

In Vitro Toxicity Evaluation of New Generic Latanost[®] and Latacom[®] as an Ophthalmic Formulation

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ABSTRACT

Aim and objective: To evaluate the safety of two new generic ophthalmic formulations, Latanost[®] (latanoprost) and Latacom[®] (latanoprost and timolol) by utilizing the three-dimensional reconstructed human cornea-like epithelium (RhCE) tissue constructs as an *in vitro* model in the assessment of ocular irritation.

Materials and methods: *In vitro* irritation test was conducted on Latanost[®] (LTN) and Latacom[®] (LTC) and their corresponding innovators, Xalatan[®] (XLT) and Xalacom[®] (XLC), respectively, by using RhCE. According to the OECD guidelines No. 492 on the testing of chemicals, the ophthalmic formulations were assessed via topical exposure of the formulations on *in vitro* RhCE tissue. Cell viability was measured by MTT assay.

Results: The mean cell viability percentage of LTN and XLT was 70.5 and 75.7%, respectively, whereas, for LTC and XLC, the percentage viability was 95.3 and 85.7%, respectively. The two new generic formulations (LTN and LTC) did not reduce the cell viability of the RhCE tissue to $\leq 60\%$. Thus, both can be considered as nonirritant.

Conclusion: Both newly developed generics are nonocular irritants.

Clinical significance: This study informs the safety assessment of new generic antiglaucoma ophthalmic solutions applicable for long-term glaucoma treatment. The formulations aim to keep eye irritation to a minimum level.

Keywords: Eye irritation, Generic, Glaucoma, Latanoprost, Timolol.

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INTRODUCTION

Glaucoma is a chronic eye disease caused by increased intraocular pressure (IOP). The disorder is characterized by progressive optic neuropathy that can lead to the death of retinal ganglion cells. About 66% of glaucoma patients have severe ocular surface diseases (OSD).¹ The symptoms of OSD include dry eye, blepharitis, Meibomian gland dysfunction, allergic eye diseases, etc. Besides affecting the wellbeing of the patients, the condition can also result in blindness.² Topical glaucoma therapies have been implicated in OSD according to several studies.^{3,4} Furthermore, the formulation was associated with deterioration of the eye dryness and irritation among glaucoma patients with preexisting OSD. The severity of OSD commonly increases with age and the frequency of using topical glaucoma therapies.⁵

Prostaglandin analogs such as latanoprost are recommended as the first-line treatment for glaucoma. However, the condition of many patients could not be sufficiently controlled via monotherapy. Thus, a fixed combination (FC) of prostaglandin analogs and β -blocker timolol is recommended. Latanoprost increases uveoscleral outflow while timolol inhibits aqueous humor production (Fig. 1). By administering the FC, it provides an additive effect to the reduction of IOP⁶ and better tolerance with a lower rate of ocular side effects such as hyperemia, ocular irritation, and keratitis.⁷

Despite the advantages, there are some concerns regarding the toxicity caused by the formulations. Prostaglandin analogs including latanoprost are reported to be associated with ocular surface problems and eye irritation.⁸ Furthermore, generic versions of latanoprost can also result in varying degrees of corneal

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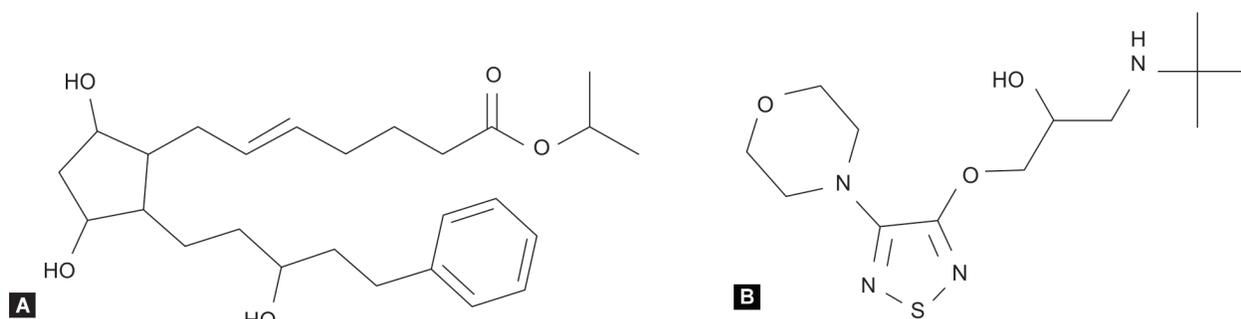
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irritation.⁹ Additionally, eye irritation can also be induced by the additives in the ophthalmic formulation.

Commonly, the generic eye drops are formulated in such a way that the active ingredient is similar to that of the innovators. However, the excipients, i.e., solubility-enhancing agents or emulsifiers used in the generics might differ considerably from the innovators. Some studies have attributed the excipients in the formulation to be causing eye discomfort and irritation



Figs 1A and B: Chemical structure of (A) latanoprost and (B) timolol

due to the presence of additional surfactants and additives in the formulation. Thus, switching to generic eye drops could be associated with the development of corneal epithelial disorder.¹⁰ In view of this, it is vital to evaluate the toxicity and ocular tolerance while developing new ophthalmic formulations. A comprehensive evaluation is needed to ensure optimal compliance with product safety and tolerability.

So far, *in vivo*, *ex vivo*, and *in vitro* test methods have been developed to evaluate the eye irritation induced by chemical substances. However, the standard reference test, an *in vivo* Draize eye test, has been heavily criticized ethically for using animals. In contrast, the *ex vivo* organotypic method employs the corneas of animals for experimental purposes. However, the findings are limiting due to inter-species differences and the possibility of inaccurate estimation of eye irritation. As a result, *in vitro* cellular models have been used in substitution of tests that rely on animals or tissues of animal origin.¹¹ An example of *in vitro* cellular model is the reconstructed human corneal-like epithelium (RhCE) that is similar in structure to the living human corneal epithelium. The viability of *in vitro* RhCE cells could be used to assess conditions such as eye irritation or serious eye damage. It is assumed that all chemical-inducing eye irritation can result in cytotoxicity to the corneal epithelium. Currently, there are a total of four test methods employing RhCE models that are validated and included in the OECD test guidelines.¹² Based on the categorization of the Globally Harmonised System (GHS), three groups of chemical substances can cause eye irritation. The first group is “GHS no category” which includes substances that do not cause any adverse effects and thus do not require labeling. The second group, GHS category 1, includes substances that can result in irreversible effects on the eye. The last group, GHS category 2, are substances that cause reversible effects in the eye.¹³ The eye irritation test performed with RhCE models can distinguish ocular irritants and corrosives (GHS Categories 1 and 2 combined) from the GHS No Category substances that are harmless and require no special labeling.

This study aimed to assess the toxicity profile of two newly developed generic ophthalmic solutions, namely Latanost® (LTN) and Latacom® (LTC). The findings would contribute vital data in the preliminary assessment of new eye drop formulations used in ocular discomfort.

MATERIALS AND METHODS

Tissue Model and Topical Glaucoma Ophthalmic Solutions

The EpiOcular™ RhCE tissues were purchased from MatTek Corporation, USA. Initially, the tissues were immersed in cell culture

inserts with serum-free media to stimulate cell differentiation to induce the formation of an organotypic, three-dimensional tissue that was similar to the corneal epithelium. All experiments were performed based on the OECD Guidelines for Testing of Chemicals No. 492. The positive and negative controls (NCs) in the experiment were methyl acetate (MatTek Corporation, USA) and sterile deionized water, respectively. Innovator products, i.e., Xalatan® (XLT) and Xalacom® (XLC) (Pfizer, USA) were purchased from local pharmacies. In contrast, the tested generic formulations were Latanost® (LTN) and Latacom® (LTC) (Duopharma Biotech Berhad, Malaysia). LTN and XLT consisted of latanoprost 0.05 mg/mL whereas LTC and XLC contained 0.05 mg/mL latanoprost and 5 mg/mL timolol.

Tissue Culture

The standard protocol of EpiOcular™ Eye Irritation Test (OCL-200-EIT) as described by Kaluzhny et al. was employed in this study.¹⁴ The RhCE tissue cultures were dispensed into the 6-well plates containing 1 mL of assay medium OLC-200-ASY in each well (MatTek Corporation, USA). They were then pre-incubated for an hour under the standard culture conditions (SCC) of 37°C, 5% CO₂, and 95% humidity. After that, the assay medium was renewed, and the tissue cultures were pre-equilibrated overnight (16–24 hours) under SCC. Following the overnight incubation, each tissue was pre-wetted with 20 µL phosphate-buffered saline (PBS) without calcium and magnesium (Bio Basic Canada Inc., Canada) for 30 minutes under SCC. This step was to ensure that the tissues were fully hydrated so that they mimic the conditions of the human eye. Then, 50 µL of both negative and positive controls and 50 µL of test formulations were applied directly to the duplicate ($n = 2$) tissues and incubated for 30 minutes under SCC. After 30 minutes of the exposure period, each pair of duplicate tissues was simultaneously taken out from the incubator and extensively rinsed with PBS to eliminate any residual test compound. After rinsing, each tissue was immediately immersed in 5 mL of an assay that had been previously warmed to room temperature in a 12-well plate and incubated for 12 minutes under SCC. Finally, the assay medium was decanted, and the tissues were transferred into a 6-well plate containing 1 mL of assay medium. Lastly, the tissues were post-incubated in the assay medium for 2 hours under SCC.

Cell Viability Test Using MTT Assay

After the exposure to either a test formulation or control, each tissue was transferred to a 24-well plate to be incubated for 3 hours at SCC. Each well contained 300 µL of 1 mg/mL MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide) medium. The MTT vital dye worked by capturing electrons during the oxidative phosphorylation of viable cells. In the process, it was

reduced by NAD(P)H-dependent microsomal enzyme and succinate dehydrogenase and became a blue formazan precipitate in viable cells.¹⁵ After 3 hours of incubation, the blue formazan salt was extracted by using 2.0 mL isopropanol (Fisher Scientific, USA) per tissue. The extraction plates were then sealed with parafilm and agitated for at least 2 hours at room temperature. After completing the extraction period, an injection needle was used to pierce the tissue to allow the extract to run into the well. The insert in the well was then discarded. A pipette was used to stir the extraction solution up and down to ensure complete mixing. Lastly, 200 µL aliquots from each well and 200 µL of isopropanol (used as blank) were transferred into a 96-well microtiter plate for optical density (OD) absorbance measurement at 570 nm. The blank corrected values were obtained by subtracting the OD of the treated tissues with the OD of the isopropanol. Finally, the percentage viability of each tissue was determined by comparing the OD of the treated tissues to the mean OD of the NC (set as 100%): % Viability = $[OD_{\text{treated tissues}}/OD_{\text{NC}}] \times 100$.

DATA ANALYSIS

The relative cell viability was expressed as the mean of two individual tissues. An irritant is identified if the relative tissue viability of the test formulations was ≤60% of the mean viability of the NCs. The cell viability data of both generic and innovator (as reference) formulations were compared using Student’s t-test (one-tailed).

RESULTS

The mean percentage of cell viability of latanoprost eye drops, LTN and XLT were 70.5 and 75.7%, respectively (Fig. 2). However, the difference between the means was not significant ($p > 0.05$). Meanwhile, the mean percentage cell viability of the latanoprost-timolol FC eye drops, LTC and XLC were 95.3 and 85.7%, respectively (Fig. 3). Again, the difference between the means was not significant ($p > 0.05$). In our study, the FC combination eye drop LTC was found

to have higher mean tissue viability than the mono-component eye drop LTN (Table 1).

DISCUSSION

The human corneal epithelium serves as an effective barrier against the external environment. It also governs the permeability of solutes, fluids, and topically-applied drug formulations.¹⁶ Any penetration of chemicals through the cornea can result in eye irritation. The RhCE model is suitable for the assessment of eye irritation caused by prodrugs such as latanoprost, an esterified prodrug of prostaglandin F2α. Kaluzhny et al. demonstrated that the RhCE tissues allow high corneal permeation of latanoprost. The tissues also facilitate esterase and amidase activities to convert latanoprost, the ester prodrug into latanoprost acid, the pharmacologically active metabolite. The mean tissue viability reported for LTN in this study was similar to a study that reported cell viability of 71.0% with commercial latanoprost 0.005% (w/v) while using the RhCE tissue model.¹⁷

In our study, higher mean tissue viability was reported with the FC combination eye drop LTC when compared with the mono-component eye drop LTN. However, contrasting results were reported in another study in which XLC was found to be more cytotoxic than the FC of latanoprost and timolol preparation on human corneal epithelial cell (HCE-T),¹⁸ an SV-40-immortalized human corneal epithelial cells. The discrepancy was likely due to the different tissue models.

Table 1: Differences in mean tissue viability between latanoprost mono-component eye drops Xalatan® and Latanost® and latanoprost-timolol combination eye drops Xalacom® and Latacom®. $p < 0.05$ indicates significant difference

Eye drops	Differences in mean tissue viability (%)	p value
Xalatan® vs Xalacom®	10.00	0.055
Latanost® vs Latacom®	24.80	0.067

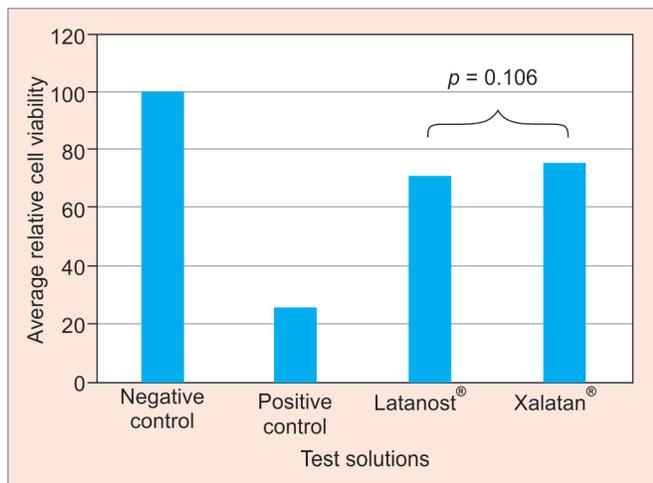


Fig. 2: Mean relative cell viability of Latanost® and Xalatan® (n = 2). Positive control (methyl acetate) and negative control (sterile deionized water) indicated test validity. Latanost® and Xalatan® are nonirritant as the mean relative tissue viability of two individual tissues exposed is not reduced <60% of the mean viability of the negative controls. The differences between the means are not significant ($p > 0.05$)

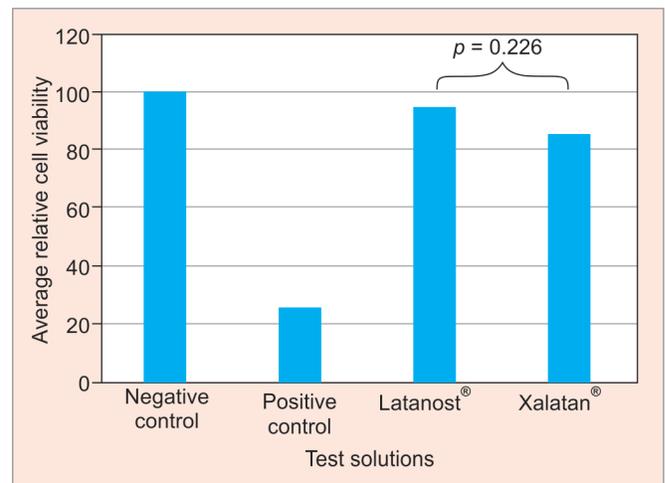


Fig. 3: Mean relative cell viability of Latacom® and Xalacom® (n = 2). Positive control (methyl acetate) and negative control (sterile deionized water) indicated test validity. Latacom® and Xalacom® are nonirritant as the mean relative tissue viability of two individual tissues exposed is not reduced <60% of the mean viability of the negative controls. The differences between the means are not significant ($p > 0.05$)

In addition, the relative cell viability of LTN and LTC was >60%. Thus, both formulations do not require further classification and labeling based on GHS. However, if the mean tissue viability is ≤60%, further tests with other *in vitro* methods must be performed for eye irritation potential prediction. This is because the RhCE test method is unable to discriminate between GHS category 1 and category 2.¹²

Besides that, the physical properties of ophthalmic formulations can affect ocular tolerability. Ideally, the optimal pH of the formulation should be pH 7.4, resembling the tear fluid pH. However, in reality, the pH of commercial topical glaucoma medications can range from 4.0 to 7.4¹⁹ because different active ingredients require different optimal pH to achieve chemical stability. Even though the eyes can tolerate a fairly wide range of pH due to the dilution and buffering capability of tears,²⁰ pH values <4 or >8 can still result in eye irritation and discomfort.²¹ Therefore, a non-isotonic formulation could lead to eye irritation.²²

The new generic eye drop formulations, LTN and LTC, do not contain additional excipients compared with the innovators. While buffering agents, i.e., sodium dihydrogen phosphate and disodium hydrogen phosphate are present in the new formulations, their pH matches the pH of the innovators, i.e., LTN (6.7) and LTC (6.0), respectively. Furthermore, the recommended physiological film osmolality level was 298 mOsm/L.²³ Thus, the tonicity agent in the formulations, i.e., sodium chloride is chosen as its concentration mimics the tear film osmolality. Lastly, the excipients in both formulations are nonirritant.

Formulators are cautious about excipients of the ophthalmic drug because of its potential to cause toxicity to corneal cells.²⁴ Many glaucoma medications contain benzalkonium chloride (BAK), a substance that could cause ocular toxicity. Because of its hazardous nature, its concentration has been kept to a minimum. Previous studies have shown that 0.005% latanoprost (0.02% BAK),²⁵ 0.5% timolol (0.005% BAK),²⁶ 0.1% brimonidine (No BAK),²⁷ 1% dorzolamide (0.005% BAK),²⁸ or 1% brinzolamide (0.01% BAK),¹⁸ there is no significant difference in cultured corneal cells. Therefore, BAK concentration at 0–0.02% (w/v) is considered safe for corneal cells. Therefore, we formulated the BAK concentration at 0.02% (w/v).

There are several limitations to be considered. Our 3D *in vitro* data do not reflect the true clinical application in humans. However, the formulations are considered safe when the ingredients are identical to that of the innovator. The topical administration is not a true representation of physiological dilution due to the potential dilution of tears. Thus, the cell toxicity might have been overestimated in the study.²⁹

CONCLUSION

This study assessed the eye irritation potential of newly developed eye drop formulations. The results show that both new formulations of LTN and LTC are nonocular irritants according to OECD guidelines.

CLINICAL SIGNIFICANCE

This study utilized a validated *in vitro* cellular model to demonstrate the safety and nonirritancy of the new generic latanoprost and latanoprost/timolol ophthalmic solutions, comparable to the innovator eye drops.

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