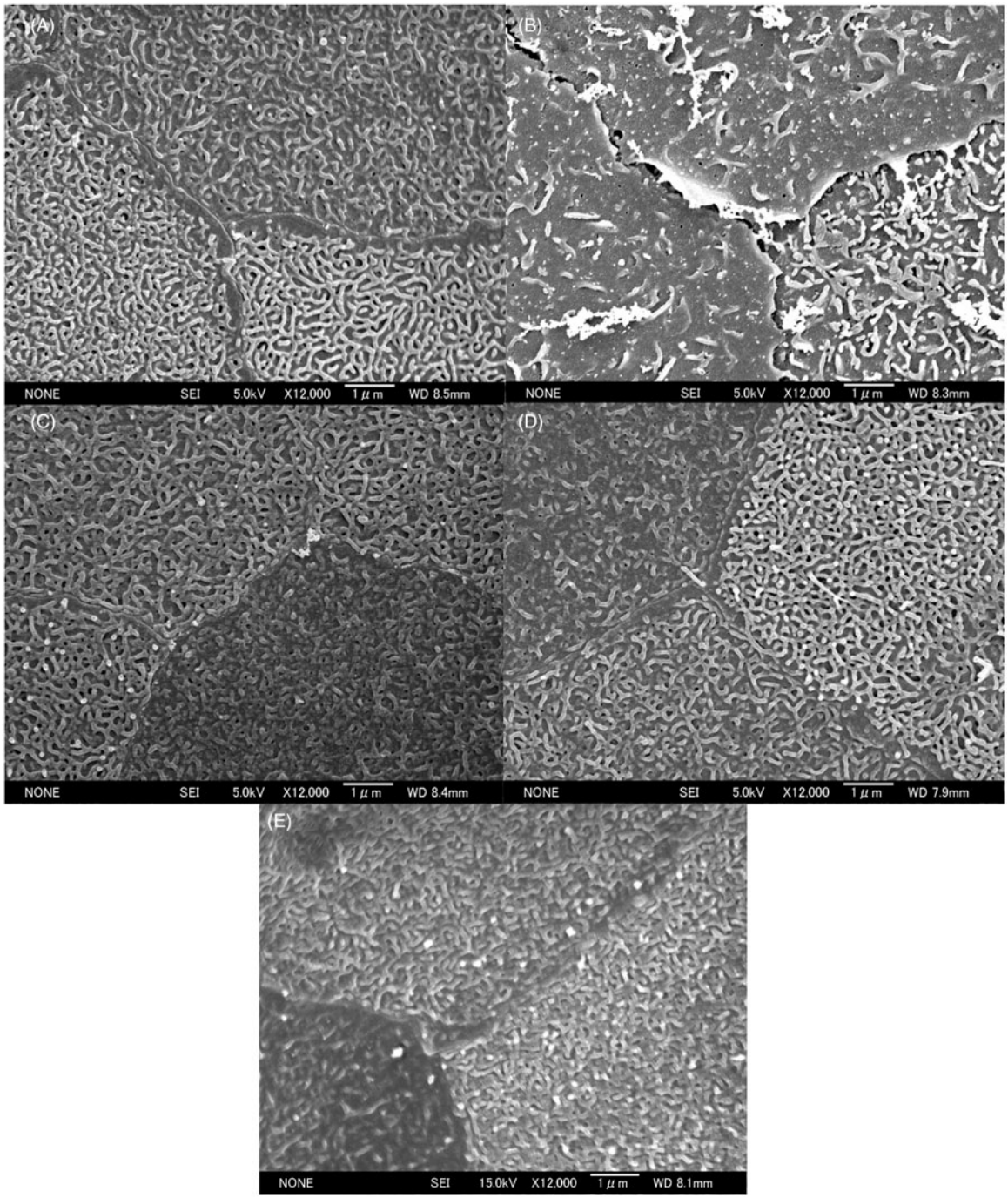

Figure 2. Scanning electron microscopy images of the corneal epithelium after 60 s of exposure to HBSS (A), Xalatan (B), Latanoprost PF BAC free (C), Latanoprost Nitten SB (D) or Latanoprost Towa (E) (12 000× magnification). Images A, C, D and E show that the corneal epithelial structures remain almost intact. Injured corneal epithelial structures including degenerated microvilli are present in B.

However, they may not have a significant effect on the TER measurement because they are highly hydrophobic and have affinity to ions. In a preliminary study, we noticed that polysorbate 80, which is a non-ionic surfactant and emulsifier, altered the electrical conductivity. Thus, we decided to measure the post-exposure TER after washing out the drugs and replacement with HBSS.

In this study, there was a significant decrease in the corneal TER after exposure of the cornea to Xalatan (Relative TER value $= 16.8 \pm 1.2\%$; $p < 0.01$) and to Latanoprost PF BAC free (Relative TER value $= 63.4 \pm 4.8\%$; $p < 0.01$). There was a less significant decrease in the corneal TER after exposure of the cornea to Latanoprost Nitten SB (Relative TER value $= 75.3 \pm 4.4\%$; $p < 0.05$) and Latanoprost Towa (Relative TER value $= 76.1 \pm 2.1\%$; $p < 0.05$). Past studies have also demonstrated that toxic effects of antiglaucoma treatments increase, depending on BAC concentration and BAC concentrations over 0.01% were associated with apparent cell death[24,25]. In previous studies, we demonstrated that BAC concentrations between 0.005 and 0.02% immediately caused acute corneal barrier dysfunction[8,9]. In this study, Xalatan contains 0.02% BAC and Latanoprost Towa contains 0.01% BAC and both have a deleterious effect on the corneal permeability. Also, the degree of this deleterious effect depends upon the BAC

concentration and this is conforming with previous studies[24,25]. Although both drugs affect negatively the corneal permeability, the effect of Xalatan is severely expressed in TER measurement and in SEM results compared to Latanoprost Towa. Latanoprost Towa drug has the lowest effect on corneal TER measurement and no deleterious effect on corneal epithelium as shown with SEM pictures. This can be explained by the relatively low concentration of its BAC and due to the presence of polysorbate 80 as an additive to the drug. In our previous study, we confirmed that polysorbate 80 has a suppressive effect on BAC activity[10]. Although Latanoprost PF and Latanoprost SB contain no BAC, both have a deleterious effect on the corneal permeability. This result is in contrast with other results that claim that Latanoprost PF slightly decreased cell viability without reaching statistical significance compared with HBSS solution[26,27].

Our result is congruous with another study that showed decreased barrier function of the cornea after Latanoprost PF exposure and also in agreement with the suggestion that latanoprost itself or other additives of Latanoprost PF ophthalmic solution may be slightly toxic to the barrier function of corneal epithelium[26]. Liang et al. concluded that PF-tafluprost did not induce any obvious cytotoxicity, showed the least expression of inflammatory or apoptotic markers and revealed preservation of membrane immune-staining of tight junction proteins in comparison with BAC-containing solutions[25]. Although Latanoprost PF and Tafluprost PF are both BAC free and preservative free, Latanoprost PF has a deleterious effect on corneal tissue[25]. This reinforces our suggestion that latanoprost itself or other additives of Latanoprost PF ophthalmic solution may be slightly toxic to the barrier function of corneal epithelium. Latanoprost SB has a less significant effect on corneal permeability than Xalatan. Latanoprost SB is BAC free but it contains sodium benzoate as a preservative. SB is generally recognized as a safe preservative in foods[28]. Previous studies concluded that Latanoprost SB did not decrease cellular viability or alter histological integrity although it did cause a mild disturbance in barrier function[26,28]. This conclusion is confirmed by our technique in this study which revealed that Latanoprost SB caused disturbance in corneal barrier function but did not affect the integrity of the corneal epithelium as shown with SEM pictures. Thus, SB should be recognized as a safer preservative for ocular formulations than BAC.

Benzalkonium chloride (BAC) was mainly used for its apparently good safety/efficacy profile. This compound is weakly allergenic and has a high rate of antimicrobial properties. Indeed, BAC efficiently destroys the cell membranes of microorganisms and seems to have a good safety profile, even though it is almost impossible for a chemical to discriminate membranes of pathogens from those of normal eye cells[13]. To reduce the ocular surface toxicity and enhance compliance of glaucoma patients, several approaches, including fixed combinations, unpreserved drops and application of less toxic preservatives have been made in the recent past[29]. Preservative-free artificial tears in reclosable containers were shown to be at risk of contamination after multiple uses over 10 h. The risk was higher in older patients and within appropriate finger manipulation, which may be the hallmark of patients with chronic eye diseases, namely, glaucoma or dry eye disease[30]. Clinically, all switch studies have confirmed that removal of BAC substantially benefitted the patient's ocular surface[29,31]. More recently, a non-preserved fixed combination of timolol and dorzolamide (Merck, Sharp & Dohme, NY) and a prostaglandin analog, tafluprost (Santen, Japan), have been developed and approved in several countries. Considerable efforts have been made in the recent past by the pharmaceutical industry to develop new antiglaucomatous compounds that would bring about efficacy, safety and compliance. Extensive research has been conducted to discover and develop less toxic preservatives than BAC and quaternary ammoniums. However, since a preservative must be a potent antimicrobial agent while not being cytotoxic, only very few agents have been proposed and are commercially available[13].

In this study, we confirmed that Latanoprost SB (containing sodium benzoate as a preservative), Latanoprost PF (BAC free) and Latanoprost Towa (containing 0.01% BAC with sodium chloride polysorbate 80 as additive) have less toxic effects on the corneal epithelium than Xalatan. Therefore, alternatives to BAC, preservatives such as sodium benzoate and additives such as polysorbate 80 must be considered in the manufacture of long-term eye drops. However, all latanoprost generics significantly decrease barrier function compared to HBSS. This result suggests that latanoprost itself may be slightly toxic to the barrier function of corneal epithelium.

Declaration of interest

The authors have no competing conflicts of interest to report.

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