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Original Article

Evaluation of maximal dosing volume for intravitreal injections in cynomolgus monkeys

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ABSTRACT — Intravitreal (IVT) injections are the current method for retinal drug delivery and have been widely used in humans, although it is well known that IVT injections often cause an increase in intraocular pressure (IOP) that closely relates to the injection volume. Because of the anatomical and physiological similarities of the monkey and human eye, cynomolgus monkeys are often used for preclinical studies on new treatments using large molecules or gene therapy requiring IVT application. However, there is only limited information on the maximal dosing volume for IVT injections in cynomolgus monkeys. To determine an appropriate maximal dosing volume for IVT injections, comprehensive ocular examinations were conducted in IVT-injected eyes of cynomolgus monkeys. Up to a dosing volume of 150 µL/eye by IVT injection, there were no IVT injection-related ocular findings in pupillary light reflex gross observations, slit lamp biomicroscopic and indirect ophthalmoscopic and fundus autofluorescent examinations, intraocular pressure, optical coherence tomography of the anterior and posterior segments of the eye, axial length measurement, electroretinogram waveforms, or histopathological examinations of treated eyes. However, leakage of the dosing solution from an eye was observed at 150 µL/eye, indicating a technical limitation for the maximal applicable volume. In addition, recovery from IOP elevation immediately after injection of 150 µL/eye was slower than after injections of 50 or 100 µL/eye. Therefore, we recommend a maximal volume of 100 µL/eye for IVT injections in cynomolgus monkeys.

Key words: Intravitreal injection, Dosing volume, Cynomolgus monkey, Toxicity study, Ophthalmological examination, New modality of drugs

INTRODUCTION

Intravitreal (IVT) injections are the current method for retinal drug delivery (Del Amo *et al*., 2017). IVT has been widely used in the delivery of anti-VEGF drugs for treatment of wet age-related macular degeneration (AMD). Agents for gene therapy of inherited retinal dystrophies are commonly applied by subretinal injections, but IVT injections have been considered as they are easier to perform and less risky than subretinal injections (Ochakovski *et al*., 2017; Ziccardi *et al*., 2019). Several gene therapies for AMD that could be applied via IVT injections are under development (Bordet and Behar-Cohen, 2019; Guimaraes *et al*., 2021). However, increased intraocular pressure (IOP) is often noticed after IVT injection during clinical use of anti-VEGF drugs, and the increases are closely related to the injection volume (Abedi *et al*., 2013; Dingerkus *et al*., 2022; Gumus *et al*., 2022). During drug development, toxicity studies should be conducted at a high dose to provide safety margins required for approval of clinical trials and later clinical use. However, many drug candidates cannot be prepared at high concen-

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trations, and therefore the highest dose used in IVT studies is often limited by the applicable dosing volume.

Toxicity studies requiring IVT injections are frequently conducted in cynomolgus monkeys because their eyes are anatomically and physiologically similar to those of humans. Furthermore, drug activity in monkeys compares well with that in humans, especially for biologics, making them a preferred model system for studies on new modalities such as large molecules and gene therapy (Short, 2021; Baldrick *et al*., 2022). However, there is limited information on the maximum dose for IVT injections in cynomolgus monkeys and the assessment of dosing effects by ophthalmologic examination. Therefore, we determined an appropriate maximal dosing volume for IVT injections in cynomolgus monkeys based on detailed analyses of the eye.

MATERIALS AND METHODS

Animals

Nine cynomolgus monkeys (male, 5-6 years of age, Cambodian origin) were used in this study. All animals were maintained in accordance with the ethics criteria contained in the bylaws of Shin Nippon Biomedical Laboratories (SNBL DSR), Ltd., Drug Safety Research Laboratories, which is accredited by AAALAC International. The study was approved by the SNBL DSR Institutional Animal Care and Use Committee and conducted in accordance with the Association for Research in Vision and Ophthalmology statement.

Environmental conditions were as follows: temperature: 23-29°C, humidity: 30-70%, ventilation: 15 times/hr, illumination: 12 hr/day of artificial light (7:00-19:00). Animals were housed 1 animal per cage (cage size: 680 mm [D] x 620 mm [W]) x 770 mm [H]).

IVT injections

After instillation of 0.4% oxybuprocaine (Santen Pharmaceutical Co., Ltd., Osaka, Japan) into the eye, the pupils were dilated with 0.5% tropicamide or a combination of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Santen Pharmaceutical Co., Ltd., Osaka, Japan) before the animals were anesthetized by intramuscular (i.m.) injection of ketamine hydrochloride (Arevipharma GmbH, Radebeul, Germany or Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan). Dulbecco's Phosphate Buffered Saline (D-PBS; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) at 50, 100, or 150 μL was injected into the vitreous body of the right eye using a syringe with a disposable 30-gauge needle. Injections were administered at a rate of 50 μL/30 sec through the conjunctiva to the center of the eye at the 6 o'clock position, approximately 2 to 3 mm from the corneal limbus, avoiding the lens and the inferior rectus (Fig. 1). The sclera and conjunctiva were squeezed with forceps for 15 sec after injection to prevent leakage of dosing solution. To prevent infections, 0.5% levofloxacin eye drops were given from 3 days before to 3 days after IVT injections.

Ophthalmic examinations

The following ophthalmic examinations were performed, and the timelines for the examinations are shown in Fig. 2:

- Pupillary light reflex (PLR) tests, gross observations, and slit lamp biomicroscopic and indirect ophthalmoscopic examinations
- Photography of the ocular fundi
- Fundus autofluorescent (FAF) examinations
- Intraocular pressure (IOP)
- Optical coherence tomography (OCT) examinations of the anterior segment
- Axial length (AL) measurements
- Electroretinography (ERG) examinations

The PLR test and gross observations were performed using a portable slit lamp (SL-15, Kowa Ltd., Aichi, Japan). The PLR test and gross observations were performed in both eyes on all examination days. The slit lamp biomicroscopic and indirect ophthalmoscopic examinations were performed in both eyes on Day -4 and in

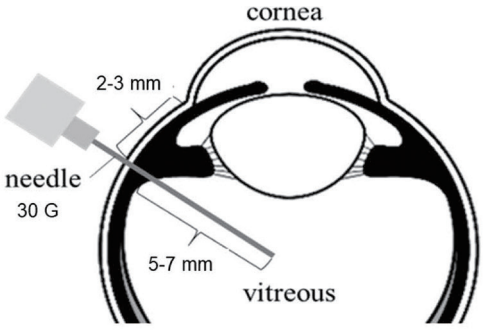

Fig. 1. Schematic drawing of injection method. D-PBS was injected into the vitreous cavity using a syringe with a disposable 30-gauge needle. Injections were made through the conjunctiva to the center of the eye at the 6 o'clock position at a distance of approximately 2 to 3 mm from the corneal limb, avoiding the lens and the junction with the inferior rectus. The needle was inserted approximately 5 to 7 mm into the vitreous cavity.

Fig. 2. Timeline of examinations. The day of IVT injections was defined as Day 0.

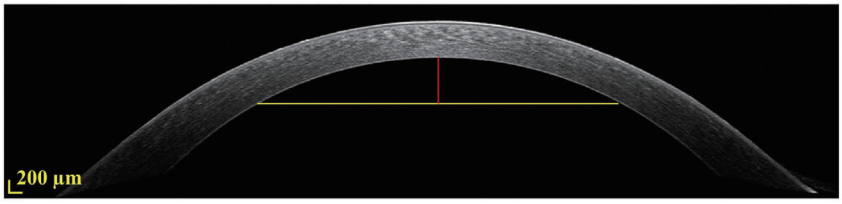

Fig. 3. Definition of anterior chamber depths. Based on images of the anterior segment, the anterior chamber depth was defined as the distance (red line) from the internal surface of corneal endothelium at the center to the point where the distance between the internal temporal and nasal surfaces of the corneal endothelium was 5000 µm (yellow line).

the right eye on Days 3 and 10. Only the right eye was used for evaluation.

FAF examinations were conducted using a Heidelberg HRA laser scanning imaging system (Heidelberg Engineering Inc., Heidelberg, Germany) in both eyes on Day -4 and in the right eye on Days 3 and 10. Only the right eye was used for evaluation.

IOP measurements were conducted in both eyes using a rebound tonometer (TonoVet tonometer TV01, Tiolat Oyj, Vantaa, Finland) after anesthetization by a 10 mg/kg i.m. injection of ketamine or when awakening with or without inducing mydriasis by instillation of a tropicamide/phenylephrine hydrochloride solution.

OCT examinations (Heidelberg Spectralis OCT2, Heidelberg Engineering Inc., Heidelberg, Germany) were conducted for the anterior and posterior segments of the eye after anesthetization by a 10 mg/kg i.m. injection of ketamine hydrochloride. Based on the anterior segment images, the anterior chamber depth was defined as the length from the center of the internal surface of the corneal endothelium to the point below where the distance between the temporal and nasal surfaces of the corneal endothelium was 5000 µm (Fig. 3). OCT examinations were conducted in both eyes on Day -4 and in the right eye on Days 0, 3, and 10. Only the right eye was used for evaluation. The analysis was performed using the inbuilt Spectralis mapping software, Heidelberg Eye Explorer (Version 6.12.3).

AL measurements were conducted using an Echoscan US-500 (Nidek Co., Ltd., Aichi, Japan) in both eyes after anesthetization by a 10 mg/kg of i.m. injection of ketamine hydrochloride. Only the right eye was used for evaluation.

ERG examinations were conducted according to the ISCEV full-field ERG standard and its 2015 and 2022 updates (McCulloch *et al.,* 2015; Robson *et al*., 2022). After the animals were adapted to the dark for at least 30 min, they were anesthetized by an i.m. injection (0.2 mL/kg, 10 mg/kg) of a mixture of ketamine hydrochloride (50 mg/mL, Ketalar for Intramuscular Injection 500 mg, Daiichi Sankyo Propharma Co., Ltd., Tokyo, Japan) and xylazine hydrochloride (Selactar 2% injection, 20 mg/mL, Bayer Yakuhin, Ltd., Osaka, Japan) in a 7:1 ratio (v/v). After ocular instillation of a mydriatic (Mydrin-P Ophthalmic Solution, Santen Pharmaceutical Co., Ltd., Osaka, Japan) under anesthesia, dilatation was confirmed, and a surface anesthetic for ophthalmology (Benoxyl Ophthalmic Solution 0.4%, Santen Pharmaceutical Co., Ltd., Osaka, Japan) and a mucous membrane-protecting agent (Scopisol Solution for Eye, Senju Pharmaceutical Co., Ltd., Osaka, Japan) were instilled into the eye. A contact lens-type electrode for monkeys (Kyoto Contact Lens, Ltd., Kyoto, Japan) was attached to the cornea. After the electric potential was stabilized, ERG examination was conducted as described in Table 1 using a retinoscope (Ganzfeld System, SG-2002, LKC Technologies, Inc., Gaithersburg, MD, USA) and an induction reaction recording device (PuREC [PC100-A, Mayo, Ltd., Aichi, Japan]):

DA 0.01 ERG was measured using the implicit time and amplitude of the b-wave. The implicit time and amplitude of DA 3 OPs were measured. Other responses were measured using the implicit time and amplitude of the a- and b-waves. The LA 31 Hz ERG was measured using the peak of implicit time and amplitude. ERG examinations were conducted in both eyes on Day -3 and in the right eye on Days 4 and 11. Only the right eye was used for evaluation.

All animals were euthanized by exsanguination under anesthetization by a combined i.m. injection of ketamine hydrochloride and medetomidine hydrochloride (Orion Corporation, Espoo, Finland) before necropsy. The eyes and optic nerves were fixed in 3% glutaraldehyde and 2.5% formaldehyde, and other tissues were routinely processed. Paraffin embedded blocks were sectioned and stained with hematoxylin and eosin (HE). The slides were examined by light microscopy.

Statistical Analysis

Anterior chamber depths (μm) in OCT, axial lengths, and ERG datasets were expressed as the mean ± standard deviation (SD). IOP data were analyzed using Dunnett's test, and anterior chamber depths (μm) in OCT, axial lengths, and ERG datasets were analyzed using a paired t-test. The significance level for both tests was 5%.

Table 1. List of ERG examinations performed.

DA 0.01 ERG
DA 3 ERG
DA 3 OPs
Light adaptation 10 min.
LA 3 ERG
LA 31 Hz ERG

RESULTS

Findings after dosing in gross observations, slit lamp biomicroscopic, and indirect ophthalmoscopic examinations

The dosing solutions were successfully administered to the vitreous body with no leakage of the administered solution, except for one eye at 150 μL (Table 2). In this eye, the subconjunctival area swelled immediately after administration, suggesting leakage of the administered solution out of the eye. There were no findings in ophthalmological examination (including PLR tests and slit lamp biomicroscopic and indirect ophthalmoscopic examinations) that would suggest endophthalmitis due to the use of D-PBS as the administration solution or otherwise caused by the administration technique. The fundus photographs on Day 10 are shown in Fig. 4.

FAF examinations

There were no findings in FAF examinations attributable to the administration of D-PBS. The FAF images are shown in Fig. 5.

IOP changes

The IOP in eyes receiving injections of 50 and 100 μL/eye returned to the normal range within 10 minutes after IVT injections (Fig. 6). In contrast, the IOP in eyes receiving injections of 150 μL/eye remained high at 10 min after injection and only returned to the normal range 30 min after injection. In the 150 μL/eye group, there was a significant difference in IOP immediately after injection compared to pre-injection ($p = 0.0302$). The dosing solution leaked from one eye after injection of 150 μL, leading to a smaller increase in the IOP for this animal compared with the other eyes in the 150 μL/eye group, indicating that a smaller volume remained in the eye.

Table 2. Incidence of IVT injection-related ocular findings in gross, slit lump biomicroscopic, and indirect ophthalmoscopic examinations in cynomolgus monkeys.

Dosing volume	Findings	Day 0	Day 3	Day 10
50 μL/eye	Normal	3/3	3/3	3/3
100 μL/eye	Normal	3/3	3/3	3/3
150 μL/eye	Normal	2/3	3/3	3/3
	Leakage of dosing solution	1/3	0/3	0/3

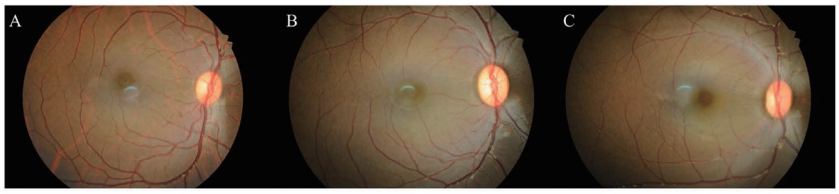

Fig. 4. Fundus photographs on Day 10 at 50 µL/eye (A), 100 µL/eye (B), and 150 µL/eye (C).

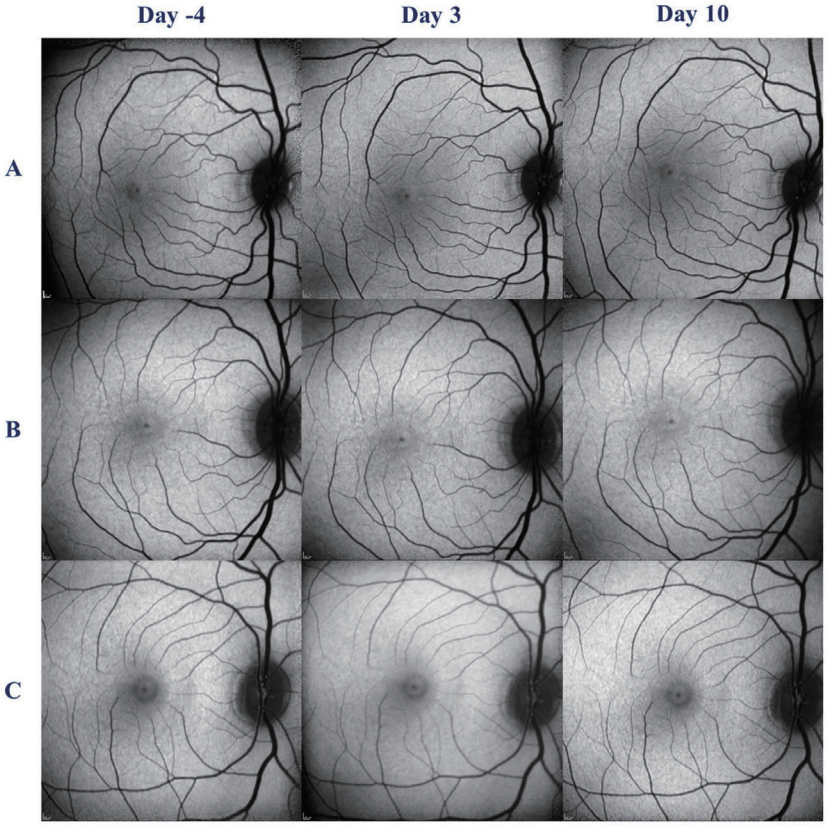

Fig. 5. FAF images on Days -4, 3, and 10 at 50 µL/eye (A), 100 µL/eye (B), and 150 µL/eye (C).

Corneal and retinal findings, anterior chamber depth by OCT, segment OCT, retinal findings by posterior segment OCT, and axial length changes

There were no findings in the cornea and retina in anterior or posterior segment OCT (Fig. 7). Anterior chamber depth was measured approximately 20 min after administration by anterior segment OCT and showed no effect of IVT administration (Table 3). The AL was also not affected by IVT administration (Table 4).

Functional changes in the retina by ERG examinations

There were no IVT injection-related ERG waveform changes (DA 0.01 ERG, DA 3 ERG, DA 3 OPs, LA 3 ERG, and LA 31 Hz ERG) in any animal (Fig. 8 and Fig. 9).

Histopathological examination

Hematoxylin and eosin (HE)-stained sections on Day 16 from cynomolgus monkey eyes injected with D-PBS at 50, 100, and 150 µL/eye. There were no IVT injection-related histopathological findings in any tissue, including

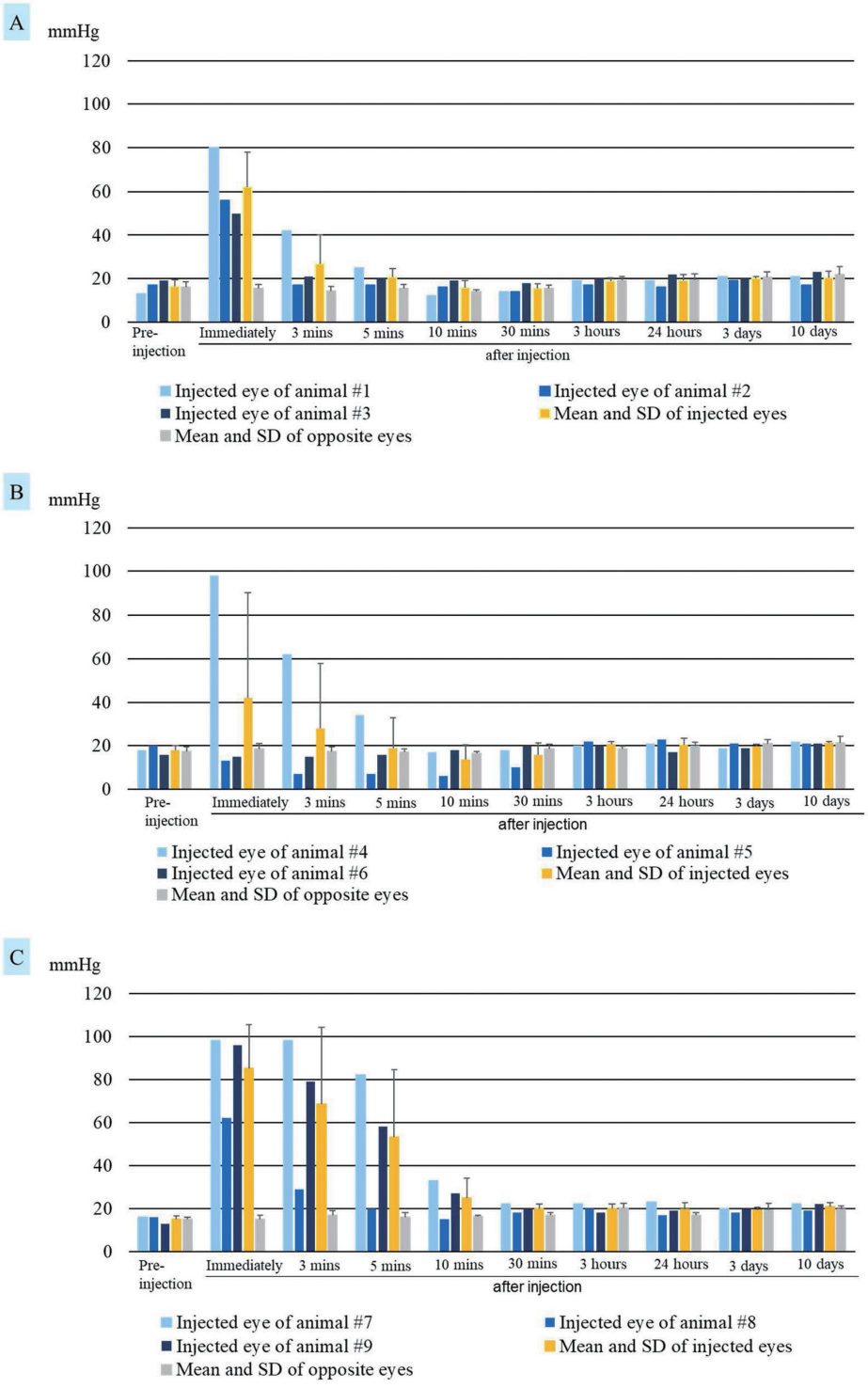

Fig. 6. IOP changes in cynomolgus monkey eyes after IVT injections of 50 (A), 100 (B), and 150 µL/eye (C). IOP was measured at the times indicated in the graph. Individual data for 3 animals per volume are shown. Data for Animal #8 (150 µL/eye group) is an outlier due to leakage of dosing solution after injection to the eye. Mean ± standard deviation (n = 3). *$p < 0.05$ (paired t-test).

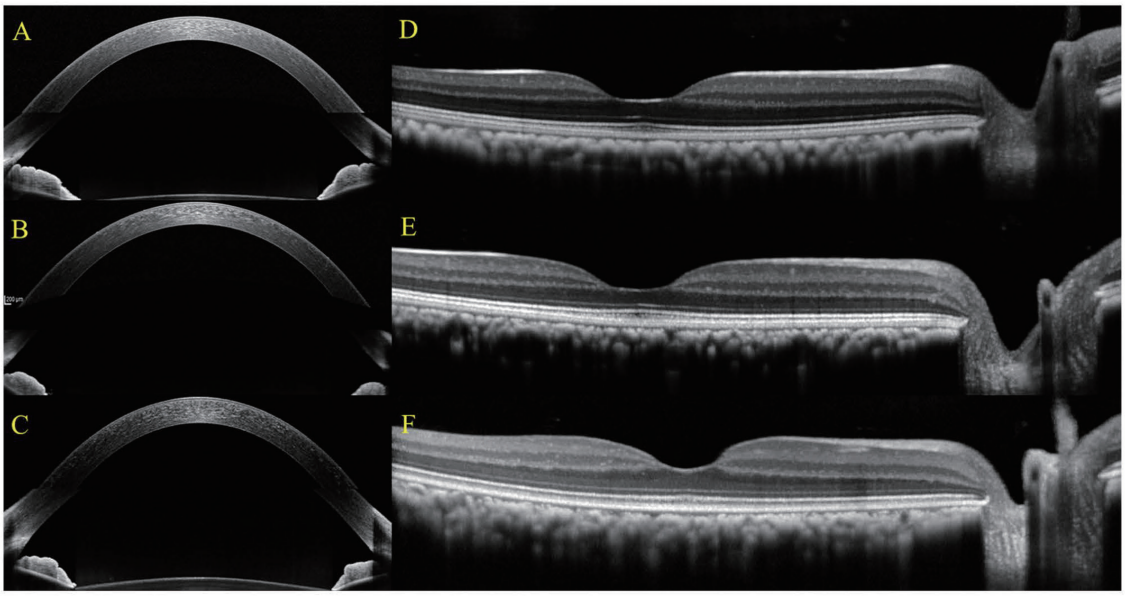

Fig. 7. Analysis of the corona after injection of D-PBS at 50, 100, and 150 µL/eye in cynomolgus monkeys. Anterior segment OCT images of the cornea on Day 3 (A-C) and posterior segment OCT images of the retina on Day 3 (D-F).

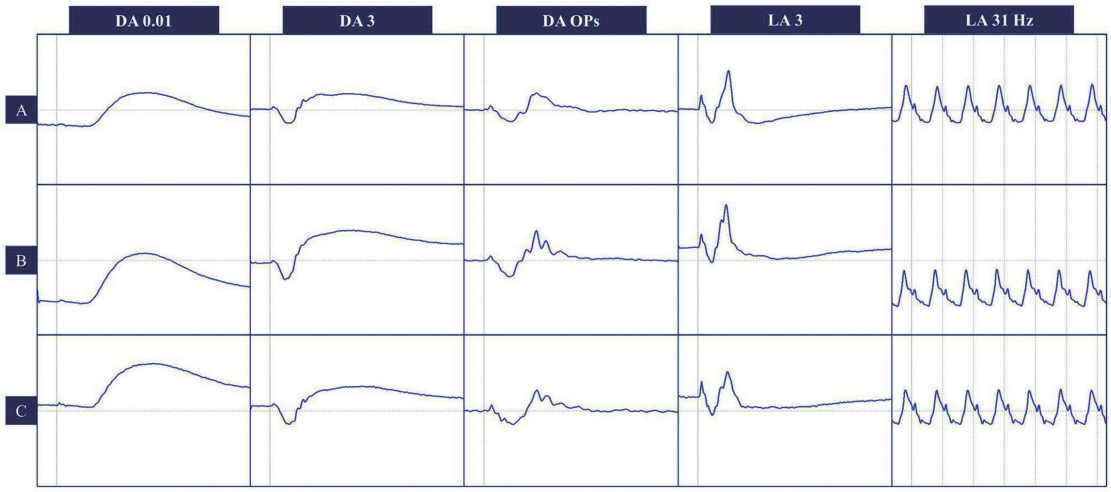

Fig. 8. Waveforms of ERG on Day 4 in cynomolgus monkeys injected with D-PBS at 50 µL (A), 100 µL (B), and 150 µL (C). There were no significant ERG waveform changes prior to or after IVT injections in any animal.

the cornea, retina, and optic nerve (Fig. 10).

DISCUSSION

According to information taken from the packaging slips available on the website of the Japanese Pharmaceuticals and Medical Devices Agency (http://www.pmda.go.jp/PmdaSearch/iyakuSearch/), the standard dose volume is 50 µL/eye for IVT drugs on the market for use in humans (ranibizumab, aflibercept, brolucizumab, and faricimab), with the largest dose volume of 100 µL/eye for triamcinolone acetonide. The safety of IVT injections

Table 3. Anterior chamber depths (μm) of cynomolgus monkey eyes with IVT injections of different dosing volumes.

Dosing volume	Day -4	Day 0 (after dosing)	Day 3	Day 10
50 μL/eye	656.33 ± 10.41	656.00 ± 20.78	644.33 ± 8.50	649.00 ± 8.89
100 μL/eye	641.00 ± 9.85	628.33 ± 9.61	640.33 ± 10.41	642.00 ± 15.39
150 μL/eye	657.33 ± 23.54	644.67 ± 11.02	664.33 ± 18.50	651.00 ± 23.39

Mean ± standard deviation (n = 3). No significant differences (Dunnet's test).

Table 4. Axial lengths (mm) of cynomolgus monkey eyes with IVT injections of different dosing volumes.

Dosing volume	Day -4	Day 3	Day 10
50 μL/eye	18.40 ± 0.42	18.38 ± 0.35	18.35 ± 0.24
100 μL/eye	18.38 ± 0.49	18.48 ± 0.35	18.41 ± 0.42
150 μL/eye	18.22 ± 0.20	18.10 ± 0.30	18.21 ± 0.29

Mean ± standard deviation (n = 3). No significant difference (Dunnet's test).

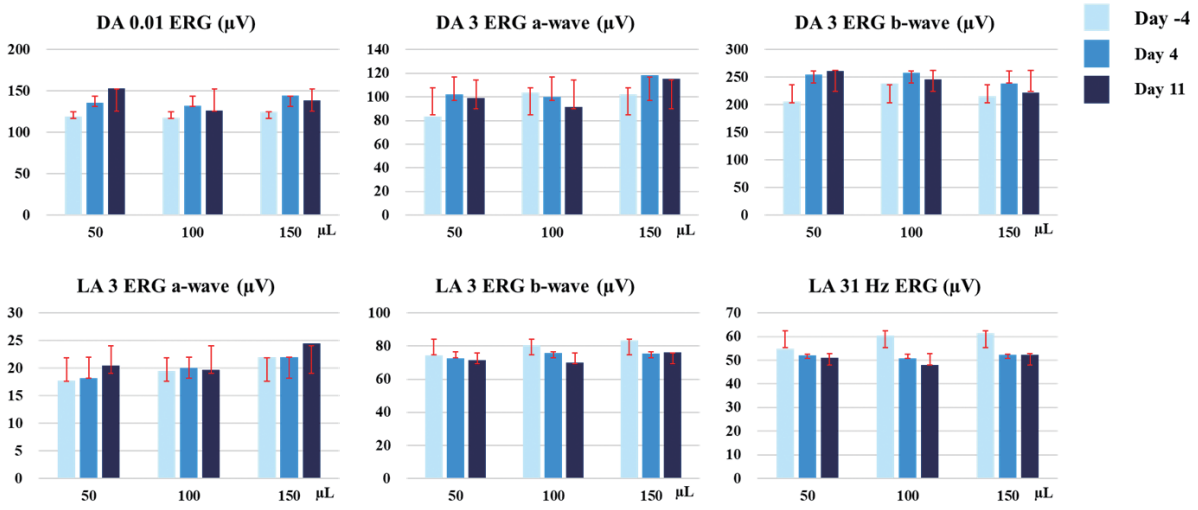

Fig. 9. Changes in amplitudes of DA 0.01 ERG, DA 3 ERG (a and b-wave), LA 3 ERG (a and b-wave), and LA 31 Hz ERG. No significant differences (Dunnet's test) in any ERG dataset.

at these dose volumes is considered established for use in humans. Similarly, dose volumes of 100 μL/eye applied in cynomolgus monkeys are likely to cause no abnormalities that could lead to visual impairments (Nork *et al.*, 2012; Shen *et al.*, 2014; Ye *et al.*, 2015; Cheung *et al.*, 2020), but there have been no systematic studies on the technical aspects of IVT injections in cynomolgus monkeys. This includes further considerations on the maximal tolerated dosage volume of IVT injections in the cynomolgus monkey. In this study, volumes of 50, 100, and 150 μL/eye were selected, and comprehensive ocular examinations consisting of general ocular examinations, PLR tests, FAF examinations, IOP measurements, OCT examinations of the anterior segment and posterior segment of the eye, AL measurements, ERG examinations, gross necropsy, and histopathological examinations after necropsy were conducted.

General ocular examinations (gross, anterior segment, intermediate transparent media, and fundus observations) using a slit lamp biomicroscope and an indirect ophthalmoscope are commonly conducted in eye examinations for nonclinical safety assessment of drugs (Brock *et al.*, 2013; Onodera *et al.*, 2015). PLR tests are easy and useful procedures to evaluate retinal function, even though those

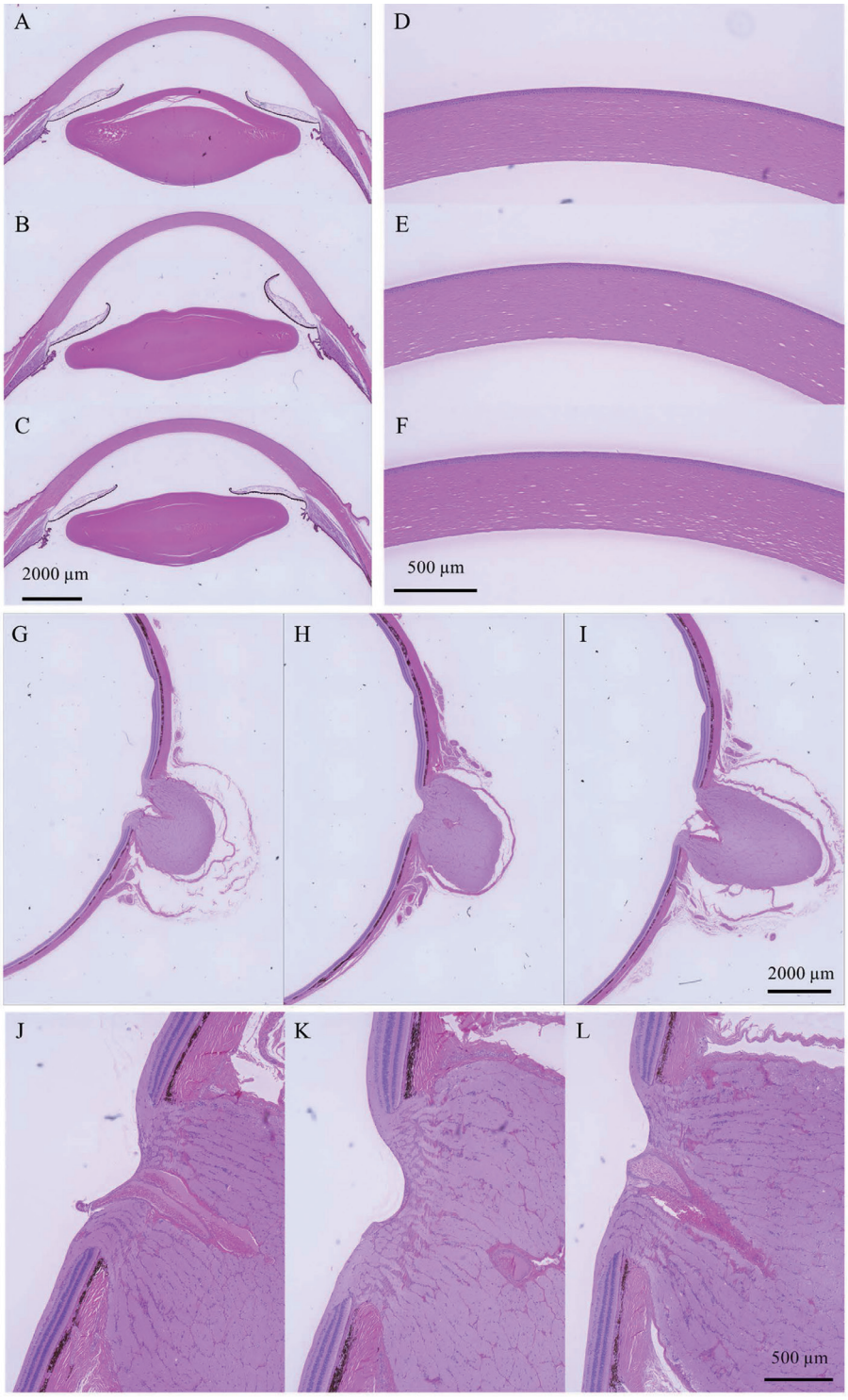

Fig. 10. Hematoxylin and eosin (HE)-stained sections on Day 16 from cynomolgus monkey eyes injected with D-PBS at 50 µL (A, D, G, and J), 100 µL (B, E, H, and K), and 150 µL (C, F, I, and L). Images A to C show the anterior segments, D to F show the corneal vertex, G to I show the retinas, and J to L show the optic nerve.

results do not directly reflect visual function (Ollivier *et al*., 2007; Onodera *et al*., 2015).

FAF imaging is based on the fluorescence from ocular endogenous fluorophores located in the retinal pigment epithelium and choroid, mainly lipofuscin and melanin, and it provides a retinal pigment epithelium health status and information about the pathogenesis and progression of retinal diseases (Calvo-Maroto and Cerviño, 2018; Yung *et al.*, 2016). The IOP also closely relates to the onset of glaucoma (Liu *et al*., 2018; Križaj, 2019), and an increase of liquid in the intravitreal cavity by IVT injection causes IOP elevation. Rebound tonometry provides an accurate, reproducible, and practical method for monitoring IOP in cynomolgus monkeys (Elsmo *et al*., 2011). OCT imaging of the posterior segment of the eye is widely used for diagnoses of diseases of the posterior vitreous, retina, and choroid, including in nonclinical safety assessment of drugs (Brock *et al*., 2013; Källberg, 2007; Soukup *et al*., 2020), whereas OCT imaging of the anterior segment is a rapidly evolving area that provides additional information on the status of the tear film, anterior chamber, and aqueous outflow pathway, as well as the morphology of the cornea (Wang *et al*., 2019; Alexopoulos *et al*., 2022; Beckmann *et al.*, 2022). Anterior chamber depths, which were assessed based on the anterior segment in OCT images, provided important information for glaucoma diagnosis (Wang *et al.*, 2019; Yang and Lin, 2019). ERG examinations in nonclinical safety assessment of drugs are a more sensitive parameter of retinal function to predict clinical outcomes (Brock *et al*., 2013). Notwithstanding any morphological or functional examinations conducted during the ante-mortem period, histopathology examinations of the eye are important procedures for nonclinical safety assessment of drugs (Shibuya *et al.*, 2015).

In this study, no IVT injection-related ocular findings were observed in slit lamp biomicroscopic and indirect ophthalmoscopic examinations, PLR tests, FAF examinations, anterior and posterior segment OCT examinations, anterior chamber depth measurements, ERG waveforms, or histopathological examinations in any animal. Based on these results, IVT injections at 150 μL/eye or less are considered to have caused no severe adverse ocular abnormalities over the 16-day period in this study. This timespan should be sufficient to assume that no permanent damage was done to the eye.

However, injecting larger volumes requires additional precautions to assure complete dosing. In one animal in the 150 μL/eye group, leakage of the dosing solution from the eye was observed, calling into question the success of administration in this animal. In addition, recovery from IOP elevation immediately after IVT injection of 150 μL/eye was delayed compared with injections of 50 and 100 μL/eye. Although these transient IOP elevations did not cause functional and morphological abnormalities, shorter IOP elevations are preferable to assure the safety of the procedure.

Therefore, we concluded based on our results that 100 μL/eye is the maximal appropriate IVT injection volume for studies in cynomolgus monkeys. We hope the results of our study will be helpful for nonclinical investigations in cynomolgus monkeys as needed to bring new modalities such as large molecules and gene therapy to the market.

Conflict of interest---- The authors declare that there is no conflict of interest.

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