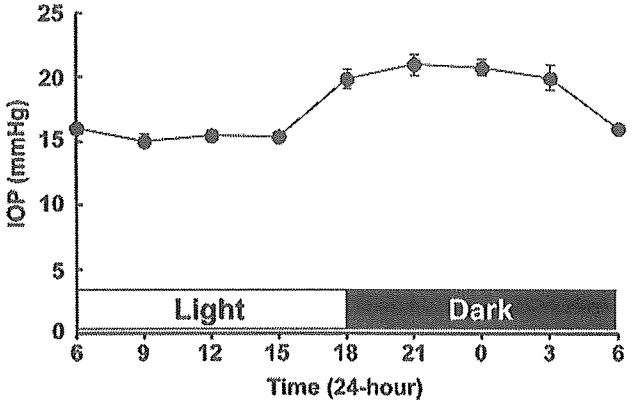

FIGURE 1. The 24-hour IOP pattern in ddY mice. The difference between the peak and trough IOP was significant ($P < 0.001$). The data are the mean mm Hg ± SEM ($n = 6$).

eration, and to evaluate the ocular hypotensive effect of a newly developed FP agonist, tafluprost, in comparison with three other clinically used PG analogues.

MATERIALS AND METHODS

Animals

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male ddY inbred mice, the most widely used strain in Japan, were obtained at 6 or 7 weeks after birth from Saitama Jikken (Saitama, Japan). All mice were housed in clear cages covered with air filters and containing white chips for bedding. The environment was kept at 21°C with a 12-hour light-dark cycle (on at 0600; off at 1800). All mice were fed ad libitum. The animals were acclimatized to the environment for at least 2 weeks before experiments.

Preparation and Application of Ophthalmic Solution

Latanoprost was purchased from Cayman Chemical Co. (Ann Arbor, MI) and dissolved in its vehicle solution, as reported previously.[14] Travoprost, isopropyl unoprostone, tafluprost, and their vehicle solutions were provided by Alcon Inc. (Fort Worth, TX), R-Tech Ueno Ltd. (Hyogo, Japan), and Santen Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. The solutions were originally prepared in their clinically used concentrations, and then serial dilutions were prepared with their corresponding vehicle solutions. With a micropipette, 3 μL of PG solution or vehicle was topically applied to one randomly selected eye in a masked manner. The untreated contralateral eye served as the control.

IOP Measurement

IOP was measured by a microneedle method in mice anesthetized by ketamine and xylazine, as described previously.[11] Briefly, a microneedle made of borosilicate glass (100-μm tip diameter) was connected to a pressure transducer (Model BLPR; World Precision Instruments, Sarasota, FL). The system pressure detected by the transducer was recorded by a data-acquisition and analysis system (PowerLab; ADInstruments, Colorado Springs, CO). In mice under general anesthesia, the microneedle was placed in the anterior chamber, and the conducted pressure was sequentially recorded in both eyes during a 4- to 7-minute time window. The effect of each drug and vehicle was calculated as the difference in IOP (ΔIOP = IOP in the treated eye − IOP in the untreated contralateral eye). The percentage of IOP reduction was defined as 100 × ΔIOP/IOP in the untreated contralateral eye (%) in each mouse. In addition, the data thus obtained underwent area-under-the-curve (AUC; percent per hour) analysis, according to the trapezoidal rule.

Assessment of 24-Hour Variation and Difference in IOP between the Eyes of the ddY Mouse

The 24-hour IOP pattern was assessed by collecting IOP measurements at 3-hour intervals, starting from 0600, as reported previously ($n = 6$).[15] One of two eyes in each mouse was selected randomly. During the IOP measurement, room lighting similar to that in the vivaria was maintained. During the dark phase, all procedures were performed under red-light illumination to eliminate the effect of lighting on IOP. For each mouse, data points were collected at 1-week intervals to eliminate any effects of the previous IOP measurement. The measurements at 0600 were obtained after turning the light on and those at 1800 were obtained before turning the light off.

Because ocular hypotensive effects were evaluated by comparison of IOP between treated and untreated contralateral eyes, the similarity of IOP in both eyes was validated in ddY mice. In mice under general anesthesia,[11] the IOP in both eyes was sequentially measured in the time window just described, and the bilateral difference was compared.

Effect of Latanoprost on Daytime and Nighttime IOP

First, to determine the period feasible for demonstrating a greater degree of ocular hypotensive effect in consideration of the 24-hour IOP variation,[15] we applied 0.005% latanoprost at 0700 or 1900, and measured IOP as described, 2 hours after administration.

Effects of PG Analogues on Mouse IOP

Tafluprost (0.0003%, 0.0015%, 0.005%, or 0.015%), latanoprost (0.001%, 0.0025%, or 0.005%), travoprost (0.001%, 0.002%, or 0.004%), isopropyl unoprostone (0.03%, 0.06%, or 0.12%), or each vehicle solution was administrated topically to one randomly chosen eye. Three investigators instilled eye drops, each without knowing what the other two were doing, and the fourth investigator, masked to the treatments, measured IOP at 1, 2, 3, 6, 9, or 12 hours after the drugs were administered, as described earlier. Thus, all measurements were performed under masked conditions. The ocular hypotensive effect of each drug was calculated, and its dose-dependency was evaluated by AUC analysis. Finally, IOP reduction by 0.005% tafluprost was compared with that produced by clinically used concentrations of the other drugs—0.005% latanoprost, 0.004% travoprost, and 0.12% unoprostone at each time point—and also by AUC analysis. Because of this invasive measurement, IOP at each time point was measured in different animals. In some mice, the measurements were repeated at 2-week intervals.

Statistical Analysis

All IOP data are shown as the mean ± SEM. The Kruskal-Wallis test was used for comparison of the 24-hour IOP variation. The Mann-Whitney test was used for comparison of the mean IOP difference and the ratio of IOP reduction between day and night. The Wilcoxon signed-ranks test was used for comparison of mean IOP between treated and contralateral eyes. The Steel test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

TABLE 1. IOP of the Right and Left Eyes in ddY Mice

	0900 h ($n = 13$)	2100 h ($n = 12$)
Right eye	14.4 ± 0.4	21.0 ± 0.7
Left eye	14.2 ± 0.4	21.1 ± 0.5

Data are shown as the mean IOP (mm Hg) ± SEM. There was no significant difference between the right and left eyes.

TABLE 2. Effect of Latanoprost (0.005%) on IOP during the Day and at Night

	n	Contralateral Eye	Treated Eye	Difference (Treated Eye) − (Contralateral Eye)	% Reduction
Day	8	13.3 ± 0.7	12.3 ± 0.7*	−1.0 ± 0.3	7.1 ± 2.3
Night	6	22.0 ± 1.8	17.1 ± 0.8**	−4.9 ± 1.1†	21.4 ± 2.8††

Data are expressed as the mean IOP (mm Hg) ± SEM.
* and **, $P = 0.017$ and 0.028 versus the contralateral eye, respectively. (Wilcoxon signed rank test).
† and ††, $P = 0.002$ and 0.007 versus the day, respectively. (Mann-Whitney test).

RESULTS

IOP of ddY Mouse

Twenty-four-hour IOP Variation in ddY Mouse. The mean IOP of mice bred in the 12-hour light–dark cycle showed a 24-hour variation (Kruskal-Wallis test, $P < 0.001$), in which IOP was lower in the day and higher at night (Fig. 1). The peak IOP measurement was 21.0 ± 0.8 mm Hg observed at 2100 and the trough IOP measurement was 15.0 ± 0.5 mm Hg at 0900 ($n = 6$). There was a significant difference between the peak and trough IOPs ($P < 0.001$).

Difference between Bilateral IOP in ddY Mice. The IOPs of ddY mouse eyes were measured at 0900 and 2100, as shown in Table 1. The mean IOP in the right eyes was 14.4 ± 0.4 mm Hg and in the left eye was 14.2 ± 0.4 mm Hg at 0900 ($n = 13$). At 2100 ($n = 12$), the pressures were 21.0 ± 0.7 and 21.1 ± 0.5 mm Hg in the right and left eye mean, respectively. There was no significant difference in IOP between the two eyes.

Data are reported as the mean ± SEM. There was no significant difference between the right and left eyes.

Effect of Latanoprost on Diurnal IOP

To evaluate the effect of latanoprost on the IOP during the day and night, we measured the IOP in normal ddY mice treated with 0.005% latanoprost at 0900 or 2100, based on the 24-hour pattern of mouse IOP (Fig. 1). During the day, the IOP of the untreated contralateral and treated eyes 2 hours after drug administration was 13.3 ± 0.7 and 12.3 ± 0.7 mm Hg, respectively, whereas at night, the IOP in the untreated contralateral and treated eyes 2 hours after administration was 22.0 ± 1.8 and 17.1 ± 0.8 mm Hg, respectively. IOP reduction by 0.005% latanoprost was 1.0 mm Hg (7.1%) during the day and 4.9 mm Hg (21.4%) at night, and the latter was statistically significant ($P = 0.002$). Further, the change (Δ) and the percent IOP reduction at night were significantly greater than those during the day ($P = 0.017$ and 0.028, respectively, Table 2). These results indicate that the effect of latanoprost on IOP is more pronounced at night when baseline IOP is higher. Hence, in the following experiments, we examined IOP reduction by PG analogues at night.

Dose–Response and Time Course of IOP Reduction by Four PG Analogues

Tafluprost. The maximum IOP reductions in the tafluprost (0.0003%, 0.0015%, 0.005%, and 0.015%)-treated groups were observed at 2 (−3.5 ± 0.7 mm Hg, 15.7% ± 2.6%), 3 (−4.0 ± 0.5 mm Hg, 17.6% ± 1.7%), 2 (−4.8 ± 0.7 mm Hg, 20.2% ± 2.0%), and 3 (−5.5 ± 1.0 mm Hg, 24.3% ± 3.3%) hours after drug administration, respectively (Fig. 2A). At 1, 2, 3, 6, and 9 hours, 0.0015%, 0.005%, and 0.015% tafluprost significantly reduced IOP compared with its effect in the vehicle-treated eye. AUC analysis of tafluprost showed a dose-dependent effect on IOP reduction, which saturated at 0.005% (Fig. 2B).

Latanoprost. The maximum IOP reductions in the latanoprost (0.001%, 0.0025%, and 0.005%)-treated groups were observed at 1 (−1.8 ± 0.3 mm Hg, 8.3% ± 1.4%), 2 (−3.1 ± 0.7 mm Hg, 15.9% ± 3.1%), and 2 (−3.9 ± 0.6 mm Hg, 18.7% ± 2.5%) hours, respectively, after drug administration (Fig. 3A). At 1, 2, and 3 hours after administration, 0.005% and 0.0025% of latanoprost significantly reduced IOP compared with that in the vehicle-treated eye. AUC analysis of the latanoprost-treated groups showed a dose-dependent effect on IOP reduction, which saturated at 0.0025% (Fig. 3B).

Travoprost. The maximum IOP reductions in the travoprost (0.001%, 0.002%, and 0.004%)-treated groups were observed at 3 (−2.9 ± 1.1 mm Hg, 12.0% ± 4.7%), 2 (−2.3 ± 0.5 mm Hg, 9.8% ± 2.2%), and 6 (−3.8 ± 0.8 mm Hg, 20.8% ± 4.6%) hours after drug administration (Fig. 4A). At 2, 3, and 6 hours, 0.004% travoprost significantly reduced IOP, when compared with its effect in the vehicle-treated eye. AUC analysis of travoprost treated groups showed a dose-dependent reduction of IOP (Fig. 4B).

Isopropyl Unoprostone. Isopropyl unoprostone had the weakest effect on IOP among the four PG analogues (Fig. 5A). The maximum IOP reduction (−2.0 ± 0.3 mm Hg, 11.2% ± 1.8%) was observed at 2 hours after the administration of 0.12% isopropyl unoprostone (Fig. 5B). At 2 hours, 0.12% unopros-

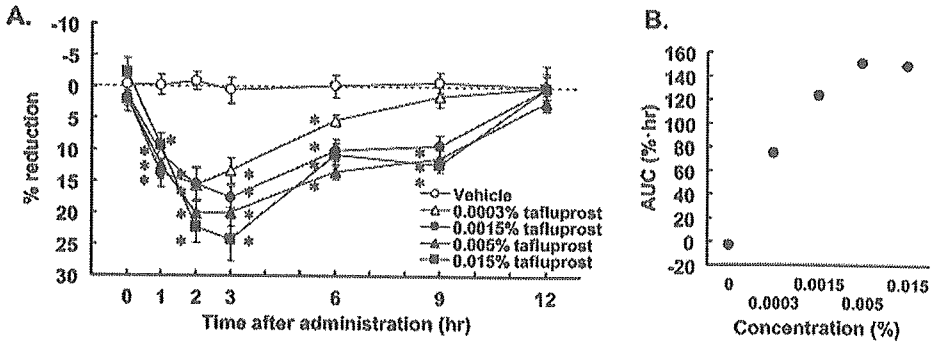

FIGURE 2. Dose-dependent IOP reduction by tafluprost in ddY mice. (A) Effect of tafluprost (0.0003%, 0.0015%, 0.005%, or 0.015%) on mouse IOP. Eye drops were instilled at 1800. The data indicate the percentage reduction in IOP due to treatment and are expressed as the mean ± SEM (n = 7–10 per time point). *$P < 0.05$ for the drug-treated versus vehicle-treated eyes, by the Steel test. (B) The ocular hypotensive effect of tafluprost, shown as the AUC (percent per hour).

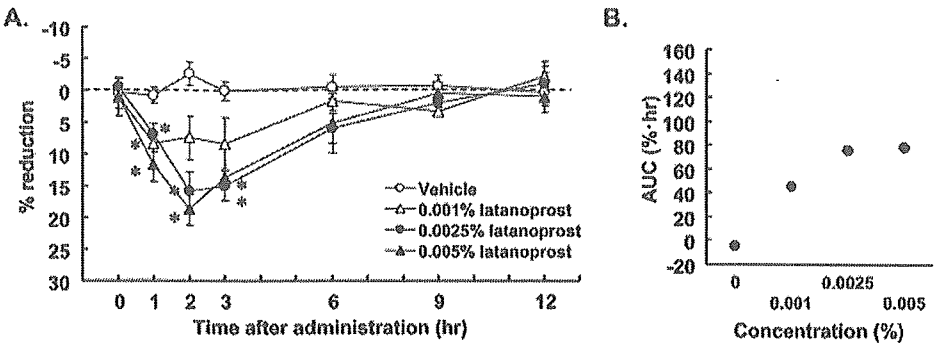

FIGURE 3. Dose-dependent IOP reduction by latanoprost in ddY mice. (A) Effect of latanoprost (0.001%, 0.0025%, and 0.005%) on mouse IOP. Eye drops were instilled at 1800. The data indicate the percent reduction in IOP due to treatment and are expressed as the mean ± SEM (n = 7–9 per time point). *P < 0.05 for the drug-treated versus vehicle-treated eyes, by the Steel test. (B) The ocular hypotensive effect of latanoprost, shown as the AUC (percent per hour).

tone significantly reduced IOP compared with its effect in the vehicle-treated eye. AUC analysis of the unoprostone-treated groups showed a dose-dependent IOP reduction, which saturated at 0.06% (Fig. 5B).

Comparison of the IOP Reduction Induced by Three PG Analogues with That Induced by Tafluprost

The baseline IOP (at 1800) in the untreated contralateral eyes in the tafluprost-, latanoprost-, travoprost-, and isopropyl unoprostone-treated groups showed no significant difference among the groups. Tafluprost 0.005% decreased IOP more than 0.005% latanoprost at 3, 6, and 9 hours (P = 0.001–0.027) or 0.12% unoprostone at 2, 3, and 6 hours (P = 0.0004–0.01). There was no significant difference between 0.005% tafluprost and 0.004% travoprost.

Among the four PGs, tafluprost showed the largest AUC and isopropyl unoprostone, the smallest (Fig. 6B).

DISCUSSION

A new FP agonist, tafluprost, and the clinically used PG analogues (except for bimatoprost)—latanoprost, travoprost, and unoprostone—significantly reduced IOP in a dose-dependent manner in ddY mouse eyes. These findings suggest mouse eyes are well suited for the pharmacological evaluation of PG analogues. Moreover, IOP reduction by these four PG analogues in mouse eyes was almost comparable to that in human eyes, as was their affinity for the FP receptor.[3,16,17]

The 24-hour variation in mouse eyes should be taken into consideration when evaluating the pressure. The IOP during the day and night varies among the many mouse strains.[18] In this study, the ddY strain showed a 24-hour pattern of IOP variation similar to that of NIH Swiss white, as previously reported.[15] These data, as well as the data from our study, demonstrate that mouse IOP is higher at night and lower in the day. Thus, to determine whether drug-induced ocular hypotension was affected by the diurnal variation of IOP, latanoprost was administered at 0700 or 1900. The results revealed a more pronounced reduction in IOP at night (21.4%) than during the day (7.1%). This finding may be compatible with those of a previous study using NIH Swiss white mice, which also showed a lesser reduction (14%) in IOP during the day than that at night (21.4%).[11,13] It is not clear whether this greater IOP reduction by latanoprost at night is simply due to circadian differences in baseline IOP or to the parameters of aqueous dynamics. In other mammals, including humans, a diurnal difference in the conventional outflow facility or aqueous production has been reported,[19,20] whereas the diurnal variation of uveoscleral outflow has not. In humans, higher baseline IOP is associated with greater IOP reduction by latanoprost,[21] which may also apply to latanoprost's effect on mouse IOP.

The PG-analogue-induced IOP reductions demonstrated in the current mouse study deserve discussion. The PG analogues currently examined, excluding unoprostone, are mainly thought to enhance uveoscleral outflow via interaction with the FP receptor.[3,10] IOP reduction by FP agonists in mouse eyes is compatible with the fact that the mouse outflow pathway is mainly dependent on uveoscleral outflow, as shown in young monkey eyes in aqueous outflow studies.[12,22] PGF2α analogues have very little effect on ocular hypotension in rabbits and cats.[23,24] Our data indicate that mouse eyes are more comparable to human eyes for the pharmacological study of drugs that enhance the uveoscleral outflow pathway.

In this study, the clinically used concentration of each drug was also tested (Fig. 6). Because the main purpose was to evaluate the usefulness of ddY mouse eyes for the pharmacological evaluation of PG analogues and a new PG analogue, tafluprost, direct comparison among all four drugs at their clinical concentrations was not a primary measure. However, the more potent FP agonist tended to reduce IOP more, almost paralleling the results obtained in humans.[17] Tafluprost is a recently developed PG derivative that has a 10-fold higher affinity for the FP receptor than does latanoprost acid.[1,2] By

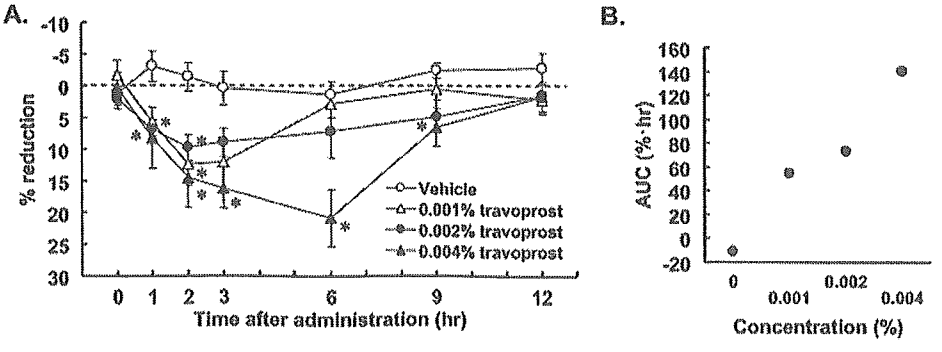

FIGURE 4. Dose-dependent IOP reduction by travoprost in ddY mice. (A) Effect of travoprost (0.001%, 0.002%, or 0.004%) on mouse IOP. Eye drops were instilled at 1800. The data indicate the percent reduction in IOP due to treatment and are expressed as the mean ± SEM (n = 6–9 per time point). *P < 0.05 for the drug-treated versus vehicle-treated eye, by the Steel test. (B) The ocular hypotensive effect of travoprost, shown as the AUC (percent per hour).

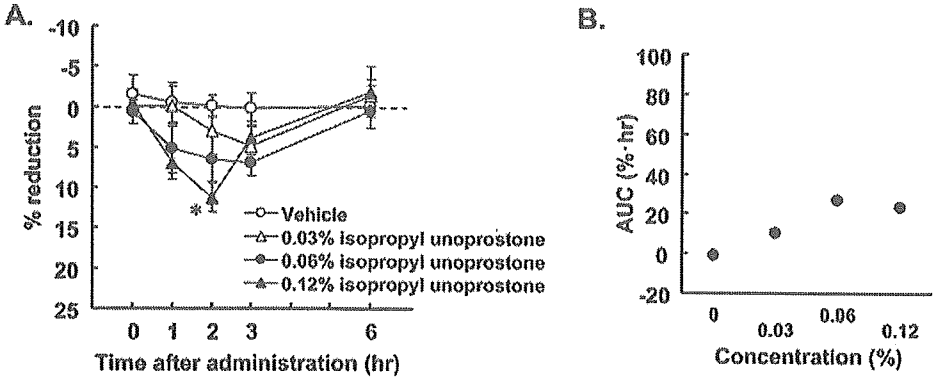

FIGURE 5. Dose-dependent IOP reduction by isopropyl unoprostone in ddY mice. (A) Effect of isopropyl unoprostone (0.03%, 0.06%, or 0.12%) on mouse IOP. Eye drops were instilled at 1800. The data indicate the percent reduction in IOP due to treatment and are expressed as the mean ± SEM (n = 9–17 per time point). *$P < 0.05$ for the drug-treated versus vehicle-treated eyes, by the Steel test. (B) The ocular hypotensive effect of unoprostone, shown as the AUC (percent per hour).

AUC analysis, tafluprost was more effective in reducing mouse IOP, and its ocular hypotensive effect lasted longer than that of latanoprost (Fig. 2). In monkey eyes, treatment with 0.0025% or 0.005% tafluprost once daily lowered IOP more effectively than latanoprost, by increasing uveoscleral outflow.[2] Moreover, tafluprost lowered the trough IOP more than latanoprost after continuous use.[1,2] These results may indicate the potential of tafluprost as a new ocular hypotensive agent in the future.

Travoprost (0.004%) showed similar ocular hypotensive potency to tafluprost in mouse eyes. Travoprost acid also has a high affinity for the FP receptor.[3] This property of travoprost may result in a longer duration of reduction of IOP than that achieved with latanoprost. In addition, the current results are consistent with the fact that IOP reduction by travoprost was greater than that by latanoprost after 2 weeks of treatment in humans.[25]

The maximum IOP reduction by isopropyl unoprostone (0.12%) in mouse eyes was approximately 50% of that with latanoprost (0.005%). This observation is consistent with previous reports in humans that unoprostone has less effect on ocular hypotension than does latanoprost.[26,27] The receptor-related mechanism of action of unoprostone has not been clarified, since this drug shows a low affinity for all prostanoid receptors.[3,7] However, there are several hypotheses to explain unoprostone-induced IOP reduction. Unoprostone free acid and further metabolites have been reported to stimulate the release of PGE2, which may play a role in IOP reduction by unoprostone.[28,29] Unoprostone free acid activates maxi-K channels to inhibit trabecular meshwork contraction, which can lead to increases in aqueous outflow.[30] Unoprostone can induce cellular responses similar to other FP agonists in cultured ciliary muscle and trabecular meshwork cells, probably by acting as a weak FP agonist.[3,16,31,32] Thus, the use of various prostanoid receptor knockout mice and the mouse IOP model established in the present study should be very useful in further studying the mechanism of IOP reduction by unoprostone.

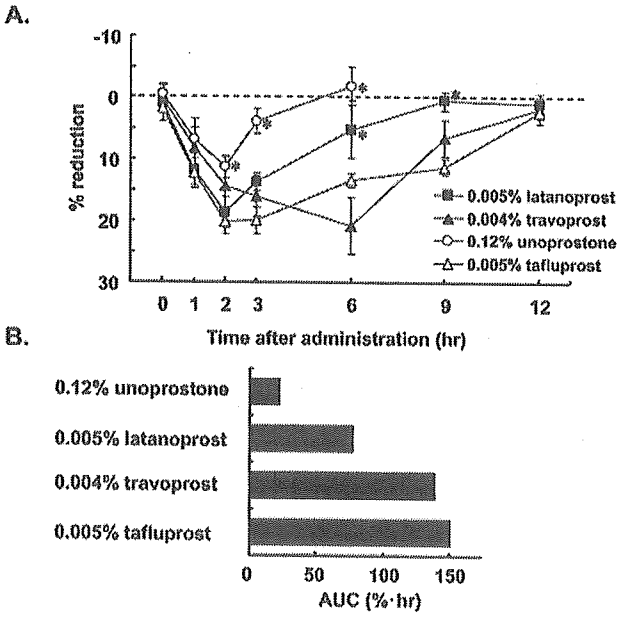

FIGURE 6. Effects of treatment with prostaglandin analogues on ddY mouse IOP. (A) Long-term effects of tafluprost (0.005%), latanoprost (0.005%), travoprost (0.004%), and isopropyl unoprostone (0.12%). Eye drops were instilled at 1800. The data indicate the percent reduction in IOP due to treatment and are expressed as the mean ± SEM (n = 6–15 per time point). *$P < 0.0167$ versus the tafluprost-treated group, by the Steel test. (B) The comparative ocular hypotensive effects of the four drugs are indicated by the AUC (percent per hour).

In conclusion, our study examined the effects of four different PG analogues on IOP in the mouse eye at night, taking 24-hour IOP variation into consideration, and clearly demonstrated the presence of dose-dependent responses in ocular hypotension to the four PG analogues. The ocular hypotensive effects of the four PG analogues in mouse eyes are comparable to those reported in humans,[17] correlating with their affinity for the FP receptor. IOP measurements using knockout mice lacking various prostanoid receptors or other receptors related to control of ocular hypotension, should be useful in investigating the mechanism of the ocular hypotensive effect of various antiglaucoma drugs. The mouse IOP model is thought to be a potential tool for basic research in the field of glaucoma.

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Neurotoxic effects of trypan blue on rat retinal ganglion cells

Yan Jin[a,b], Saiko Uchida[a], Yasuo Yanagi[a], Makoto Aihara[a,*], Makoto Araie[a]

[a]*Department of Ophthalmology, University of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*
[b]*Department of Ophthalmology, The second hospital, Jilin University, 218 fiqiang street, Changchun 130041, China*

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Abstract

To facilitate the peeling of internal limiting membrane or epiretinal membrane in vitreoretinal surgery, trypan blue (TB) and indocyanine green (ICG) have been used. However, the cytotoxicity of these dyes have been concerned. The aim of this study was to investigate the neurotoxic effects of TB on rat retinal ganglion cells (RGCs) and compare the effect of TB with ICG. Rat RGCs were purified by a two-step immunopanning procedure. In short-time exposure experiments, purified RGCs cultured for 3 days were exposed to 600 mg L^{-1} TB, 1500 mg L^{-1} TB and 1500 mg L^{-1} ICG for 10 sec to 30 min. The number of viable RGCs was counted after 12 hr in culture. In long-time exposure experiments, purified RGCs were cultured for 3 days in TB solutions ranging from 2 to 800 mg L^{-1} or in ICG solutions at concentrations from 2 to 250 mg L^{-1}. Then the number of viable cells was counted. Exposure to 600 mg L^{-1} TB, 1500 mg L^{-1} TB and 1500 mg L^{-1} ICG for short time from 10 sec to 30 min caused a time-dependent damage to RGCs. There was no significant difference in cytotoxicity to RGCs between TB and ICG in short-time exposure. In long-time exposure, TB as well as ICG showed neurotoxic effect on RGCs in a dose-dependent manner. Fifty percentage inhibitory concentration (IC50) of TB to RGCs was calculated as 115 mg L^{-1}, while that of ICG was 33 mg L^{-1}. In conclusion, TB induced neurotoxic effect on RGCs in a dose- and time-dependent manner. During the short-time exposure in surgery, there is likely no difference in neurotoxic effect on RGCs between TB and ICG. However, once the dyes are left in the eyes after surgery, TB may show less toxicity on RGCs than ICG.

Keywords: retinal ganglion cells; trypan blue; indocyanine green; vitrectomy

1. Introduction

In the past several years, the cytotoxicity of the dyes used in vitreoretinal surgery to facilitate the peeling of internal limiting membrane (ILM) or epiretinal membrane (ERM) has been concerned (Li et al., 2003; Stalmans et al., 2003a,b; Ashikari et al., 2003; Machida et al., 2003; Iriyama et al., 2004). Indocyanine green (ICG) is used to stain ILM and facilitate its removal (Kadonosono et al., 2000; Burk et al., 2000; Kwok et al., 2001). Although several clinical studies demonstrated that good visual function can be attained after ICG-assisted vitrectomy (Da et al., 2001; Weinberger et al., 2002; Wolf et al., 2003; Kwok et al., 2003), other studies reported that some of the patients treated with ICG in vitrectomy had visual field defects (Haritoglou et al., 2002; Uemura et al., 2003; Gass et al., 2003). In experimental studies, it has been reported that ICG caused cytotoxity to cultured human retinal pigment epithelium (RPE) cells in a dose- and time-dependent manner (Ho et al., 2003; Yam et al., 2003). ICG also showed a toxic effect on retinal ganglion cells (RGCs) in a dose-dependent manner after long time exposure both in vitro and in vivo (Iriyama et al., 2004).

Recently trypan blue (TB) has been introduced to intraocular surgery. TB at concentrations of 0·06% (600 mg L^{-1}) or 0·15% (1500 mg L^{-1}) has been proved to be efficient to facilitate the removal of ILM and ERM in vitrectomy (Feron et al., 2002; Li et al., 2003; Stalmans et al., 2003a,b). Animal study showed no histological abnormalities 1 month after intravitreal injection of 0·06% TB, although higher concentration (0·2%) was found to be toxic to photoreceptors (Veckeneer et al.,

* Corresponding author. Address: Makoto Aihara, Department of Ophthalmology, University of Tokyo School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 8660; fax: +81 3 3817 0798.
E-mail address: aihara-tky@umin.ac.jp (M. Aihara).

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2001). In vitro, it has been reported that exposure of 0·3% TB for 5 min had no toxic effect on cultured human RPE cells (Stalmans et al., 2003a,b). TB used in clinical doses has not shown toxic effects on cultured RPE cells and glial cells (Jackson et al., 2004). These studies support that TB is a safe and efficient dye for staining in surgery (Feron et al., 2002; Li et al., 2003; Stalmans et al., 2003a, b). However, the effect of TB on RGCs has not been clarified. Because RGCs are likely to be more susceptible to ICG than RPE cells in vitro (Iriyama et al., 2004), TB may induce RGCs damage at concentrations that inducing no RPE cells damage. In this study, we thus investigated the effect of TB on purified rat RGCs and compared it with the effect of ICG.

2. Materials and methods

2.1. Preparation of TB and ICG solutions

For short-time exposure experiments, 30 mg TB (Wako, MW=960·81, Wako pure chemical industries, Osaka, Japan) was dissolved with 3 ml B27 complete medium (neurobasal medium (Invitrogen) with 1 mM L-glutamine (Sigma), B27 supplement (Invitrogen), 40 ng ml^{-1} human recombinant BDNF (Sigma), 40 ng ml^{-1} rat recombinant CNTF (Sigma), 10 μM forskolin (Sigma), 10 μg ml^{-1} gentamicin) and then further diluted with B27 complete medium to give TB solutions of 600 and 1500 mg L^{-1}. These concentrations were chosen on the basis of previous clinical studies (Feron et al., 2002; Li et al., 2003; Stalmans et al., 2003a,b). ICG solution of 10 000 mg L^{-1} was first prepared by dissolving 25 mg ICG (Diagnogreen; MW= 774·96, Daiich pharmaceutical, Tokyo, Japan) with 2·5 ml B27 complete medium. Then 1500 mg L^{-1} ICG solution was obtained by further dilution with B27 complete medium.

For long-time exposure experiments, 50 mg TB was dissolved with 2 ml distilled water, which was further diluted with distilled water to give TB solutions with the concentration of 0·2, 1·0, 2·5, 5, 10, 20, 25×10^3 mg L^{-1}. These solutions were further diluted with B27 complete medium to final concentrations of 2, 10, 25, 50, 100, 200, 250, 400, 800 mg L^{-1}. For ICG solutions, 25 mg ICG was dissolved with 1 ml of distilled water, which was further diluted with distilled water to obtain ICG solutions with concentrations of 200, 1000, 2500, 5000, 10 000, 250 000 mg L^{-1}. These solutions were further diluted with B27 complete medium to final solutions of 2, 10, 25, 50, 100, 250 mg L^{-1}. Considering the possible relationship with the cell viability (Iriyama et al., 2004), pH and osmolarity of the TB and ICG solutions used in the study were measured and shown in Table 1.

2.2. Purification and culture of RGCs

RGCs were purified by a two-step immunopanning procedure as described previously (Iriyama et al., 2004; Uchida et al., 2003). The dissociated cells of retinas from 8-day-old Wister rats were incubated in flasks (Nunc A/S, Roskilde, Denmark) coated with anti-rat macrophage monoclonal antibody (1:50; Chemicon, Temecula, CA) to exclude macrophages, and then incubated in tubes (Corning, Acton, MA) coated with anti-rat Thy1·1 monoclonal antibody (1:300; Chemicon). Adherent RGCs on tubes were collected by centrifugation at 600 rpm for 5 min, and seeded on poly-L-lysine and laminin coated glass coverslips at a density of 1000 cells/well. The purified RGCs were cultured in B27 complete medium. The cultures were

Table 1
pH and osmolarity of TB and ICG solutions

Short-time exposure experiments (TB and ICG)										
	TB							ICG		
Concentration (mg L^{-1})	600				1500			1500		
pH	7·35				7·31			7·40		
Osmolarity (mOsm)	233				238			228		
Long-time exposure experiments (TB)										
Concentration (mg L^{-1})	0	2	10	25	50	100	200	250	400	800
pH	7·37	7·34	7·34	7·35	7·34	7·33	7·33	7·32	7·35	7·30
Osmolarity (mOsm)	230	226	225	225	225	226	227	225	227	222
Long-time exposure experiments (ICG)										
Concentration (mg L^{-1})	2		10		25		50		100	250
pH	7·38		7·36		7·35		7·34		7·33	7·34
Osmolarity (mOsm)	219		214		224		225		227	226

For long-time exposure, TB was diluted with distilled water, which was further diluted with B27 complete medium. For short-time exposure, TB and ICG were diluted with B27 complete medium.

incubated in a humid chamber at 37°C with 5% CO_2 balanced with air.

2.3. TB and ICG exposure experiments

For short-time exposure experiments, purified RGCs cultured for 3 days were exposed to 600 mg L^{-1} TB, 1500 mg L^{-1} TB and 1500 mg L^{-1} ICG diluted in B27 complete medium for 10 sec, 2 min, 5 min, 15 min and 30 min. As a control, purified RGCs were exposed to B27 complete medium with identical procedure. Thereafter, the cells were washed in phosphate-buffered saline (PBS) for 1 time and then cultured in B27 complete medium for 12 hr. After that, the viable cells were stained by 1 μM calcein-AM (Molecular Probes, Eugene, OR) and all surviving RGCs on each glass coverslip were counted at 200× magnification under an inverted fluorescence microscope. A surviving RGC was defined as a cell with calcein-AM-stained cell body and a neurite outgrowth of at least 2 cell diameters from the cell body. The number of RGCs after 3 days culture in B27 complete medium was set at 100%. The fluorescent images of the stained RGCs were captured by a laser scanning microscope (Fluoview; Olympus, Tokyo, Japan).

In long-time exposure experiments, purified RGCs were cultured for 3 days in 400 μl of TB solutions at concentrations from 2 to 800 mg L^{-1} or ICG solutions ranging from 2 to 250 mg L^{-1}. As control, RGCs were cultured in 400 μl of B27 complete medium for 3 days. Then the number of viable RGCs was counted by aforementioned methods.

2.4. Statistics

Mann–Whitney's *U*-test with Bonferroni correction was used to compare the number of RGCs in short-time exposure experiments. Values of $P<0{\cdot}05$ were considered statistically significant.

3. Results

3.1. Short-time exposure experiments

After short time exposure, 600 mg L^{-1} TB, 1500 mg L^{-1} TB and 1500 mg L^{-1} ICG showed a time-dependent toxicity to RGCs (Fig. 1). When RGCs were exposed to 600 mg L^{-1} TB, 1500 mg L^{-1} TB or 1500 mg L^{-1} ICG for 2 min or longer time, the percentage of viable cells was reduced significantly. There was no significant difference in neurotoxicity among these 3 groups ($P>0{\cdot}05$).

3.2. Long-time exposure experiments

After long-time exposure, TB showed toxic effect on purified RGCs in a dose-dependent manner (Fig. 2).

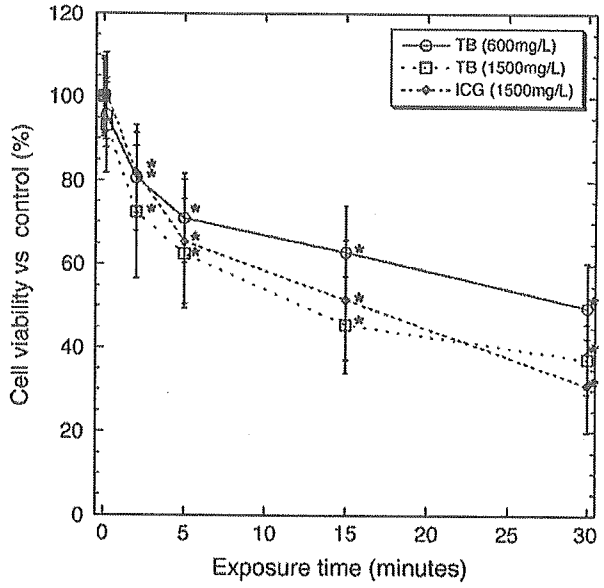

Fig. 1. Effect of TB and ICG on RGCs in short-time exposure experiments. Percentage of viable RGCs were enumerated after exposure to 600 mg L^{-1} TB, 1500 mg L^{-1} TB and 1500 mg L^{-1} ICG for 10 sec, 2 min, 5 min, 15 min and 30 min followed by one time wash in PBS. The number of viable RGCs in control was set as 100%. Data represent the mean ± SD ($n=6$). Short-time exposure to TB or ICG induced a toxic effect on RGCs in a time-dependent manner. Two minutes and longer time exposure to TB or ICG significantly reduced the number of RGCs. There were no difference among different dyes groups ($P>0{\cdot}05$). *, Bonferroni corrected $P<0{\cdot}05$ with Mann–Whitney's *U*-test, compared with control group.

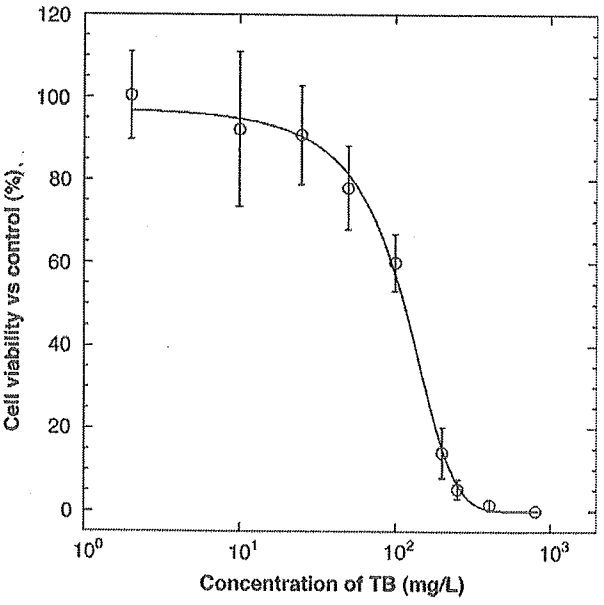

Fig. 2. Effect of TB on RGCs in long-time exposure experiments. RGCs were cultured in B27 complete medium containing TB at concentrations of 2, 10, 25, 50, 100, 200, 250, 400, 800 mg L^{-1} for 3 days. Viable RGCs were counted after culture. The number of RGCs cultured in B27 complete medium was set as 100%. Data represent the mean ± SD ($n=6$). A long-time exposure to TB induced toxic effect on RGCs in a dose-dependent manner.

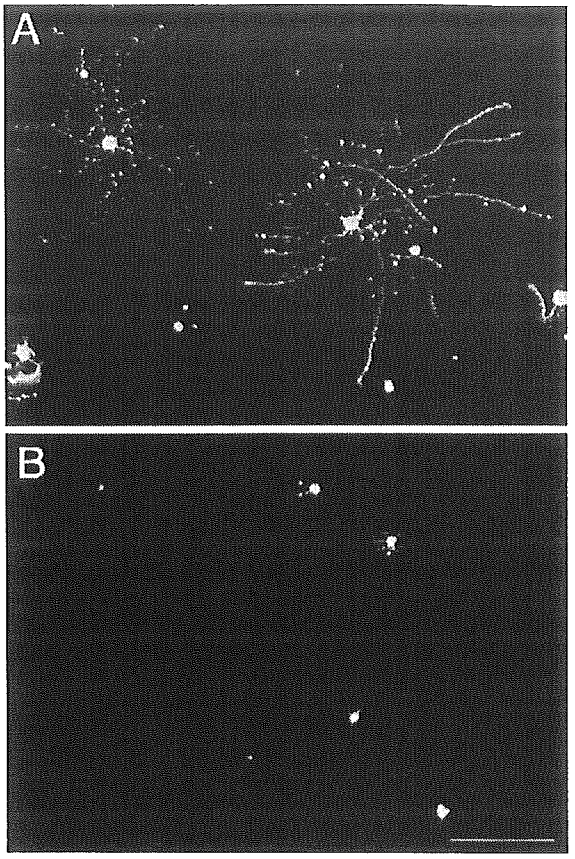

Fig. 3. Fluorescence images of RGCs cultured for 3 days. The cell bodies and neurites of RGCs were stained by calcein-AM. (A) RGCs in B27 complete medium extend long neurites. (B) RGCs in B27 complete medium containing 200 mg L^{-1} TB showed shorter and less neurites. Scale bar, 200 μm.

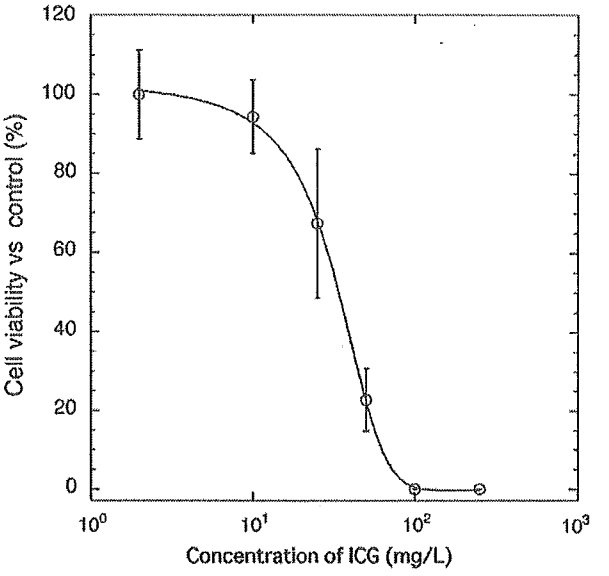

Fig. 4. Effect of ICG on RGCs in long-time exposure experiments. RGCs were cultured in B27 complete medium containing ICG at concentrations of 2, 10, 25, 50, 100, 250 mg L^{-1} for 3 days. Viable RGCs were counted after culture. The number of RGCs cultured in B27 complete medium was set as 100%. Data represent the mean ± SD ($n=6$). A long-time exposure to ICG induced toxic effect on RGCs in a dose-dependent manner.

Significant effect of TB was observed at a concentration of 100 mg L^{-1} and more evident at concentrations from 200 to 800 mg L^{-1}. RGCs in control group extended long neurites (Fig. 3A). At concentration of 200 mg L^{-1}, TB reduced the cell number significantly and RGCs showed shorter and less neurites (Fig. 3B). IC50 of TB to RGCs was calculated as 115 mg L^{-1} (120 μM).

ICG also caused a dose-dependent damage to RGCs (Fig. 4). After long time exposure, significant effect of ICG was observed in 25 mg L^{-1} solution and more evident in 50 mg L^{-1} solution (Fig. 4). When the dose of ICG solutions was higher than 100 mg L^{-1}, there were no alive RGCs. IC50 of ICG to RGCs was calculated as 33 mg L^{-1} (43 μM).

4. Discussion

In clinical studies, 600 or 1500 mg L^{-1} TB was injected into air-filled vitreous cavity and kept for 1 or 2 min to stain ILM or ERM (Feron et al., 2002; Li et al., 2003; Stalmans et al., 2003a,b). One major issue is whether the dye exerts toxicity to RGCs during the surgery. On the basis of previous studies, TB solutions at concentrations of 600 and 1500 mg L^{-1} were used in the short-time exposure experiments. The results showed TB had a short-time toxic effect on RGCs in a time-dependent manner, suggesting that TB may induce significant damage on RGCs when exposure time is longer than 2 min. Additionally, ICG were used to compare the toxic effect on RGCs with TB in short-time exposure. Concentrations of ICG ranging from 500 to 5000 mg L^{-1} have been employed during surgery (Kadonosono et al., 2000; Burk et al., 2000; Kwok et al., 2001; Haritoglou et al., 2002; Uemura et al., 2003; Gass et al., 2003). The results showed that there were no difference between these two dyes. In this study, only one concentration (1500 mg L^{-1}) of ICG was used in order to compare the cytotoxicity with the same concentration of TB for short-time exposure. The clinical dose of ICG is different in some reports ranged from 500 to 5000 mg L^{-1}. Moreover, procedure of ICG or TB injection into the vitreous cavity to stain the membrane has variations in each operator. Thus, the actual dose and time of dyes to which RGC are exposed during the surgery is hard to be examined. Therefore, there is a limitation to mimic the clinical condition in in vitro experiments. Various factors to stain the membrane with these dyes will be considered in future experiments to determine the dose-response neurotoxic effect and time to prevent neurotoxic effect on RGC. However, our result suggests that reduction of the exposure time is highly

recommended because shorter staining time is less likely to induce RGC damage during surgery.

Another in vitro study reported that TB had no toxic effect on cultured human RPE cells even at a concentration of 3000 mg L^{-1} (0·3%) after 5 min exposure (Stalmans et al., 2003a,b). Our results demonstrated that TB at lower concentrations (600 and 1500 mg L^{-1}) and with shorter exposure time (2 min) showed toxicity to RGCs, implying that RGCs are highly susceptible to TB. Furthermore, because the RGC layer is adjacent to vitreous cavity, RGCs are likely to be exposed to higher concentrations of dye than RPE cells during surgery. Therefore, careful assessment of RGCs damage as well as RPE cells damage is recommended in TB-assisted vitrectomy.

Another major issue is whether the residual dye in the eye after surgery induces RGCs damage. Toxic effect of ICG on RGCs after long time exposure was reported (Iriyama et al., 2004). Our results of long-time exposure experiments demonstrated that TB induced a dose-dependent toxicity to RGCs. TB at concentrations lower than 50 mg L^{-1} did not damage RGCs. But TB at concentrations higher than 100 mg L^{-1} showed significant toxic effect on RGCs. It should be noted that in intraocular surgery, TB is used at 6 to 15 fold higher concentrations than can induce RGCs damage. If TB remains in vitreous cavity at higher concentrations for a long time after its application, RGCs damage might occur. During surgery, the operator should wash the vitreous cavity thoroughly and minimize the residual TB.

The osmolarity of the dye solutions were examined for its possible relationship with the viability of cells (Stalmans et al., 2002; Haritoglou et al., 2003). In long-time exposure experiments, the osmolarity of TB and ICG solutions ranged from 214 to 238 mOsm, which were similar to that of ICG solutions used in previous study (Iriyama et al., 2004). Hypoosmolarity of TB solutions compared with physiological saline (ca. 280 mOSm) can be explained by the fact that the osmolarity of neurobasal medium used as TB solvent medium is lower than other kinds of solvent medium (Brewer et al., 1993). Because of similar osmolarity in different concentrations of TB solutions, it is likely that the toxic effect of TB on RGCs is dose-dependent and not correlated with osmolarity.

TB has been considered to be a better alternative for staining ILM and ERM compared with ICG (Feron et al., 2002; Li et al., 2003). According to our long-time exposure experiments, IC50 of TB to RGCs is about 4 times of that of ICG, suggesting that once these dyes are left in the eyes, TB may show less toxicity on RGCs than ICG, although it deserves to mention that ICG may be diluted in a lower concentration in the eye than TB by irrigation fluid and the concentration of the dye in vitreous and vitreoretinal interface may be variable (Kadonosono et al., 2000; Horio and Horiguchi, 2004).

In summary, we have demonstrated that TB had toxic effect on RGCs in a dose- and time-dependent manner. It is confirmed that direct exposure to TB at clinical concentrations for longer than 2 min may damage RGCs. Moreover, if TB used in surgery is not thoroughly removed, the residual dye may cause toxic effect on RGCs The use of TB for a time as short as possible and extensive wash is recommended in TB-assisted vitrectomy.

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Short Communication

Neuroprotective effect of calcium channel blocker against retinal ganglion cell damage under hypoxia

Hideyuki Yamada[a,*], *Yi-Ning Chen*[b], *Makoto Aihara*[b], *Makoto Araie*[b]

[a]*Department of Ophthalmology, Tokyo National Hospital Organization, 3-1-1 Takeoka, Kiyose, Tokyo 204-8585, Japan*
[b]*Department of Ophthalmology, Tokyo University Medical School, Bunkyo, Tokyo 113-8654, Japan*

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ABSTRACT

The purpose of this study was to determine whether iganidipine, nimodipine and lomerizine, potentially useful calcium channel blockers for ophthalmic treatment, have direct retinal neuroprotective effects against hypoxic damage in experimental in vitro model. We used purified retinal ganglion cells (RGCs) from newborn rats. RGCs were incubated in controlled-atmosphere incubator in which oxygen levels were reduced to 5% normal partial pressure and cell viability was assessed. We also examined the effect of calcium channel blockers on the calcium ion concentration in RGC under hypoxic stress by calcium imaging. Iganidipine, nimodipine and lomerizine (0.01–1 μM) increased the RGC viability. Increase in intra-RGC calcium ion concentration by hypoxic damage was reduced by these calcium channel blockers. In conclusion, iganidipine, nimodipine and lomerizine were effective against hypoxic RGC damage in vitro. This neuroprotective effect was thought to be mediated by blocking calcium ion influx into RGC. These findings suggest that iganidipine, nimodipine and lomerizine have a direct neuroprotective effect against RGC damage related to hypoxia.

Retinal ganglion cell (RGC) is damaged in various ocular diseases, including glaucoma. Although not always confirmed, many investigators reported beneficial effects of systemic calcium channel blocker on the prognosis at least in a part of glaucoma patients (Bose et al., 1995; Gaspar et al., 1994; Kitazawa et al., 1989; Netland et al., 1993). It has been suggested that the beneficial effect was mainly due to increase in ocular blood flow (Tomita et al., 1999; Yamamoto et al., 1998) or direct effect to RGC survival (Boehm et al., 2003).

RGC culture system is a useful experimental model to assess the direct effect of calcium channel blockers to RGC (Otori et al., 2003). Using retinal cell or pure RGC culture model, neuroprotective effect of calcium blockers against neuronal damage induced by glutamate has been discussed (Otori et al., 2003; Toriu et al., 2000). However, several recent studies cast doubt on the primary role of glutamate in glaucoma (Carter-Dawson et al., 2002; Honkanen et al., 2003; Levkovitch-Verbin et al., 2002). Tissue hypoxia has been postulated to occur in glaucomatous eye in the basis of blood flow and histopathologic studies (Chung et al., 1999; Cioffi and Wang, 1999; Flammer et al., 2002; Flammer, 1994; Osborne et al., 1999; Tezel and Wax, 2004). In addition, it is reported that hypoxia lead to selective RGC death (Luo et al., 2001) in vitro.

Thus, we hypothesized that the damage of RGC by hypoxia would be at least partly caused by intracellar

* *Corresponding author.* Fax: +81 424 94 2168.
E-mail address: yamachan-tky@umin.ac.jp (H. Yamada).

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calcium increase through calcium channel. To verify this hypothesis, we have developed a hypoxia damage model using purified RGC culture system (Goto et al., 2002; Inatani et al., 2001; Kashiwagi et al., 2001; Otori et al., 2003; Otori et al., 1998; Pielen et al., 2004; Tezel and Yang, 2004) and investigated direct neuroprotective effect of calcium channel blockers on hypoxia-induced cell death.

Iganidipine hydrochloride, a new dihydropyridine-derivative calcium channel blocker, is relatively water soluble and is the only dihydropyridine-derivative calcium channel blocker presently available that is easily prepared as an ophthalmic solution (Ishii et al., 2003). Instillation of iganidipine increased the blood flow in the ipsilateral optic nerve head (ONH) in rabbit (Ishii et al., 2004; Waki et al., 2000) and monkey (Ishii et al., 2004), and inhibited the endothelin-1-induced contraction of retinal vessels (Ishii et al., 2003).

Nimodipine is a dihydropyridine calcium channel blocker and clinically used to treat cerebral vasospasm and migraine headaches. Several researchers recently reported that nimodipine oral administration had beneficial effect in visual field testing, color vision (Piltz et al., 1998) and contrast sensitivity (Boehm et al., 2003).

Lomerizine, a dual L/T-type calcium channel blocker (Hara et al., 1999), has been clinically used for treatment of migraine (Hara et al., 1995). It has shown selective enhancement of retinal and ONH blood flow in humans and rabbits (Tamaki et al., 2003) and neuroprotective effect in rabbits (Danielisova

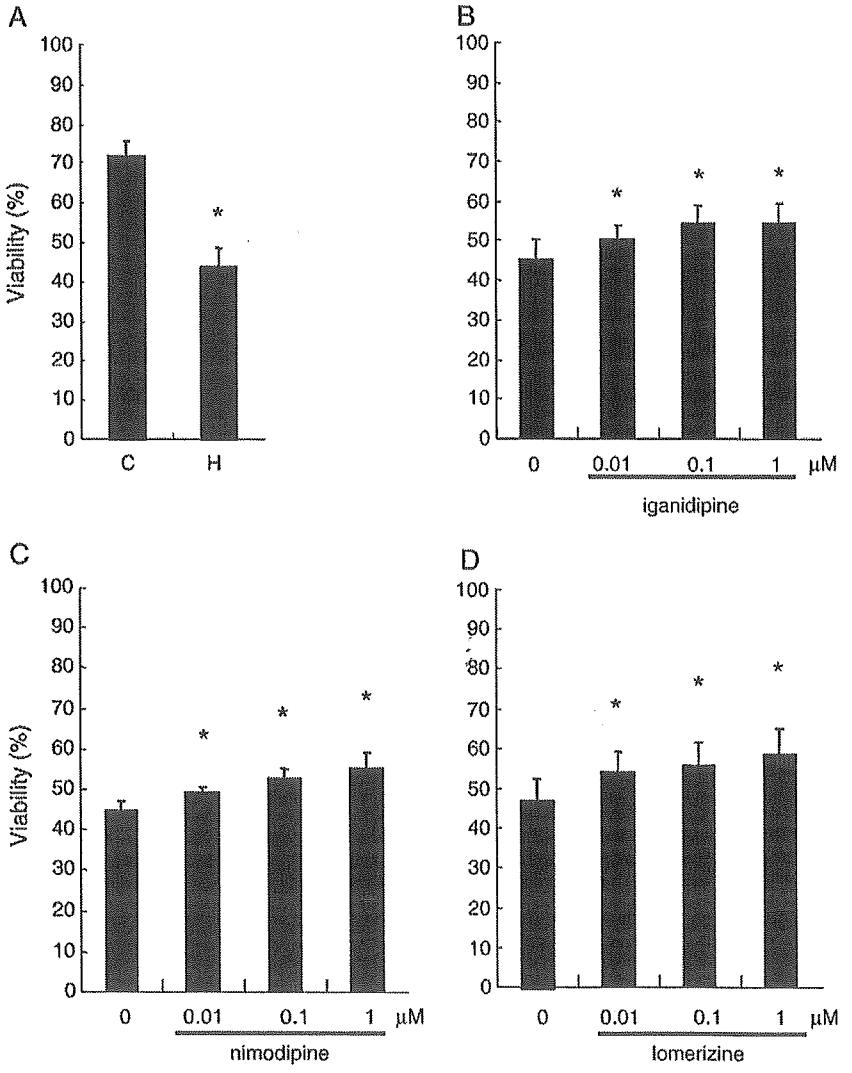

Fig. 1 – Effect of iganidipine, nimodipine and lomerizine to hypoxia-induced RGC loss. (A) C: Survival rate (viability) of RGC under normoxia, H: survival rate under hypoxic condition for 12 h. $n = 10$, Mann–Whitney U test. *$P < 0.01$ versus C. (B) Survival rate of RGC with iganidipine. RGCs were cultured under hypoxic condition for 12 h in the medium including iganidipine. $n = 8$. (C) Survival rate of RGC with nimodipine. RGCs were cultured under hypoxic condition for 12 h in the medium including nimodipine. $n = 8$. (D) Survival rate of RGC with lomerizine. RGCs were cultured under hypoxic condition for 12 h in the medium including lomerizine. $n = 8$. ANOVA followed by Dunnett's multiple comparison test for panels B, C and D. *$P < 0.01$ versus 0 μM. Viability is indicated as mean ± SD.

and Chavko, 1994) and rats (Yamashita et al., 1993) without showing any significant effects on the systemic blood pressure or heart rate (Gotoh et al., 1995; Nakashima and Kanamaru, 1989; Tamaki et al., 2003).

Therefore, Iganidipine, nimodipine and lomerizine are potentially useful calcium channel blockers for ophthalmic treatment. We assessed the protective effect of these drugs using RGC hypoxia damage model.

RGC cultures were obtained from retina of 5- to 7-day-old Sprague–Dawley rats according to a two-step immunopanning procedure (Barres et al., 1988; Goto et al., 2002; Inatani et al., 2001; Kashiwagi et al., 2001; Otori et al., 2003; Otori et al., 1998; Pielen et al., 2004). Briefly, retinas were dissociated into cell suspension, using Papain Dissociation System (Worthington Biochemical Corp). 50 ml flasks and 50 ml tubes were incubated with anti-rat macrophage antibody (1:50 dilution, Chemicon) and anti-rat and mouse Thy1.1 antibody (1:300 dilution, Chemicon) in phosphate buffer saline (PBS), respectively at 4 °C overnight. Antibodies were removed and cell suspension was incubated in flask for 1 h. Suspensions containing cells that did not adhere to the flask were treated for 1 h in tubes coated with Thy1.1 antibody. Cells adhering to the tube (RGCs) were resuspended in serum-free Neurobasal medium (GIBCO) supplemented with 2% B27, BDNF (40 ng/ml), CNTF (40 ng/ml) and Forskolin (10 μM) and seeded onto 13mm auto-craved cover slips, placed within 24-well plates. Cover slips were coated with 0.05 mg/ml of poly-L-lysine (GIBCO) overnight, rinsed twice with Hanks' buffered saline solution (HBSS) and then coated for 2 h with 1 μg/ml of laminin (GIBCO). RGCs were cultured for 3 days under normoxia condition (20%O_2, 5%CO_2, 37 °C) before each experiment.

On day 3, one or combination of the following drugs was added to the each culture: (1) iganidipine (Senjyu, Japan), (2) nimodipine (Sigma), (3) lomerizine (Nippon Organon, Japan), (4) ionomycin (5 μM; SIGMA), (5) EDTA (10 μM; SIGMA). Immediately after adding drugs, plates were transferred to a controlled-atmosphere incubator in which oxygen levels were reduced (hypoxic condition; i.e., a 5%CO_2, 90%N_2 and 5%O_2 mix). The concentration of O_2 was decided based on the result of preliminary experiments.

The viability of the purified RGC cell cultures was counted by LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). For calcium imaging, the medium of RGC culture was changed with HEPES–Tyrode Buffer (composition in mM: 140.0 NaCl, 2.7 KCl, 1.0 $CaCl_2$, 12.0 $NaHCO_3$, 5.6 D-glucose, 0.37 NaH_2PO_4 and 25.0 HEPES, with pH 7.4 at 25 °C) containing 0.1% BSA and 5 μM fura-2/AM, a fluorescent calcium ion indicator. Then, these RGCs were incubated under hypoxic condition for further 60 min. The cells were subsequently washed three times with the HEPES–Tyrode Buffer to remove the dye. The resultant fluorescent images were analyzed with a fluorescence analyzer (Argus 50, Hamamatsu Photonics). The fluorescence ratio (F340/F380) was obtained by dividing, after background subtraction, the 340-nm by the 380-nm images on a pixel-by-pixel basis. The mean ratio over the area of each RGC, well separated from the edge of other RGCs, was measured. Results are indicated as mean ± SD. Data were analyzed by Mann–Whitney *U* test for comparison of two groups or one-way analysis of variance (ANOVA), followed by Dunnett's test for comparison of three or more groups, with $P < 0.05$ being considered to indicate significance.

The viability of RGCs was 71.9 ± 3.5% in the normal condition ($n = 10$). To examine the protective effect of iganidipine, nimodipine and lomerizine, RGC culture was incubated under the hypoxic condition for 12 h. The viability of control RGC (no drug in medium) was decreased to 44.0 ± 4.5% ($n = 10$) (Fig. 1A, $P < 0.01$). Iganidipine ($n = 8$), nimodipine ($n = 8$) and lomerizine ($n = 8$) ameliorated hypoxic damage (Figs. 1B–D). Application of iganidipine (0.01–1 μM), nimodipine (0.01–1 μM) and lomerizine (0.01–1 μM) reduced RGC death in a dose-dependent manner ($P < 0.01$).

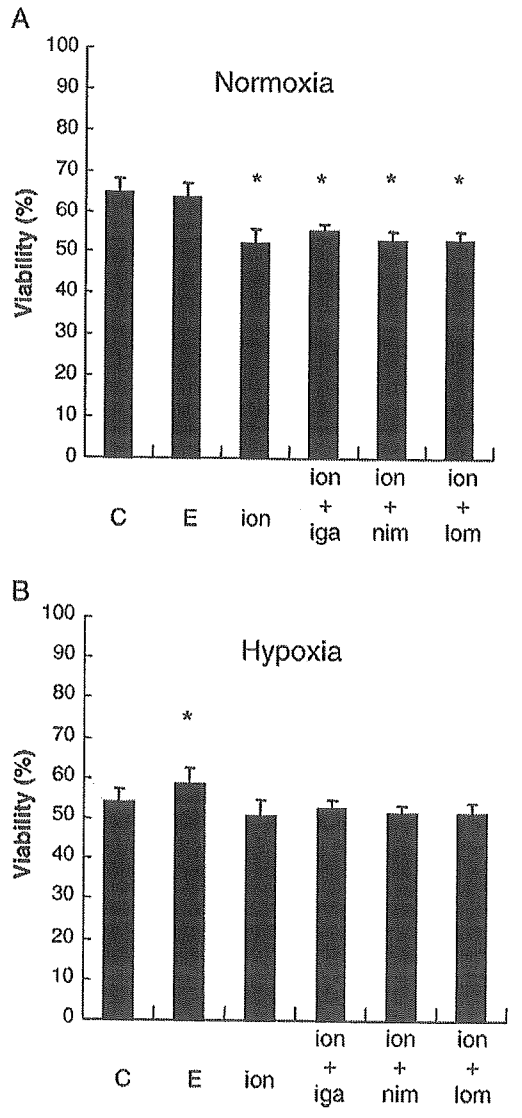

Fig. 2 – Effect of EDTA, ionomycin and calcium blockers to RGC survival rate under normoxic and hypoxic condition. (A) RGC survival rate (viability) under normal condition for 12 h. (B) Viability under hypoxic condition for 12 h. C = control, E = EDTA, ion = ionomycin, iga = iganidipine, nim = nimodipine, lom = lomerizine. ANOVA followed by Dunnett's multiple comparison test. *P < 0.01 versus control. n = 4. Viability is indicated as mean ± SD.

EDTA, which is calcium ion chelator, reduced RGC loss under hypoxic condition ($n = 4$) (Fig. 2B, $P < 0.01$). Ionomycin, which is calcium ionophore, increased RGC loss even under normoxic condition (Fig. 2A, $P < 0.01$). However, its toxicity was not blocked by calcium channel blockers (Fig. 2A).

To examine the effect of these calcium channel blockers on the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in RGC under hypoxic stress, RGC was cultured under hypoxic condition for 5 h and calcium imaging was performed at the earlier timing than viability experiment because the raise of $[Ca^{2+}]_i$ was supposed to precede RGC death.

Fig. 3 shows the F340/F380 ratios obtained by calcium imaging of RGCs ($n = 6$). Calcium ion concentration in RGC increased under hypoxic condition ($P < 0.01$). Calcium channel blockers (at 0.01 μM and 1 μM) reduced increase of calcium ion concentration in RGC under hypoxic condition ($P < 0.01$).

The new aspects of this study are that hypoxia-induced RGC death is at least partly related to increase of $[Ca^{2+}]_i$ and that calcium channel blockers can protect directly RGC death from hypoxic injury.

There are several points to be discussed in the present study. First, it should be considered if the RGC viability was influenced by anaerobic respiration in our culture system. To examine this, it will be helpful to analyze the energy metabolism (e.g., lactate production) (Ben-Yoseph et al.,

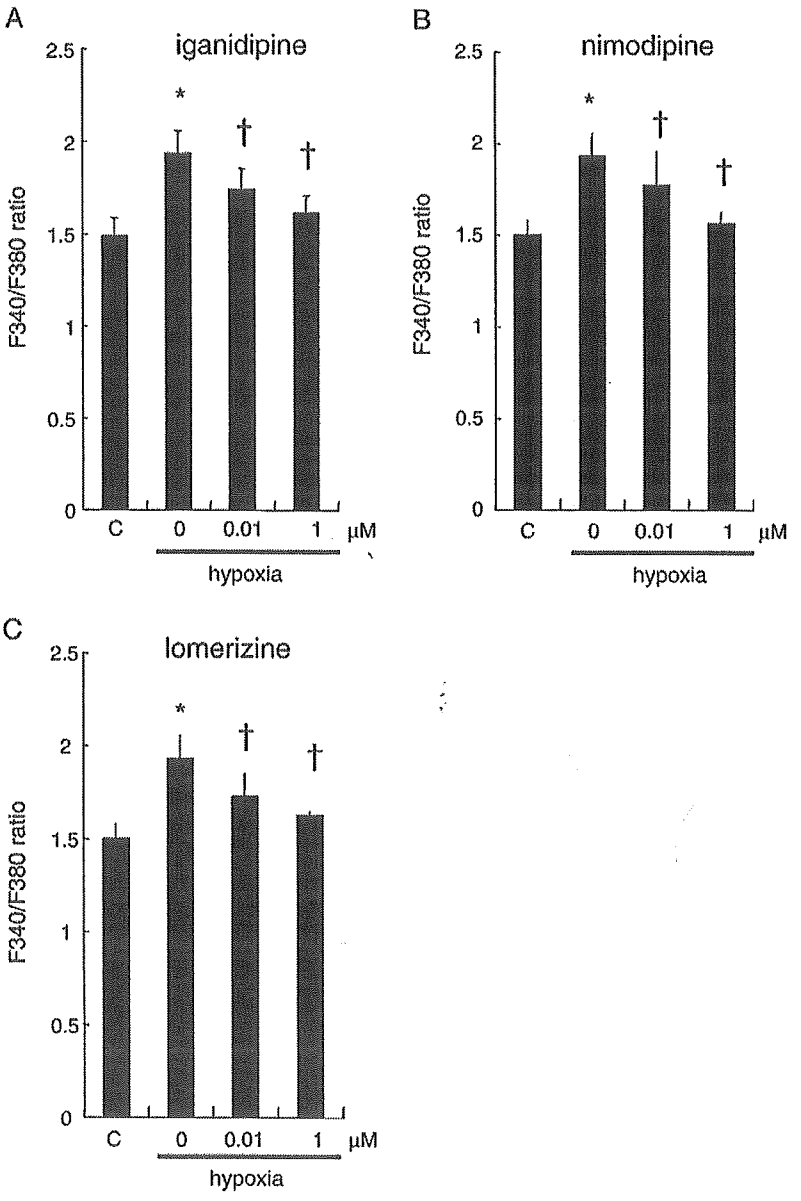

Fig. 3 – Fluorescence ratio (F340/F380) of RGC under normoxia and hypoxia. The effect of calcium channel blockers to fluorescence ratio was also indicated. (A) Iganidipine, (B) Nimodipine, (C) Lomerizine. C = control under normoxia. ANOVA followed by Dunnett's multiple comparison test. *$P < 0.01$ versus C, †$P < 0.01$ versus 0 μM. $n = 6$. Fluorescence ratio is indicated as mean ± SD.

1993; Cox et al., 1983). Unfortunately, RGC pure culture system that we adopted yielded too small amount of RGC to analyze the energy metabolism. Another culture system should be taken to examine this point. The second point is that iganidipine, nimodipine and lomerizine at the same concentration indicated almost equal neuroprotective effect at the similar extent in spite of their different properties for calcium channels. Though lomerizine exerts its neuroprotective effect partly via t-type channel expressed in the neuron (Hara et al., 1999; Mori et al., 1996), lomerizine indicated no advantage compared to other drugs. This fact suggests that t-type channel is not involved in the hypoxic RGC damage pathway, or relatively small magnitude of protective effect might indicate that the hypoxia stimulation was excessive or too small to assess the difference of effect among calcium blockers. It is also interesting whether hypoxia truly influenced the apoptosis process or function (e.g. evoked potentials) of RGC. Further extensive and careful examination should be planed to assess these points to clarify the mechanism of its neuroprotective effects.

The previous reports have shown that 10^{-8}~10^{-7} tissue concentration can be reached by administration of iganidipine, nimodipine and lomerizine (Ishii et al., 2003; Nakashima and Kanamaru, 1989; Sakai, 1994; Yan et al., 1993). Thus, these drugs may have a potential to be used clinically as ophthalmic neuroprotective drugs in consideration of their systemic side effects. The systemic side effect (for example, dizziness, headache, flushing, edema and/or systemic hypotension) is thought to be less frequent during nimodipine (Battistella et al., 1990) and lomerizine (Hara et al., 1999) treatment. Thus, these agents may be used for ophthalmic long-term oral treatment without compliance problems. Iganidipine can be given as an ophthalmic solution, which should be free of systemic effects.

In conclusion, our study indicates the possibility of direct neuroprotective effect of iganidipine, nimodipine and lomerizine on RGC death against hypoxic damage. Although further studies are required to clarify the direct mechanism of neuroprotection of calcium channel blockers, these calcium channel blockers may be candidates of ophthalmic drugs for neurodegenerative ocular diseases related to hypoxia.

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