

Effect of Topical Latanoprost–Timolol Combined Therapy on Retinal Blood Flow and Circulation of Optic Nerve Head Tissue in Cynomolgus Monkeys

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Purpose: To evaluate the effects of topical latanoprost and timolol in combined therapy on retinal blood flow and tissue circulation in the optic nerve head (ONH) of the cynomolgus monkey.

Methods: Latanoprost (30 μ L, 0.005%) was instilled once daily and timolol (30 μ L, 0.5%) twice daily for 6 days into 1 eye, and physiological saline into the other eye to serve as control. Blood velocity through retinal veins was determined using Kowa Laser Speckle Blood Flow Meter. The ONH tissue blood velocity (NB_{ONH}) was determined using the Laser Speckle Tissue Circulation Analyzer. Retinal blood flow and NB_{ONH} determinations were carried out before the first instillation and 4 hours after the first instillation on the 2nd experimental day, and the last instillation at the same sites at the same time of day. The intraocular pressure (IOP) was also measured periodically.

Results: After the first instillation, on the 2nd experimental day, the retinal blood flow decreased compared with the baseline, but 6-day instillation caused no significant change from the baseline. Six-day instillation increased the NB_{ONH} in the treated eyes by 10% from the baseline and by 7% from that in the fellow control eye. After 6-day instillation, the IOP was lowered by 7.8 ± 2.7 mm Hg and 3.6 ± 4.3 mm Hg in the treated and control eyes, respectively.

Conclusions: Combined 6-day instillation of latanoprost once daily and timolol twice daily has no significant effect on the retinal blood flow, but significantly increases the ONH tissue blood velocity in monkey eyes. **Jpn J Ophthalmol 2000;44:227–234** © 2000 Japanese Ophthalmological Society

Key Words: Cynomolgus monkey, latanoprost, optic nerve head circulation, retinal blood flow, timolol.

Introduction

The pathogenesis of glaucomatous optic nerve head (ONH) damage is still not well-understood. Previous studies have indicated that compromise of the tissue circulation in the ONH may play a role in glaucomatous injury of the ONH, although intraocular pressure (IOP) has been consistently found to be one of the most important risk factors.^{1–3} Therefore, the possible effects of antiglaucomatous agents on the circulation of the ONH tissue are of great clinical importance.

Timolol, a nonselective β -adrenergic antagonist, has probably been the most widely used antiglaucomatous agent. β -Adrenergic receptors generally mediate relaxation of vascular smooth muscles, and systemic introduction of β -adrenergic antagonists induces vasoconstriction in various tissues.^{4,5} Latanoprost (13,14-dihydro-17-phenyl-18,19,20-trinor-prostaglandin $F_{2\alpha}$ -isopropyl-ester) is a recently developed prostaglandin (PG)-related compound and is a potent ocular hypotensive drug in humans.^{6–9} Clinically, the combined use of latanoprost and timolol is often required.^{10–13} Therefore, it would be useful to evaluate the effect of these two compounds in combined use on the circulation of ONH tissue. Studies in monkeys and humans focusing on the

Received: March 12, 1999

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pharmacological mechanism of latanoprost showed that this agent reduces IOP by enhancing non-pressure-dependent uveoscleral outflow.^{14,15} It may be possible that, when used in combination with latanoprost, more timolol is carried in uveoscleral flow to the posterior segment of the eye, thereby decreasing the peripheral blood flow through the β_2 -blocking effect.^{4,5,16} Another possibility is that the combined use of latanoprost and timolol might increase the ONH blood flow by increasing ocular perfusion pressure (OPP) through a substantial decrease in IOP.

In this study, we investigated the combined effect of topical latanoprost and timolol on retinal blood flow and ONH tissue circulation in monkey eyes using noninvasive methods utilizing the laser speckle phenomenon.¹⁷⁻²⁰

Materials and Methods

Determinations of Retinal Blood Flow

The blood flow through the large retinal vessel was evaluated using a Laser Speckle Blood Flow Meter (BP-1000 α ; Kowa, Nagoya), which has been described elsewhere.^{17,18} An apparatus consisting of a fundus camera (Fx-50c; Kowa) equipped with the He-Ne laser (wavelength: 632.8 nm; Spectra Physics, Mountain View, CA, USA), a photomultiplier (Hamamatsu Photonics, Hamamatsu), a photon-counting unit, and a digital correlator were used for determination of blood flow in the retinal vessel. An attached Plasma-Coupled Device line image sensor (S2304; Hamamatsu Photonics) was used for measurement of retinal vessel diameter. The reflection of the He-Ne laser was detected by the photomultiplier, and a photon-counting unit processed the signals detected. The digital correlator and microcomputer calculated the autocorrelation function of the processed signals. The lag time corresponding to half the height of the correlation function was defined as the correlation time (τ_c), and the retinal blood velocity (mm/s) was evaluated as a reciprocal of the correlation time ($1/\tau_c$).¹⁷ At the same time, a halogen light from the fundus camera as a light source was irradiated on the ocular fundus. The reflected light from the fundus was magnified approximately five times by the lenses and focused on the line image sensor. The diameter of the target retinal vessel was determined with the image processor and then corrected for refraction and the axial length of the subject's eye, using Litemann's method, modified to BF-1000 α .²¹ Retinal blood flow (nL/s) was calculated from the retinal blood velocity and the diameter of vessel.

The time required for these processes was 1.0 sec and, during which the retinal image was sent to the microcomputer eight times, and the average blood flow was adopted.

Determinations of Tissue Circulation in ONH

Tissue circulation in the ONH was evaluated using the laser speckle method of which details were reported elsewhere.^{19,20} An apparatus consisting of a fundus camera (TRC-WT3[®]; Topcon, Tokyo) equipped with a diode laser (wavelength: 808 nm) was used for the determination. The scattered laser light was visualized on an image sensor (100 \times 100 pixels, BASIS type; Canon, Tokyo). The difference between the average of the speckle intensity (I_{mean}) and the speckle intensity for successive scanning of the image speckles at the pixels on the sensor plane was calculated, and the ratio of I_{mean} to this difference was defined as normalized blur (NB). NB is nearly equivalent to the reciprocal of speckle contrast described by Fercher and Briers^{22,23} and is thought to be indicative of tissue blood velocity.^{19,20} In this experiment, NB was determined for 5 seconds, during which there was no eye movement, five times, each after a 30-second interval, and the average of three measurements excluding the maximum and the minimum values was calculated (NB_{ONH}).

Drugs

The ophthalmic solution of 0.005% latanoprost (PhXA41; Xalatan Pharmacacia & Upjohn, Uppsala, Sweden) was kindly supplied by the company, and the ophthalmic solution of 0.5% timolol was purchased from Banyu Pharmaceutical Company (Tokyo).

Experimental Procedures

Eight adult cynomolgus monkeys (3-7 kg) were used and handled in accordance with the ARVO Resolution on the Use of Animals in Research. All examinations were carried out using the monkey chair, reported previously.²⁴ The animals were entrained to a light schedule of alternating 12-hour periods of light and dark (light on at 4:00 AM) for at least 3 weeks prior to use. After dilating the pupil with one drop 0.4% tropicamide (Mydrin M[®]; Santen Pharmaceutical, Osaka), the retinal blood flow was recorded from the superior temporal retinal vein at a distance of one disc diameter away from the ONH. The image speckles from the largest square field in the ONH free of visible surface vessels were recorded to measure the NB_{ONH} (Figures

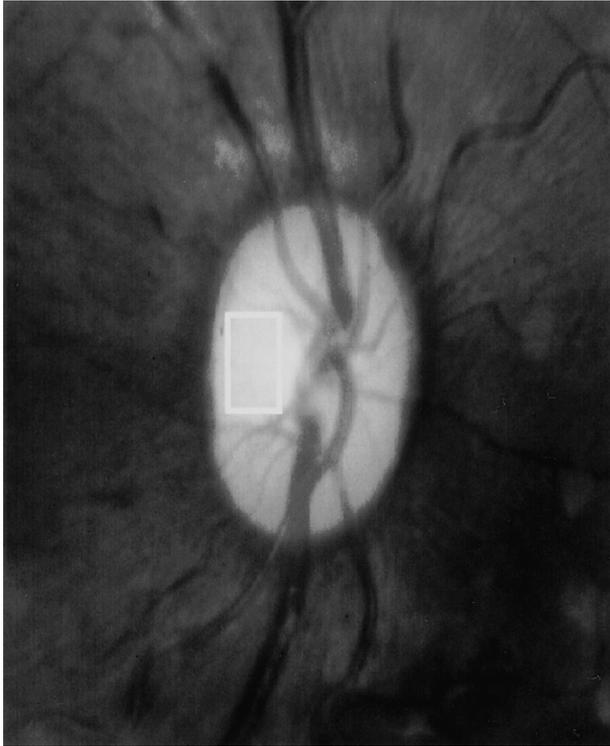


Figure 1. Determination field of normalized blur (NB) in optic nerve head (ONH) tissue. Image speckles from largest square field (0.42×0.42) free of visible surface vessels in temporal field on ONH were recorded to determine NB in ONH tissue (NB_{ONH}). Visible surface vessels near measurement field were used as markers for measurements. Actual measurement field is enclosed within white box to avoid visible surface vessels.

1, 2). Color fundus photographs were taken to record the site of retinal blood flow and NB_{ONH} measurement and the visible surface vessels near the measurement field were used as markers.

At 7:00 AM on the first day, the monkeys were removed from the cage, unsedated if possible, and placed in the monkey chair. One hour later, IOP measurement in both eyes (baseline IOP at 8:00 AM) was performed with a calibrated applanation pneumotonometer (Alcon Labs, Fort Worth, TX, USA) after instillation of topical anesthesia (0.4% oxybuprocaine hydrochloride (Benoxil®; Senju, Osaka)). Then the animals were returned to the cage and 4 hours later (at 12:00 PM), under general anesthesia induced by ketamine hydrochloride (Ketalar®; San-kyo, Tokyo) at a dose of 8–10 mg/kg intramuscularly, the determination of retinal blood flow and NB_{ONH} were carried out as described above in both eyes. Then, IOP measurement in both eyes (base line IOP at 12:00 PM) was performed and the blood pres-

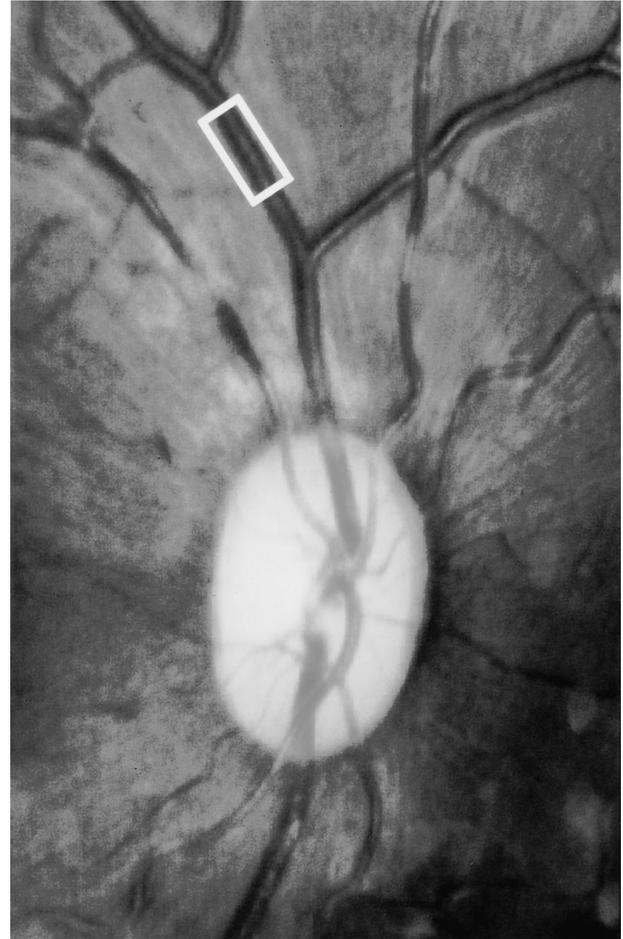


Figure 2. Determination field of blood flow in retinal vein. Retinal blood flow was recorded from superior temporal retinal vein at distance of one disk diameter away from optic nerve head. Actual measurement field is enclosed within white box.

sure and pulse rate were measured simultaneously with a sphygmomanometer for an infant (SP-8800; Nihon Koden, Tokyo). The mean blood pressure (BP_m) was calculated according to the formula: $BP_m = BP_d + 1/3(BP_s - BP_d)$, where BP_d and BP_s are diastolic and systolic brachial blood pressure, respectively. The OPP was calculated according to the formula: $OPP = 2/3BP_m - IOP$.²⁵ Arterial O_2 saturation (SaO_2) was checked using the pulse oxygen meter (OLV-1200; Nihon Koden, Tokyo), and body temperature was monitored with a Thermopit (IT-500M; Nipro, Osaka).

At 8:00 PM on the experiment day, 30 μ L of latanoprost (0.005%) was instilled into one eye of each monkey, and the physiological saline into the other eye to serve as control. From the 2nd to the 6th experimental day, 30 μ L of latanoprost was instilled

once daily at 8:00 PM and 30 μ L of 0.5% timolol was instilled twice daily at 8:00 AM and 8:00 PM into the latanoprost-treated eye and saline into the other eye. On the 7th experimental day, 30 μ L of timolol was instilled at 8:00 AM.

From the 2nd to the 7th experimental day, the IOP measurement without general anesthesia was carried out at 8:00 AM before timolol instillation. At 12:00 PM on the 2nd and 7th experimental days, the retinal blood flow and NB_{ONH}, IOP, blood pressure, pulse rate, SaO₂, and temperature measurements were carried out as above. All measurements were performed by an investigator masked to the treatment.

Calculation and Statistical Analysis

The results are presented as mean \pm standard deviation. Paired Student's *t*-test and unpaired *t*-test were used to evaluate statistical significance. For multiple comparisons, Bonferroni's correction was used to calculate *P* values. Significance levels of *P* < .05 were considered as statistically significant.

Results

Systemic condition parameters during the experiment are shown in Table 1. A significant reduction in the pulse rate on the 2nd (27%) and the 7th (18%) experimental day was observed. Other parameters showed no significant changes.

A significant reduction in the IOP measured at 8:00 AM in the treated eyes was seen during the experimental period. The IOP levels in the treated eyes were significantly lower than those in saline-treated eyes. On the 7th experimental day, the IOP in the treated eye was lower by 28% and 21% than the baseline and that in the fellow control eye, respectively. The IOP level in the saline-treated fellow

Table 1. Systemic Condition Parameters Before and After Topical Instillation of Latanoprost and Timolol Combined

	Time (Day)		
	1	2	7
BP _m (mm Hg)	65.3 \pm 9.4	63.2 \pm 12.4	65.4 \pm 9.2
Pulse rate (beats/min)	169.4 \pm 23.5	123.9 \pm 33.9 (<i>P</i> = .008)*	139.5 \pm 20.6 (<i>P</i> = .016)*
BT ($^{\circ}$ C)	37.6 \pm 0.5	37.7 \pm 0.7	37.5 \pm 0.8
SaO ₂ (%)	99.2 \pm 0.3	98.2 \pm 0.8	99.1 \pm 0.5

Data are mean \pm standard deviation (*n* = 8). BP_m: arterial blood pressure, BT: body temperature, SaO₂: Saturation of arterial O₂.

**P* value by paired *t*-test with Bonferroni's correction for difference from baseline.

control eye was also significantly lower than the baseline between the 3rd and 7th experimental days, showing 10% decrease on the 7th day (Figure 3). The IOP level at 12:00 PM when the retinal blood flow and NB_{ONH} were measured was significantly lower than the baseline at 12:00 PM and that in the saline-treated fellow control eyes. The IOP in treated eyes showed a 33.6% and a 44.3% decrease on the 2nd and 7th experimental days, respectively. On the 7th experimental day, it was also significantly lower than in saline-treated fellow control eyes. The IOP in saline-treated fellow control eyes was lower than the baseline by 24% and 20% on the 2nd and 7th experimental days, respectively (Figure 4). The OPPs in both eyes were significantly higher than the baseline on the 7th experimental day, showing 30% and 15% increase in the treated and the fellow control eye, respectively. The bilateral difference in the OPP was also significant on the 7th day (Figure 5).

Retinal vein diameter showed no significant changes during the experimental period. On the other hand, the blood velocity and blood flow through the retinal vein only in the treated eyes showed a 23% reduction on the 2nd experimental day, while no significant change from the baseline was seen on the 7th experimental day (Figure 6). Differences from the baseline in the NB_{ONH} are summarized in Figure 7. A significant increase in NB_{ONH} was observed only in the treated eye on the 7th experimental day. It was 10% and 7% higher than the

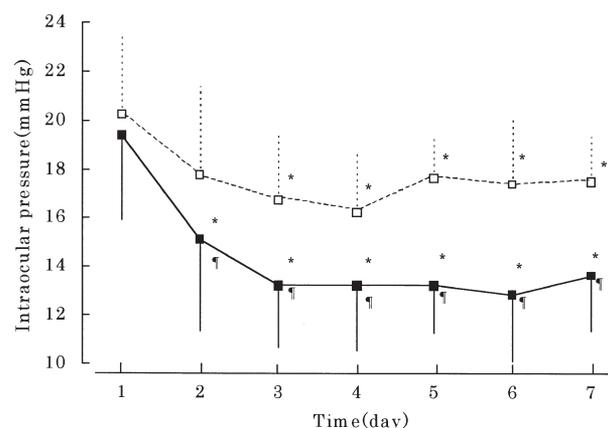


Figure 3. Time course (day) of intraocular pressure at 8:00 AM after topical instillation of latanoprost and timolol combined (■) or saline (□). Each plot represents mean value and error bars represent standard deviation. **P* < .05 by paired *t*-test with Bonferroni's correction for difference from baseline. ‡*P* < .05 by paired *t*-test with Bonferroni's correction for the difference from fellow eyes.

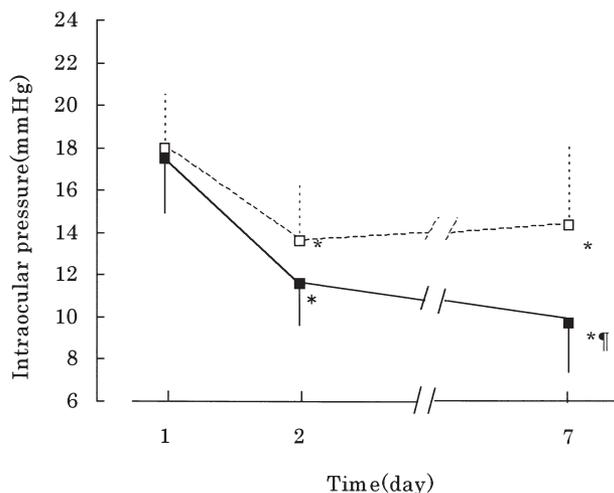


Figure 4. Time course of intraocular pressure at 12:00 AM after topical instillation of latanoprost and timolol combined (■) or saline (□). Each plot represents mean value and error bars represent standard deviation. * $P < .05$ by paired t -test with Bonferroni's correction for difference from baseline. ¶ $P < .05$ by paired t -test with Bonferroni's correction for the difference from fellow eyes.

baseline and that in the saline-treated fellow control eye, respectively.

Discussion

In monkey eyes, latanoprost or timolol reduces the IOP by about 20%.^{14,15,26-28} In the present study,

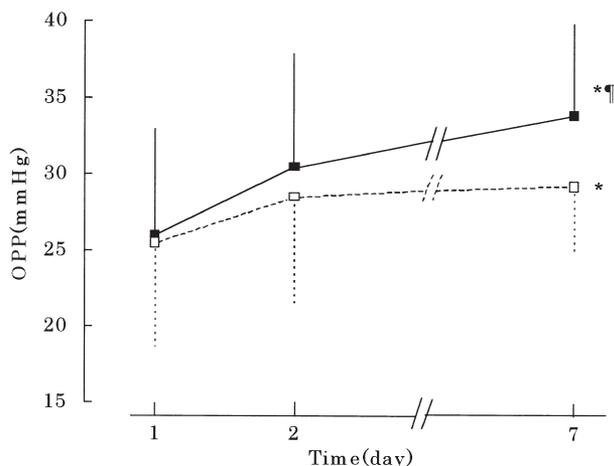


Figure 5. Time course of ocular perfusion pressure (OPP) at 12:00 AM after topical latanoprost and timolol combined (■) or saline (□). Each plot represents mean value and error bars represent standard deviation. * $P < .05$ by paired t -test with Bonferroni's correction for differences from baseline. ¶ $P < .05$ by unpaired t -test for with Bonferroni's correction for the difference from fellow eyes.

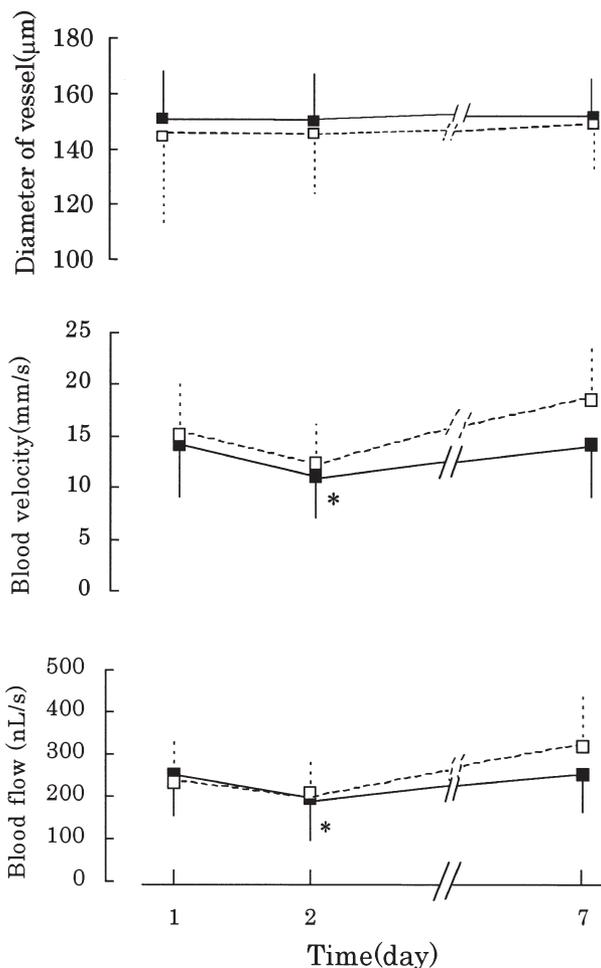


Figure 6. Time course of diameter, blood velocity, and blood flow of retinal vein after topical instillation of latanoprost and timolol combined (■) or saline (□). Each plot represents mean value and error bars represent standard deviation. * $P < .05$ by paired t -test with Bonferroni's correction for the difference from fellow eyes.

we observed as much as a 40% reduction in the IOP levels by the combined use of latanoprost and timolol, suggesting an additive ocular hypotensive effect of these drugs in monkey eyes. In human eyes, it was reported that the combined use of latanoprost and timolol resulted in additive reduction in the IOP,¹⁰⁻¹² which is consistent with our current observations. Whether or not latanoprost or timolol has any effect on ocular blood flow has been studied intensively in humans and experimental animals, but the results are still controversial. Nicolela et al²⁹ reported that 12 hours after a single timolol instillation, the end diastolic velocity in the human ophthalmic artery was reduced, whereas repeated instillations did not show any significant change.

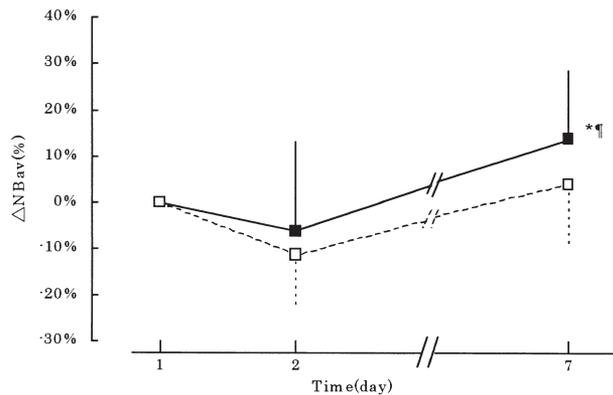


Figure 7. Time course of NB_{ONH} after topical instillation of latanoprost and timolol combined (■) or saline (□). Each plot represents mean value and error bars represent standard deviation. * $P < .05$ by paired t -test with Bonferroni's correction for the difference from baseline. ¶ $P < .05$ by paired t -test with Bonferroni's correction for the difference from fellow eyes.

They also observed that latanoprost did not show any effects upon the central retinal artery, short posterior ciliary arteries, or ophthalmic artery.

In the present study, use of latanoprost and timolol combined showed no significant effect on the retinal blood velocity and blood flow on the 7th day, whereas on the 2nd day they were significantly lower than the baseline only in the treated eyes. Although there was no significant decrease in the blood pressure level, the pulse rate was reduced significantly in the present experiment, which is attributed to the systemic β -blocking effect of timolol. The β -blocking action of timolol will decrease the pulse rate and may have a vasoconstrictive effect in the retinal vessel,³⁰⁻³² thereby provoking a decrease in the retinal blood flow. On the other hand, Grunwald reported that topical timolol caused an increase in the retinal blood flow in humans through an increase in the ocular perfusion pressure associated with a decrease in the IOP.³³⁻³⁵ A similar mechanism may be possible in the action of timolol in the monkey eye. However, since latanoprost was also instilled in combination with timolol in this study, the ocular effect of timolol might be somewhat modified. Further, the weight of the cynomolgus monkey is approximately 1/15 that of the human, and this difference might also modify the systemic effects of timolol. A reduction in the pulse rate should result in an increase in the stroke volume through the autoregulatory mechanism.^{36,37} Discrepancy between the present results and those in humans³³⁻³⁵ or between the results on the 2nd and

7th experimental days may be explained by the above differences in the experimental conditions or the mechanisms of timolol affecting the retinal blood flow.

In the present study, an increase in the ocular perfusion pressure was observed both in the treated and the fellow control eyes, while an increase in the NB_{ONH} was observed only in the treated eyes. This finding suggests that the increase in the NB_{ONH} could not be attributed only to an increase in the ocular perfusion pressure. The PGs may be important in the regulation of the local blood flow. Certain PGs, such as PGI₂ and PGE₂, function as potent vasodilators in many species.³⁸ Although PGF_{2 α} usually constricts arteries,³⁹⁻⁴¹ in some tissues it functions as a vasodilatory factor, which increases blood flow.⁴²⁻⁴⁵ For example, it has been reported in canine uterine arteries that a low concentration of PGF_{2 α} (5×10^{-8} mol/L) exerted vasodilatory effects via PGI₂.⁴² Astin et al⁴⁵ reported in white rabbits, that a single instillation of PGF_{2 α} resulted in activation of nitric oxide synthase, thereby dilating the vessels in the conjunctiva. Latanoprost is not a PGF_{2 α} itself but is a derivative with very selective FP-receptor agonistic activity.²⁶ It may be possible that latanoprost per se possesses vasodilatory effects at some concentration level. There is evidence suggesting that a small amount of the topically instilled drug can penetrate to the posterior or retrobulbar parts of the eye.^{46,47} The difference between the effect on the retinal blood flow and that on the NB_{ONH} after latanoprost and timolol instillation is probably explained by anatomical and possible pharmacological difference between the retinal artery and the short posterior ciliary arteries which feed the ONH.⁴⁸

In summary, we observed that 1-week instillation of topical latanoprost and timolol combined, increased the tissue circulation in the ONH in the monkey eye, although its mechanism has not yet been ascertained. It must be kept in mind that the result in the monkey should not be directly extrapolated to humans. However, combined use of topical latanoprost and timolol is expected to be prescribed often and the current observations would be relevant in studying human eyes.

This paper was published in the *Nippon Ganka Gakkai Zasshi (J Jpn Ophthalmol Soc)* 104: 3-10, 2000. It appears here in a modified form after peer review and editing for this journal.

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