Current Eye Research

ISSN: 0271-3683 (Print) 1460-2202 (Online) Journal homepage: http://www.tandfonline.com/loi/icey20

Acute Corneal Toxicity of Combined Antiglaucoma Topical Eyedrops

Yasser Helmy Mohamed, Masafumi Uematsu, Naoko Onizuka, Ryotaro Ueki, Daisuke Inoue, Azusa Fujikawa, Hitoshi Sasaki & Takashi Kitaoka

To cite this article: Yasser Helmy Mohamed, Masafumi Uematsu, Naoko Onizuka, Ryotaro Ueki, Daisuke Inoue, Azusa Fujikawa, Hitoshi Sasaki & Takashi Kitaoka (2016): Acute Corneal Toxicity of Combined Antiglaucoma Topical Eyedrops, Current Eye Research, DOI: 10.3109/02713683.2015.1122811

To link to this article: http://dx.doi.org/10.3109/02713683.2015.1122811

Published online: 22 Mar 2016.

Submit your article to this journal

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=icey20

Download by: [Nagasaki University], [yasser mohamed] **Date:** 23 March 2016, At: 18:23

CURRENT EYE RESEARCH, 2016
http://dx.doi.org/10.3109/02713683.2015.1122811

Taylor & Francis
Taylor & Francis Group

Acute Corneal Toxicity of Combined Antiglaucoma Topical Eyedrops

Yasser Helmy Mohamed[a,b], Masafumi Uematsu[a], Naoko Onizuka[a], Ryotaro Ueki[a], Daisuke Inoue[a], Azusa Fujikawa[a], Hitoshi Sasaki[c], and Takashi Kitaoka[a]

[a]Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan; [b]Department of Ophthalmology, EL-Minia University Hospital, EL-Minia, Egypt; [c]Department of Hospital Pharmacy, Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan

ABSTRACT

Purpose: To investigate the corneal toxicity of three combined antiglaucoma topical eyedrops using transepithelial electrical resistance (TER) and scanning electron microscopy (SEM).

Methods: Corneal TER changes after a 60-s exposure to latanoprost/timolol with 0.02% benzalkonium chloride (BAC), travoprost/timolol with polyquaternium-1, and dorzolamide/timolol with 0.005% BAC 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 latanoprost/timolol with 0.02% BAC. Travoprost/timolol with polyquaternium-1 and dorzolamide/timolol with 0.005% BAC did not produce any significant decrease in the corneal TER as compared to HBSS control eyes. SEM revealed that superficial cells of corneas treated with latanoprost/timolol with 0.02% BAC were damaged and exhibited degenerated microvilli. Conversely, the superficial cells of corneas exposed to travoprost/timolol with polyquaternium-1 or dorzolamide/timolol with 0.005% BAC appeared normal and had normal microvilli under SEM examinations.

Conclusion: The corneal toxicity of latanoprost/timolol with 0.02% BAC is greater than that of travoprost/timolol with polyquaternium-1 and dorzolamide/timolol with 0.005% BAC. Latanoprost/timolol contains 0.02% BAC, which may be responsible for the corneal toxicity.

ARTICLE HISTORY
Received 2 June 2015
Revised 30 September 2015
Accepted 13 November 2015

KEYWORDS
Cornea; electrophysiology; preservative; scanning electron microscopy; toxicology

Downloaded by [Nagasaki University], [yasser mohamed] at 18:23 23 March 2016

Introduction

Primary open-angle glaucoma and ocular hypertension are generally treated with topical antiglaucomatous drugs as the first choice of treatment to reduce the risk of progressive visual field loss.[1] Although monotherapy is the first line of choice, fixed-combination therapies are effective for patients who require more than one therapy to control intraocular pressure. This approach simplifies the treatment regimen and improves adherence of patients considerably. Another potential benefit of combining medications in one bottle is the decreased number of exposures to both active ingredients and preservatives, such as benzalkonium chloride (BAC).[2] The majority of topical ophthalmic products are preserved with BAC, which has numerous detrimental side effects, including ocular surface toxicity.[3] Chronic topical glaucoma therapy can lead to alterations in both tear film and fluorescein staining of the corneal surface and to an increase in inflammatory cytokines, among other deleterious effects. These ocular surface changes are thought to be caused by BAC.[2,4]

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.[5,6] We developed a method of measuring the TER of live rabbit cornea. In this method, the cornea is not damaged by the experimental procedure and the TER is stable before drug administration. In previous studies, after developing a new *in vivo* method of measuring the TER of rabbit corneas, we demonstrated that BAC concentrations between 0.005% and 0.02% immediately caused acute corneal barrier dysfunction.[6,7]

Therefore, the aim of this study is to detect the differences of the corneal irritancy among three anti-glaucoma fixed-combination eyedrops with different types of preservations by using our technique. The first one is fixed anti-glaucoma combination eyedrop (latanoprost 0.005%/timolol maleate 0.5%) and contains 0.02% BAC as a preservative. The second is travoprost 0.004%/timolol maleate 0.5% fixed-combination ophthalmic solution preserved with polyquaternium-1 0.001% (Polyquad, PQ) instead of BAC, and the third is dorzolamide1%/timolol maleate 0.5% fixed-combination ophthalmic solution preserved with 0.005% BAC.

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 fixed-combination eyedrops, latanoprost/

CONTACT Masafumi Uematsu uematsu-ngs@umin.ac.jp Department of Ophthalmology and Visual Sciences, Graduate School of Biomedical Sciences, Nagasaki University, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.

© 2016 Taylor & Francis

Downloaded by [Nagasaki University], [yasser mohamed] at 18:23 23 March 2016

timolol (Xalacom, Pfizer, New York, NY), travoprost/timolol (Duotrav, Alcon Laboratories, Inc., Fort Worth, TX), and dorzolamide/timolol (Cosopt, Merk Sharp & Dohme, Kenilworth, NJ) were used in this study.

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 with 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 solution was gently poured into the ring, and the overflow was aspirated. After an exposure period of 60 s, the rings were washed out with 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.[5–7] In the present study, the influences of commercial solutions of latanoprost/timolol preserved with 0.02% BAC, travoprost/timolol preserved with PQ, and dorzolamide/timolol preserved with 0.005% BAC eyedrop fixed combinations on corneal TER changes are determined. The sample size for the corneal TER study was set at 3, which we found to be sufficient for statistical analyses in our previous TER studies.[5–7]

Scanning electron microscopy (SEM)

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 corneas, the rabbits were immediately sacrificed by 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 by using an auto fine coater (JEOL JFC-1600, JEOL, Tokyo, Japan). After processing, the surface of the corneal epithelium was observed under a scanning electron microscope (Hitachi S2360, Hitachi, Ibaragi, Japan).

Statistical analysis

All results were expressed as the mean ± standard error of three experiments. Statistical comparisons were performed with 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 TER

The mean corneal TER for the live rabbits used in this study was 826.1 ± 60.4 ohm cm^2. Figure 1 shows the TER after corneal exposure to HBSS and fixed-combination eyedrops. There was no change in the corneal TER after exposure to HBSS (relative TER value = 101.3 ± 2.8%). There was a significant decrease in the corneal TER after exposure to latanoprost/timolol (relative TER value = 15.6 ± 3.8%) ($p < 0.01$). In contrast, travoprost/timolol (relative TER value = 82.2 ± 7.9%) and dorzolamide/timolol (relative TER value = 91.0 ± 1.0%) did not produce a significant decrease in the corneal TER as compared to HBSS control eyes.

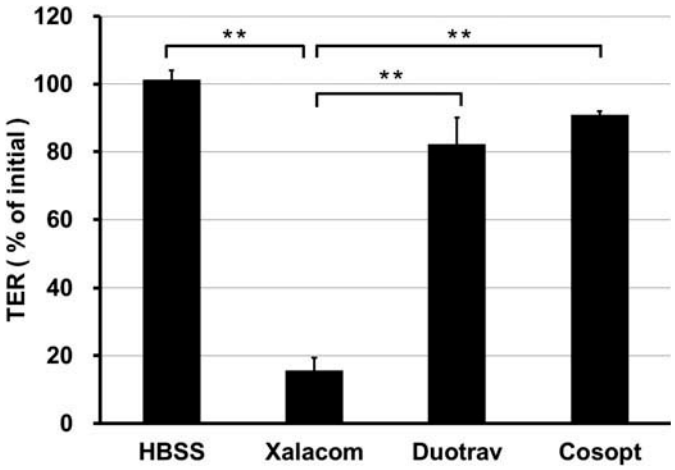

Figure 1. Corneal TER changes after exposure to HBSS, latanoprost/timolol, travoprost/timolol, or dorzolamide/timolol for 60 s. Data represent the percentage compared to the pre-exposure value. Each value is the mean ± SE ($n = 3$). **$p < 0.01$ as compared with latanoprost/timolol (Tukey test).

Downloaded by [Nagasaki University], [yasser mohamed] at 18:23 23 March 2016

Scanning electron microscopy

As shown in Figure 2, the superficial cells of the cornea of control eyes exposed to HBSS solution were normal in appearance with normal microvilli. In contrast, the superficial cells were damaged and exhibited degenerated microvilli in corneas exposed to latanoprost/timolol fixed-combination eyedrops. Conversely, the superficial cells of corneas exposed to travoprost/timolol or dorzolamide/timolol fixed-combination eyedrops appeared normal with normal microvilli.

Discussion

Long-term use of topical medication is needed to treat glaucoma. Unless patients can use eyedrops with comfort, safety, and convenience, treatment adherence is not guaranteed and treatment efficacy is severely reduced. Long-term antiglaucoma eyedrop therapy requires not only efficacy and safety, but also good tolerability for improved patient comfort, and hence better compliance. Multiple studies have indicated that the toxicity of antiglaucoma eyedrops 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.[3,8] BAC is used to prevent bacterial contamination in multi-dose bottles during the treatment period. Such bactericidal agents are necessary for patient safety because the multi-use containers for eyedrops often lead to improper use.[9] However, concerns have been raised about the cytotoxicity of BAC, which is a known irritant; BAC could potentially damage the delicate ocular surface in the millions of patients who use eyedrops routinely over many years.-[10] BAC also disrupts the precorneal tear film and may cause adverse effects including dry eye, tearing, burning, and foreign body sensations.[8] 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 used.[11,12] One of the most commonly prescribed classes of hypotensive agents are fixed-combination therapies containing PGAs plus the beta-blocker timolol, which is used primarily as second-line monotherapy after initial PGA monotherapy has failed. Fixed-combination therapies are a cost-effective way to treat glaucoma, and they simplify the treatment regimen considerably. Another potential benefit of combining medications in one bottle is the decreased number of exposures, on a daily basis, to both active ingredients and preservatives contained in most multi-dose topical ophthalmic preparations.[2]

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.[13,14] Alternative methods include evaluation of toxicity in cultured ocular cells,[15] direct confocal microscopic analysis,[16] and other approaches using isolated animal corneas.[17,18] Corneal drug permeability has been evaluated by diffusion experiments in vitro.[19] The epithelial barrier function in humans has been examined by measuring the permeability of fluorescence.[20,21] Drug toxicity must be rapidly evaluated because topically instilled drugs become rapidly diluted with tears.[22] However, ocular surface changes are difficult to elicit within a short period using the previous described methods. We previously described a method of assessing

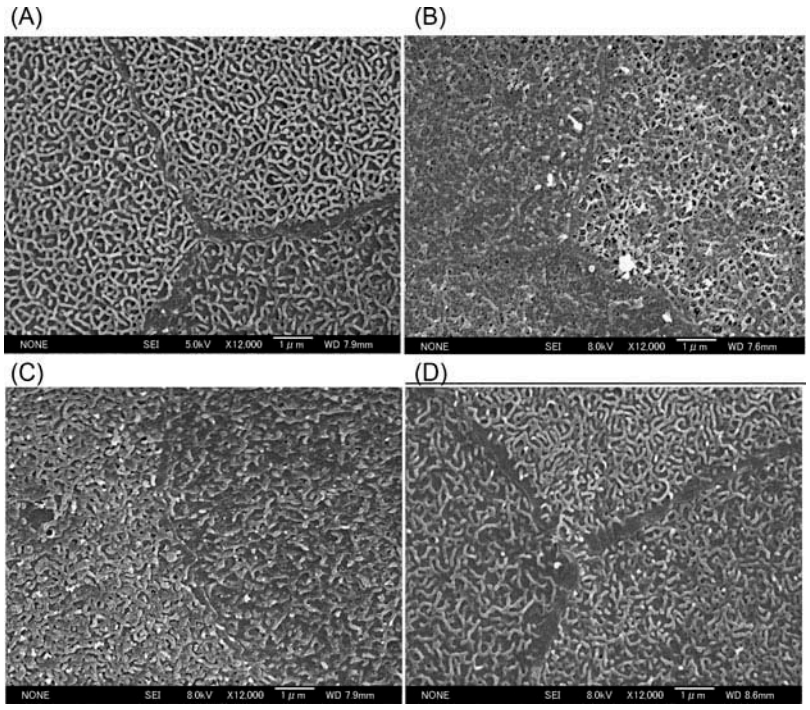

Figure 2. Scanning electron microscopy images of the corneal epithelium after 60 s of exposure to the solutions (12 000× magnification). The superficial cells of the cornea of control eyes exposed to HBSS solution were normal in appearance with normal microvilli (A). The superficial cells were damaged and exhibited degenerated microvilli in corneas exposed to latanoprost/timolol (B). The superficial cells of corneas exposed to travoprost/timolol (C) or dorzolamide/timolol (D) appeared normal with normal microvilli.

Downloaded by [Nagasaki University], [yasser mohamed] at 18:23 23 March 2016

acute corneal change after drug instillation by measuring TER *in vivo* within seconds.[5] In general, TER reflects the barrier function of the 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 a useful indicator of corneal toxicity.[5–7,23]

Our corneal TER measurement system *in vivo* uses custom-designed thin stick electrodes and a volt-ohm meter to 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 eyedrops.[5–7] In the present study, the results of the TER measurement showed that latanoprost/timolol with 0.02% BAC had acute potential damage to the corneal epithelial barrier function. Conversely, there were no remarkable TER measurement changes to corneal epithelial barrier function when travoprost/timolol with PQ and dorzolamide/timolol with 0.005% BAC were used. The toxicity of latanoprost/timolol with 0.02% BAC was different from fixed-combination eyedrops preserved in 0.001% PQ (travoprost/timolol). This result is consistent with other studies[2,24] that found that travoprost plus timolol fixed combination preserved with PQ (travoprost/timolol) had greater corneal and conjunctival cell survival than the latanoprost/timolol with 0.02% BAC. BAC alone has significant *in vitro* cytotoxicity to cultured ocular epithelial cells. The substitution of BAC with PQ resulted in significantly higher percentages of live conjunctival and corneal cells.[25] Although timolol may have mild effect on corneal epithelium barrier function,[21] we have not examined the effect of timolol because the all examined drugs contain the same concentration (0.5%) of timolol in this study.

BAC is a quaternary ammonium compound that acts as a surfactant, disrupting bacterial cell membranes and ultimately leading to bacterial cell death. PQ is a polymer of many quaternary ammonium structures and is classified as a polycationic preservative.[25] PQ has less ability to penetrate mammalian cells and, therefore, is less likely to cause cytotoxic effects.[2] Additionally, PQ does not increase corneal permeability as BAC is known to do,[26] and this lack of increased corneal permeability is a property that significantly reduces its cytotoxic effect as compared with BAC. Also, Ammar et al. found a protective effect of travoprost, but not latanoprost, against BAC-induced toxicity.[2] Although dorzolamide/timolol is preserved with 0.005% BAC, it has no corneal toxicity comparable to that of latanoprost/timolol with 0.02% BAC. This lack of toxicity may be due to the lower concentration of BAC, or it may be due to the presence of additives, such as D-mannitol, in dorzolamide/timolol fixed-combination eyedrops. Our results are consistent with the results of Nagai et al. who suggested that the presence of D-mannitol as an additive in dorzolamide/timolol conceals the deleterious effect of BAC toxicity.[27] 0.02% BAC within latanoprost/timolol combination drug may have an advantage of increased absorption of the drug into the eye to get a high therapeutic concentration in the anterior chamber, and thus enhances its therapeutic effect.

In this study, we confirmed the acute effects of these combination drugs on the corneal TER measurements and SEM. We have ongoing studies about the chronic effects of these combination drugs on the corneal surface. In addition, the regeneration ability of the corneal epithelium after acute and chronic use of these drugs will be determined.

We concluded from the present study that the corneal toxicity of latanoprost/timolol with 0.02% BAC is much more than that of travoprost/timolol and dorzolamide/timolol. Travoprost/timolol is preserved with a non-BAC system (PQ). Therefore, these results suggest that the use of PQ is related to the low cytotoxicity of travoprost/timolol. On the other hand, the low concentration of BAC (0.005%) and the presence of the D-mannitol additive reduced the corneal toxicity of dorzolamide/timolol in the present study. Travoprost/timolol and dorzolamide/timolol may be less damaging to the ocular surface of glaucoma patients receiving long-term eyedrop therapy.

Declaration of interest

The authors of this work report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. All authors disclose no financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

References

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006;90:262–267.
2. Ammar DA, Noecker RJ, Kahook MY. Effects of benzalkonium chloride- and polyquad-preserved combination glaucoma medications on cultured human ocular surface cells. Adv Ther 2011;28(6):501–510.
3. Baudouin C. Detrimental effect of preservatives in eyedrops: implications for the treatment of glaucoma. Acta Ophthalmol 2008;86:716–726.
4. Horsley MB, Kahook MY. Effects of prostaglandin analog therapy on the ocular surface of glaucoma patients. Clin Ophthalmol 2009;3:291–295.
5. Uematsu M, Kumagami T, Kusano M, Yamada K, Mishima K, Fujimura K, et al. Acute corneal epithelial change after instillation of benzalkonium chloride evaluated using a newly developed in vivo corneal transepithelial electric resistance measurement method. Ophthalmic Res 2007;39(6):308–314.
6. Kusano M, Uematsu M, Kumagami T, Sasaki H, Kitaoka T. Evaluation of acute corneal barrier change induced by topically applied preservatives using corneal transepithelial electric resistance in vivo. Cornea 2010;29(1):80–85.
7. Onizuka N, Uematsu M, Kusano M, Sasaki H, Suzuma K, Kitaoka T. Influence of different additives and their concentrations on corneal toxicity and antimicrobial effect of benzalkonium chloride. Cornea 2014;33(5):521–526.
8. Georgiev GA, Yokoi N, Koev K, Kutsarova E, Ivanova S, Kyumurkov A, et al. Surface chemistry study of the interactions of benzalkonium chloride with films of meibum, corneal cells lipids, and whole tears. Invest Ophthalmol Vis Sci 2011;52:4645–4654.
9. Baudouin C, Labbé A, Liang H, Pauly A, Brignole-Baudouin F. Preservatives in eyedrops: the good, the bad and the ugly. Progr. Retin Eye Res 2010;29:312–334.
10. Kim MS, Choi CY, Kim JM, Chang HR, Woo HY. Microbial contamination of multiply used preservative-free artificial tears packed in reclosable containers. Br J Ophthalmol 2008;92:1518–1521.

11. Hommer A. A double-masked, randomized, parallel comparison of a fixed combination of bimatoprost 0.03%/timolol 0.5% with non-fixed combination use in patients with glaucoma or ocular hypertension. Eur J Ophthalmol 2007;17:53–62.
12. Pisella PJ, Pouliquen P, Baudouin C. Prevalence of ocular symptoms and signs with preserved and preservative free glaucoma medication. Br J Ophthalmol 2002;86:418–423.
13. Draize JH, Woodard G, Calvery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944;82:377–390.
14. York M, Steiling W. A critical review of the assessment of eye irritation potential using the Draize rabbit eye test. J Appl Toxicol 1998;18:233–240.
15. Kruszewski FH, Walker TL, DiPasquale LC. Evaluation of a human corneal epithelial cell line as an in vitro model for assessing ocular irritation. Fundam Appl Toxicol 1997;36:130–140.
16. Furrer P, Plazonnet B, Mayer JM, Gurny R. Application of in vivo confocal microscopy to the objective evaluation of ocular irritation induced by surfactants. Int J Pharm 2000;207:89–98.
17. Igarashi H, Northover AM. Increases in opacity and thickness induced by surfactants and other chemicals in the bovine isolated cornea. Toxicol Lett 1987;39:249–254.
18. Prinsen MK. The chicken enucleated eye test (CEET): a practical (pre)screen for the assessment of eye irritation/corrosion potential of test materials. Food Chem Toxicol 1996;34:291–296.
19. Sasaki H, Yamamura K, Mukai T, Nishida K, Nakamura J, Nakashima M, et al. Enhancement of ocular drug penetration. Crit Rev Ther Drug Carrier Syst 1999;16:85–146.
20. Joshi A, Maurice D, Paugh JR. A new method for determining corneal epithelial barrier to fluorescein in humans. Invest Ophthalmol Vis Sci 1996;37:1008–1016.
21. Ishibashi T, Yokoi N, Kinoshita S. Comparison of the short-term effects on the human corneal surface of topical timolol maleate with and without benzalkonium chloride. J Glaucoma 2003;12:486–490.
22. Jordan A, Baum J. Basic tear flow. Does it exist? Ophthalmology 1980;87:920–930.
23. Nakashima M, Nakamura T, Teshima M, To H, Uematsu M, Kitaoka T, et al. Breakdown evaluation of corneal epithelial barrier caused by antiallergic eyedrops using an electrophysiologic method. J Ocul Pharmacol Ther 2008;24:43–51.
24. Liang H, Brignole-Baudouin F, Pauly A, Riancho L, Baudouin C. Polyquad-preserved travoprost/timolol, benzalkonium chloride (BAK)-preserved travoprost/timolol, and latanoprost/timolol in fixed combinations: a rabbit ocular surface study. Adv Ther 2011;28(4):311–325.
25. Tripathi BJ, Tripathi RC, Kolli SP. Cytotoxicity of ophthalmic preservatives on human corneal epithelium. Lens Eye Toxic Res 1992;9:361–375.
26. Lopez Bernal D, Ubels JL. Quantitative evaluation of the corneal epithelial barrier: effect of artificial tears and preservatives. Curr Eye Res 1991;10:645–656.
27. Nagai N, Murao T, Oe K, Ito Y, Okamoto N, Shimomura Y. In vitro evaluation for corneal damages by anti-glaucoma combination eyedrops using human corneal epithelial cell (HCE-T). Yakugaku Zasshi 2011;131:985–991.

Downloaded by [Nagasaki University], [yasser mohamed] at 18:23 23 March 2016