Evaluation of Rabbit Model for Glaucoma Study: Drug Interaction in a Rabbit Model Instilled with Ophthalmic Preparation containing Latanoprost and Timolol

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Studies in rabbits have been performed to evaluate the intraocular pressure (IOP)-reducing effect of various anti-glaucoma eye drops and eye drops of latanoprost (LP), which is a selective agonist of prostaglandin F receptor (FP). However, the sensitivity and localization of FP receptors in the rabbit eye are different from those in humans, and studying the relationship between IOP regulation and FP receptors in rabbits is important for the evaluation of anti-glaucoma eye drops. In this study, we investigated whether stimulation of FP receptors in rabbits affects the regulation of aqueous humour production via β receptors by using the LP and the β-blocker timolol (TM). Ocular hypertension was induced in the rabbits by the infusion of a 5% glucose solution (15 mL/kg). Although no reduction in IOP was observed after the instillation of saline and 0.005% LP, 0.5% TM eye drops significantly reduced IOP. The IOP-reducing effect, as measured by area under the curve (AUC ∆IOP) in rabbits treated with TM eye drops, was 81.3% that of LP 0.005%/TM 0.5% fixed combination (LTFC) eye drops, and the TM concentration in the aqueous humour following the instillation of LTFC eye drops was similar to that of TM eye drops. These results show that the stimulation of FP receptors affects the production of aqueous humour via β receptors in rabbits, meaning the rabbit model is not suitable for the evaluation of anti-glaucoma eye drops with FP receptor activity, since this drug effect was not observed in humans.

Key words — rabbit, drug interaction, glaucoma, prostaglandin F receptor, β receptor

INTRODUCTION

Glaucoma is a devastating disease that causes damage to the optic nerve and loss of visual function; it is the main cause of blindness in adults.1,5 Previous studies have shown that increased intraocular pressure (IOP) is the major risk factor for glaucoma and its progression.2,3 With regard to therapy for glaucoma, topical medications are usually chosen as the primary treatment since reducing IOP is reportedly the most effective treatment approach.4,5 Monotherapy with prostaglandin F2α (FP) analogue latanoprost (LP) (Xalatan® eye drops 0.005%, Pfizer Japan Inc, Tokyo, Japan) is the most commonly prescribed medication.6 LP eye drops increase aqueous humour outflow in humans either by enhancing the pressure-sensitive (presumably trabecular or conventional) outflow pathway or by increasing pressure-insensitive (uveoscleral) outflow.7,9 Two mechanisms have been suggested for the increase in aqueous humour outflow caused by LP eye drops: (I) LP stimulates the FP receptor, and the stimulated FP receptor increases the production of extracellular matrix (ECM). The metabolism of ECM causes degradation of collagen in the uveoscleral space, resulting in increased aqueous humour outflow.
(II) LP stimulates the FP receptor in the smooth muscle of the ciliary body, causing smooth muscle relaxation and intercellular space expansion, resulting in enhanced aqueous humour outflow.\(^{(10)}\)

The rabbit has been used as an experimental model to evaluate the IOP-reducing effect of various anti-glaucoma eye drops. In some studies, the rabbit model is also used for evaluating LP eye drops.\(^{(11, 12)}\) However, the sensitivity and localization of FP receptors in the rabbit eye is different from those in humans.\(^{(11, 12)}\) Therefore, researching the relationship between IOP regulation and FP receptors in the rabbit is important when using an animal model for the evaluation of anti-glaucoma eye drops. However, there is little evidence for FP receptor-mediated IOP regulation in rabbits.

In IOP regulation, the balance between production and outflow of aqueous humour is important. \(\beta\)-receptors regulate aqueous humour production, and eye drops containing the topical \(\beta\)-blocker timolol maleate (TM) (Timoptol\textsuperscript{a} ophthalmic solution 0.5\%, Santen Pharmaceutical Co Ltd, Osaka, Japan) reduce IOP by reducing aqueous humour formation.\(^{(13)}\) TM has been used as therapy for glaucoma patients. Fixed combination therapies are also used for patients who require more than one therapy to control IOP, and LP 0.005\%/TM 0.5\% fixed combination (LTFC) eye drops (Xalacom\textsuperscript{a} combination eye drops, Pfizer Inc) have recently become available in Japan. This approach simplifies the treatment regimen and improves patient compliance considerably, and randomized clinical trials have demonstrated that LTFC eye drops instilled once daily reduce IOP more effectively than either of the two components used alone (TM or LP eye drops).\(^{(14-16)}\)

In this study, we investigated whether the stimulation of FP receptors in the rabbit eye using LP, TM, and LTFC eye drops influences the production of aqueous humour \textit{via} the \(\beta\) receptor.

**MATERIALS AND METHODS**

1. **Animals and Reagents**

   Male Japanese albino rabbits (2.5-3.0 kg) were housed under the following conditions: 25\(^\circ\)C room temperature, 7:00 am - 7:00 pm fluorescent light. The rabbits were allowed free access to food (CR-3 commercial diet, Clea Japan Inc, Tokyo) and water. All procedures were performed in accordance with the Kindai University (formerly Kinki University) Faculty of Pharmacy Committee Guidelines for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. A commercially available 0.5\% TM eye drops (Timoptol\textsuperscript{a} ophthalmic solution 0.5\%, \(\beta\)-blockers) was purchased from Santen Pharmaceutical Co Ltd (Osaka, Japan). The commercially available 0.005\% LP eye drops (Xalatan\textsuperscript{a} eye drops 0.005\%, prostaglandin) and LTFC eye drops (Xalacom\textsuperscript{a} combination eye drops 0.005\%, prostaglandin) were provided by Pfizer Japan, Inc (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

2. **Measurement of IOP in Rabbits Receiving a Rapid Infusion of Isotonic Glucose**

   The experiment was carried out according to our previous report.\(^{(17)}\) Ocular hypertension was induced by the rapid infusion of a 5\% glucose solution (isotonic glucose solution; 15 mL/kg body weight) within 20 sec into the marginal ear vein of a rabbit. Saline (control), TM eye drops, LP eye drops, or LTFC eye drops (30 \(\mu\)L) were instilled 10 min prior to the infusion of the isotonic glucose solution. For the combination of
TM eye drops and LP eye drops (30 μL), the TM eye drops was instilled 10 min prior to the infusion of the isotonic glucose solution, and the LP eye drops was instilled 5 min or 15 min prior to the infusion (when two drugs were used, they were applied 5 min apart). IOP in rabbits was measured with an electronic tonometer (Medtronic SOLAN, Jacksonville, FL, USA) under surface anesthesia (0.4% Benoxil). ΔIOP (mmHg) was analyzed as the difference in IOP between rabbits with or without the rapid infusion of the 5% glucose solution (a decrease in ΔIOP shows a high ocular hypotensive effect). The area under the curve (AUC\_\text{ΔIOP}) of ΔIOP versus time (minutes) (the area under the ΔIOP-time curve) was calculated according to the following equation (Eq 1):

\[
AUC_{\Delta IOP} = \int_0^t \Delta IOP dt
\]

where \( t \) is the time after infusion of the isotonic glucose solution. AUC\_\text{ΔIOP} was determined according to the trapezoidal rule up to the last ΔIOP measurement point.

3. In Vivo Transcorneal Penetration of Ophthalmic Preparations containing TM

The in vivo transcorneal penetration of TM from TM eye drops or LTFC eye drops was determined as described previously. Rabbits were anesthetized with isoflurane, and a topical anesthetic (0.4% Benoxil) was instilled into each eye 3 min before sampling of the aqueous humor. A 29 gauge injection needle was inserted into the eye to obtain aqueous humor samples (5 μL each), and the rabbits were left to stabilize for 30 min. Then, 30 μL of eye drops were instilled into the eyes. TM concentrations in the samples were determined by the following HPLC method. A solution (10 μL) containing propyl p-hydroxybenzoate (internal standard) was injected onto a Mightysil RP-18 (3 μm, column size: 2.0 mm × 50 mm) column (Kanto Chemical Co, Inc, Tokyo, Japan) using a Shimadzu LC-10AD system. The column temperature was maintained at 35°C by a column oven CTO-6A (Shimadzu Corp, Kyoto, Japan). The mobile phase consisted of 25 mM phosphate buffer (pH 7) containing 30% methanol and 10% acetonitrile at a flow rate of 0.2 mL/min. The wavelength for detection was 294 nm.

The area under the TM concentration-time curve (AUC\_\text{TM}) was calculated according to the following equation (Eq 2):

\[
AUC_{\text{TM}} = \int_0^t C_{\text{TM}} dt
\]

where \( t \) is a time after eye drop instillation (0-90 min), and \( C_{\text{TM}} \) is the TM concentration at time \( t \). AUC\_\text{TM} was determined according to the trapezoidal rule up to the last TM concentration measurement point.

4. Statistical Analysis

Statistical differences were evaluated by unpaired Student’s \( t \)-test and or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison. \( P \) values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

1. Changes in IOP by Combination of LP and TM in Rabbit Models with Experimentally Induced Ocular Hypertension

The rabbit model has been used for the evaluation of eye drops; however, the sensitivity and localization of FP receptors in the rabbit eye is different from those in human eyes, and there is little evidence for the effect of FP receptor stimulation on IOP regulation in the rabbit model.
Therefore, studying the relationship between IOP regulation and FP receptor activation in rabbits is important for validating the use of this model in evaluating anti-glaucoma eye drops. In this study, we investigated whether the stimulation of the FP receptor in rabbit eyes affects the production of aqueous humour via β receptor activity by using commercially available LP, TM, and LTFC eye drops (standard formulations and doses used in clinical practice were used in this study).

Intravenous administration of an isotonic glucose solution (5%) is a simple and reproducible technique to increase IOP for the screening of anti-glaucoma agents. The mechanism of IOP enhancement in the intravenous administration of an isotonic glucose solution is as follows: the infusion of isotonic glucose increases the body fluid volume, resulting in the enhancement of aqueous humour formation (positive feedback). Figure 1 shows the changes in IOP following the instillation of TM eye drops and LP eye drops in rabbits receiving a rapid infusion of isotonic glucose. A rapid elevation in IOP is induced by the rapid infusion of an isotonic glucose solution through the marginal ear vein, reaching a maximum level 5 minutes after the infusion. Although the pattern of change in IOP in rabbits instilled with saline or LP eye drops was similar, TM eye drops significantly suppressed the elevation in IOP as compared to that in rabbits instilled with saline (Fig 1). It is known that TM eye drops decrease IOP by reducing aqueous humour formation in human and rabbit eyes, and LP eye drops increase aqueous humour outflow in humans either by enhancing the pressure-sensitive (presumably trabecular or conventional) outflow pathway or by increasing pressure-insensitive (uveoscleral) outflow. However, LP-induced increase in aqueous humour outflow is not observed in the rabbit, since the sensitivity and localization of FP receptors in the rabbit eye is different from those of in humans eyes. Thus, the results of Fig 1 support the findings of previous reports. Figure 2 shows the effect of LP eye drops on the reduction in IOP in rabbits also instilled with TM eye drops;

**Fig 1** Changes in IOP in Rabbits Instilled with LP Eye Drops or TM Eye Drops. Ocular hypertension in rabbits was induced by the rapid infusion of a 5% glucose solution into the marginal ear vein. 30 μL of eye drops was instilled 10 min prior to the infusion. Saline (○), rabbits treated with saline. LP (●), rabbits treated with LP eye drops. TM (▲), rabbits treated with TM eye drops. The data are presented as the means ± SE of 8-10 independent rabbits. * 1P < 0.05, vs Saline for each category.

**Fig 2** Comparison of IOP in Rabbits Instilled with Combinations of LP Eye Drops and TM Eye Drops. Ocular hypertension in rabbits was induced by the rapid infusion of a 5% glucose solution into the marginal ear vein. 30 μL of eye drops was instilled 10 min prior to the infusion. or, when two drugs were used, they were applied 5 min apart. TM (○), rabbits treated with TM eye drops. TM before LP (●), rabbits treated with TM eye drops before the instillation of LP eye drops. TM after LP (▲), rabbits treated with TM eye drops after the instillation of LP eye drops. LTFC (◆), rabbits treated with LTFC eye drops. The data are presented as the means ± SE of 8-12 independent rabbits. * 2P < 0.05, vs TM for each category.
Table 1 shows the AUC of various eye drop preparations used in Figs 1 and 2.

The AUC for rabbits treated with TM eye drops was 81.3% of those treated with LTFC eye drops (Table 1). In addition, we measured TM concentrations in the aqueous humour after the instillation of TM eye drops or LTFC eye drops (Fig 3). The peak TM concentration was observed 40 min after the instillation of either eye drops, and the peak concentrations were similar. The AUC values for TM eye drops and LTFC eye drops were 901 ± 92 μM·min and 914 ± 87 μM·min, respectively (means ± SE, n = 5).

These results show the addition of LP to TM eye drops prior to the instillation of LP eye drops attenuates the ocular hypotensive effect of TM in rabbit eyes.

Moreover, the IOP of rabbits treated with TM eye drops after the instillation of LP eye drops was higher than that of rabbits treated with TM eye drops prior to the instillation of LP eye drops. The AUC of rabbits treated with TM eye drops before the instillation of LP eye drops was 86.2% that of rabbits treated with TM eye drops after the instillation of LP eye drops (Fig 2 and Table 1).

Randomized clinical trials in humans have demonstrated that LTFC eye drops instilled once daily reduce IOP more effectively than either of the two components used alone (TM or LP eye drops). From these previous reports and these results, it is suggested that the IOP-regulatory mechanism via FP receptors differs between human patients and rabbit models, and the rabbit may not be suitable as the model for evaluating anti-glaucoma eye drops modulating IOP via the FP receptor. On the other hand, these drug interactions between LP and TM in rabbits with experimentally induced ocular hypertension are interesting findings. It was previously known that TM decreases aqueous formation by inhibiting the β-receptor, and that LP increases trabecular and uveoscleral outflow. Taken together, based on our findings, we hypothesize that LP may decrease the sensitivity of the β-receptor to the effects of TM, and the changes in trabecular and uveoscleral outflow caused by LP may be related to the attenuation of aqueous formation by TM. However, this remains a hypothesis, and the mechanism for this effect requires examination in the future.

In the present study, we demonstrated that stimulation of FP receptors affected the production of aqueous humour via β receptor activity in the rabbit model, however this drug interaction has not been observed in humans. Although the

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**Table 1** Ocular Hypotensive Effect of Combinations of LP and TM Eye Drops

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>LP</th>
<th>TM</th>
<th>TM before LP</th>
<th>TM after LP</th>
<th>LTFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{AUC} (mmHg·min)</td>
<td>256 ± 39*2</td>
<td>243 ± 49*2</td>
<td>135 ± 28*1</td>
<td>156 ± 28*1</td>
<td>181 ± 23*1</td>
<td>166 ± 21*1</td>
</tr>
</tbody>
</table>

AUC_{AUC} values were calculated according to equation 1 (see MATERIALS AND METHODS). The data are presented as means ± SE of 8-12 independent rabbits. *1 P < 0.05, vs Saline. *2 P < 0.05, vs TM.
rabbit model is used in the evaluation of LP eye drops,\textsuperscript{11, 12} the rabbit may not be suitable as the model for evaluating anti-glaucoma eye drops with IOP regulation via FP receptor activity. These findings provide significant information that can be used to select animal models for future development and evaluation of anti-glaucoma eye drops.

**Conflict of Interest**

The authors declare no conflict of interest.

**References**