

The ocular toxicity and pharmacokinetics of simvastatin following intravitreal injection in mice

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Abstract

• **AIM:** To investigate the retinal toxicity and pharmacokinetics of simvastatin intravitreally injected into mice.

• **METHODS:** Forty-eight 6-8-week-old C57BL/6J mice were used in this study. Simvastatin was intravitreally injected into the right eye of each mouse; the left eye was injected with vehicle and was used as a control. Bilateral dark-adapted electroretinography (ERG) was performed 1 and 7d following injection. Histology was examined using a combination of light, fluorescence and electron microscopy. High-performance liquid chromatography (HPLC) was used to determine the decay in the retinal simvastatin concentration.

• **RESULTS:** ERG revealed no significant changes in the simvastatin-injected eyes compared to control. Histologic studies showed normal retinal morphology in eyes injected with simvastatin up to a final vitreal concentration of 200 $\mu\text{mol/L}$. No significant changes in the number of photoreceptors, bipolar cells or ganglion cells were found. The retinal simvastatin concentration decayed exponentially, with a half-life of 1.92-2.41h.

• **CONCLUSION:** Intravitreal injection of up to 200 $\mu\text{mol/L}$ simvastatin produced no signs of adverse effects in the mouse retina. Simvastatin reaches the retina shortly after intravitreal injection and has a short half-life.

• **KEYWORDS:** simvastatin; retina; electroretinography; high-performance liquid chromatography; electron microscopy; intravitreal injection

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INTRODUCTION

Statins, potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have widely been prescribed for the treatment of hyperlipidemia since 1987^[1] and have recently been demonstrated to possess an expanding spectrum of activities, including anti-inflammatory, anti-angiogenic, anti-oxidant^[2], immunosuppressive^[3] and neuroprotective^[4-6] effects. Statins have been reported to be beneficial to patients with a variety of ocular diseases such as diabetic retinopathy^[7], age-related macular degeneration (AMD)^[8] and retinal ischemia and to potentially be useful in glaucoma^[9-12].

Simvastatin, the semisynthetic statin tested in the present study, is one of the most potent of all the identified statins; simvastatin inhibits the diabetes-induced increases in vascular endothelial growth factor (VEGF) expression, protects the blood-retinal barrier and suppresses the progression of streptozotocin-induced diabetic retinopathy in rats^[13-15]. A recent study has shown that simvastatin activated the anti-oxidative defense protein HO-1 in cultured human retinal pigment epithelial (RPE) cells^[16] and that this activity contributed to its cytoprotective effect against AMD. Considering the potential adverse side effects of intravitreally-injected steroids [e.g. intraocular pressure (IOP) elevation, cataract] and anti-VEGF drugs (e.g. intolerance, cost, and unknown deleterious cumulative systemic effects) commonly used in the treatment in AMD or diabetic retinopathy, simvastatin might be a potential alternative drug candidate as several reports have suggested that simvastatin inhibits the progression of these two diseases^[17-20]. It has also been shown that simvastatin enhanced retinal ganglion cell (RGC) survival and protected visual functions in rodent models of acute retinal ischemia/reperfusion injury^[21-23] and optic nerve lesioning^[24]. Simvastatin inhibits the Rho/Rho-kinase pathway and thus might have therapeutic potential in the prevention of cicatricial contraction of proliferative membranes *in vivo* study. And thus, simvastatin might provide a new strategy for the treatment and prevention of the development of proliferative vitreoretinal diseases^[25].

In most of the literature, statins were administered orally or injected intraperitoneally. From a clinical perspective, some ocular conditions would be better managed if the intraocular statin concentration was increased more efficiently *via* direct intravitreal injection; such conditions include when ocular conditions are acute or when systemic side effects should be avoided^[26-27]. From a research perspective, intravitreal injection is superior to systemic delivery routes because the other eye can serve as an internal control in certain experimental settings^[28].

Our objective was to study the retinal toxicity of simvastatin to mice using electroretinography (ERG) and electron microscopy. These results are pivotal in guiding future laboratory and clinical studies on the dosage and delivery of simvastatin. This study is particularly relevant to mouse research models, as such disease models are widely available and are easily combined with genetic manipulations or surgical interventions^[29]. The long-term goal of this line of investigation is to utilize mouse models to investigate the potential neuroprotective effects of simvastatin against a variety of retinal diseases such as glaucoma and AMD^[30-32].

MATERIALS AND METHODS

Animal Preparations Forty-eight 6-8 weeks old C57BL/6J mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA) were used for the experiments. The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the animal welfare guidelines of the IACUC of Baylor College of Medicine.

Intravitreal Injection Because vitreal simvastatin concentrations of 5 and 15 $\mu\text{mol/L}$ have been shown to be effective and did not produce sign of toxicity in previous animal studies^[24-25], we selected higher vitreal simvastatin concentrations of 50 and 200 $\mu\text{mol/L}$ for this retinal toxicity study to provide at least 10 \times margin over the previous studies.

Simvastatin (#S6196, Sigma-Aldrich Corp. St. Louis, MO, USA) was converted to its active form according to the manufacturer's instructions^[33], diluted in sterile buffered balanced salt solutions (pH 7.5; Alcon Laboratories, Inc., Fort Worth, TX, USA), and then filtered using a 0.22- μm PVDF filter (Millipore, Billerica, MA, USA). Aliquots were stored at -20°C before use. The mice were first anesthetized by intraperitoneally injecting weight-based doses of ketamine (95 mg/mL) and xylazine (5 mg/mL). Then, a single drop of 0.5% proparacaine hydrochloride (Alcon Laboratories, Inc., Fort Worth, TX, USA) and 1% tropicamide were applied to both eyes. One microliter of 0.5 or 2.0 mmol/L simvastatin was injected into the vitreous of the right eye under a stereomicroscope using a Nanofil syringe fitted with a 33-gauge beveled needle (WPI, Sarasota, FL, USA). One microliter of an equivalent vehicle solution was injected into

the left eye as a control. Assuming that the injected 1 μL of solution was diluted into the 10 μL of vitreous fluid in the eye^[34-35], the vitreal concentrations of simvastatin were 50 and 200 $\mu\text{mol/L}$ for the injected 0.5 and 2.0 mmol/L solutions, respectively. The needle was inserted behind the limbus through the pars plana at an oblique angle to avoid damaging the crystallized lens. To prevent the injected solution from escaping the eye when the needle was withdrawn, the needle tip was held in the eye for 30s after the injection to facilitate mixing.

Electroretinography Recordings *In vivo* scotopic ERG was recorded bilaterally from the mice 1 and 7d after intravitreal injection. A pre-injection measurement was performed on a different cohort of mice as a reference. Prior to each ERG assessment, the mice were allowed to adapt to the dark for at least 2.5h. Under dim redlight, the mice were prepared for ERG testing as described previously^[30]. The signals were amplified using a Grass P122 amplifier and were band-pass filtered from 0.1 to 1000 Hz (Grass Instruments, West Warwick, RI, USA). The data were acquired using a National Instruments data acquisition board (USB-6216, National Instruments, TX, USA) at a sampling rate of 10 kHz. The traces were analyzed using custom codes written in MATLAB (MathWorks, Natick, MA, USA)^[30].

The flashes used for scotopic b-wave measurements were generated using cyan light-emitting diodes calibrated with a photometer (ILT1700, International Light, MA, USA) and were converted to the unit photoisomerizations/rod, where 1 scot cd $\text{m}^2=581$ photoisomerizations/rod/s^[30]. To remove oscillatory potentials before fitting, the scotopic b-wave was digitally filtered using the `filtfilt` function in MATLAB (low-pass filter; $F_c=60$ Hz). The positive and negative scotopic threshold responses (STRs) were measured under stimuli of various intensities as described previously^[30]. The cone ERG recording was performed according to a paired-flash method using xenon flashes^[36]. An initial conditioning flash saturated both rods and cones 2s before a probe flash. The ERG signal recorded by the probe flash is attributed to responses driven by the cones because cones recover faster than rods. In this manuscript, the stimulus intensities are presented on a log scale, where $1\text{E}+0=1$ photoisomerizations/rod. All statistical analyses were performed using paired *t*-tests in SPSS version 20 (IBM).

Pharmacokinetic Analysis *via* High-performance Liquid Chromatography The ocular concentration of simvastatin in the current study was measured using the retinal tissue instead of the vitreous humor, which is commonly used in rabbit pharmacokinetic models^[37-39]. This experimental approach is advantageous because the retina itself is typically the target of treatment.

The injected eyes were enucleated at 1, 3, 6, 12, 24 or 48h to measure the retinal concentration of the drug. For each of the above time points, at least three microcentrifuge tube samples were analyzed. Each sample contained four whole retinæ extracted from four eyes. First, 100 µL of phosphate buffered saline (PBS) at 4°C was added to the sample before the samples were homogenized using amotorized mortar (#47747-370, VWR International LLC, Radnor, PA, USA) for a total of 6min. Then, 900 µL of acetonitrile at 4°C was added to the sample. The mixture was subsequently vortexed for 2min and then ultra-centrifuged at 68 000× g for 20min at 4°C. The supernatant was transferred to auto-sampler vials and then concentrated to a volume of 50-80 µL using a SpeedVac. The resulting samples contained 50% acetonitrile and 0.1% (v/v) trifluoroacetic acid (TFA).

The simvastatin standards (Sigma-Aldrich Corp., St. Louis, MO, USA) were dissolved in 100 µL of 50% acetonitrile containing 0.1% (v/v) TFA and were vortexed for 1min. All samples were filtered through a 0.45 µm Acrodisc® 3 CR PTFE filter to remove insoluble particles before injection into a C18 high-performance liquid chromatography (HPLC) column (Grace Davison Discovery Sciences, 4.6×250 mm, 5-micron, Vydac 218TP C18).

The samples were analyzed using Shimadzu gradient HPLC (Shimadzu Co., Kyoto, Japan) in a system of 0.1% aqueous TFA (buffer A) versus 0.1% TFA in acetonitrile (buffer B) at a flow rate of 1 mL/min. A 100 µL volume of each sample was injected into the C18 column, which was pre-equilibrated with 50% buffer B. Simvastatin was eluted with a two-step gradient of acetonitrile: 50% acetonitrile containing 0.1% TFA for 10min followed by 80% acetonitrile containing 0.1% TFA for an additional 10min. Simvastatin was monitored by measuring the absorbance at 238 nm using a Shimadzu SPD-M20A UV/VIS photodiode array detector (Shimadzu Co.) interfaced to a computer running Shimadzu LCsolution software. The standard curve, which was created using eight different concentrations of the simvastatin standard, was linear from 2 to 640 pmol (correlation coefficient 0.99988). The detection limit using this method was estimated to be approximately 1.5 pmol (signal-to-noise ratio greater than 2).

Pharmacokinetic data were then analyzed using Phoenix WinNonlin software version 5.3 (Certera, St. Louis, MO, USA). The following equation was used for the one-compartment model:

$$C(t) = \frac{\text{Dose}}{V_d} \times \exp^{-Kt}$$

where C(t) denotes the quantity of simvastatin at time t, V_d denotes the volume of distribution, and $K(h^{-1})$ denotes the elimination rate constant. In addition, data were analyzed using a non-compartmental model for reference.

Retinal Histology On the 7th day following injection, anesthetized mice were euthanatized *via* cervical dislocation,

and their eyes were enucleated after ERG recording was conducted. For histologic studies, a large full-thickness incision was made in the cornea, and the anterior segment was removed. Then, the eye cups were fixed in 3% glutaraldehyde in phosphate buffer. The tissue was then washed in 1 mol/L sodium phosphate buffer, pH 7.3, and post-fixed in 1% osmium tetroxide for 1h at room temperature. The dehydrated tissue was infiltrated with acetone and Poly/Bed 812 plastic resin. The tissue was then embedded in plastic block molds with 100% Poly/Bed 812. Sections (1 µm thick) were generated on an ultramicrotome, placed on glass slides and stained with toluidine blue for light microscopy. The areas of interest were trimmed, and 80-nm-thick ultra-thin sections were sliced using a Leica Ultracut R Ultramicrotome, mounted on 100-mesh copper grids, and stained with 2% uranyl acetate and Reynold's lead citrate. The specimens were imaged using a Zeiss CEM 902 electron microscope to study the retinal ultrastructure. For immunohistochemistry (IHC) and cell counting studies, the eyes were carefully dissected to isolate whole retinæ, which were then incubated in 4% paraformaldehyde (Electron Microscopy Science, Fort Washington, PA, USA) in Dulbecco's phosphate-buffered saline (DPBS, pH7.4, Invitrogen, LaJolla, CA, USA) at room temperature for 45min for fixation. An indirect antibody method was adopted for IHC. First, retinæ were sliced into vertical 40-µm-thick sections using a microtome (Vibratome; Leica Microsystems, Bannockburn, IL, USA) and then blocked with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in TBS [DPBS containing 0.5% Triton X-100 (Sigma) and 0.1% sodium azide (Sigma), pH 7.2] at 4°C overnight to reduce nonspecific labeling. The free-floating sections were incubated in primary antibodies diluted in TBS containing 3% donkey serum at 4°C for 4d. Controls lacking primary antibodies were also processed. After several rinses, the sections were transferred and incubated in a TBS solution containing 3% normal donkey serum and donkey-hosted secondary antibodies conjugated with Cy3 (1:200, Jackson ImmunoResearch) or Alexa Fluor 488 (1:200 dilution, Molecular Probes, Eugene, OR) at 4°C overnight. A fluorescent nuclear dye, TO-PRO3 (1:3000 dilution, Molecular Probes, Eugene, OR, Cat. No. T3605), was applied together with the secondary antibodies.

After rinsing several times, the sections were mounted with Vectashield medium (Vector Laboratories, Burlingame, CA, USA), cover slipped and, finally, observed under a confocal laser scanning microscope (LSM 510; Zeiss, Thornwood, NY, USA). Images were acquired using Zeiss LSM software and a 40× or a 63× oil-immersion objective. Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA) was used to crop images and to apply uniform brightness and contrast adjustments.

In the present study, rod bipolar cells were immuno-labeled with a mouse antibody against PKCα (1:250 dilution; BD

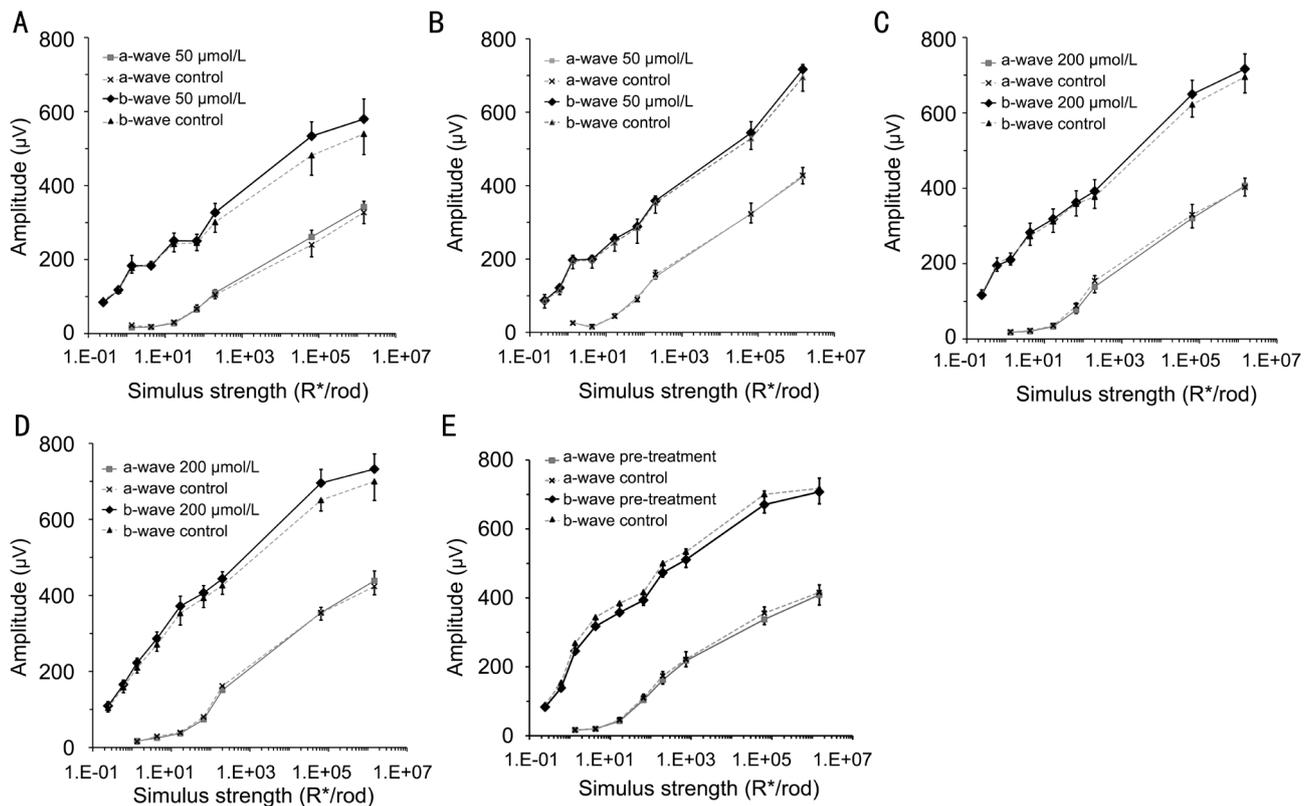


Figure 1 Changes in the amplitudes of the scotopic a-wave and b-wave against an increasing stimulus intensity measured after injection of simvastatin A: Day 1 after injection of 50 µmol/L simvastatin; B: Day 7 after injection of 50 µmol/L simvastatin; C: Day 1 after injection of 200 µmol/L simvastatin; D: Day 7 after injection of 200 µmol/L simvastatin; E: Pre-injection.

Transduction Labs, San Jose, CA, USA, Cat. No. 610107)^[40]. Photoreceptor nuclei in the outer retina and ganglion cell nuclei in the inner retina were stained using the fluorescent nuclear dye TO-PRO3^[41]. Cone cell bodies were immunolabeled with a rabbit antibody against GNAT2 (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA, Cat. No. SC-390)^[42-43]. The dimensions were measured using built-in tools in Zeiss LSM software.

To determine whether injection of 200 µmol/L simvastatin produced any toxicity to the retina and caused any retinal cell loss, the thicknesses of the two retinal nuclear layers were gauged, and the numbers of cone soma, rod bipolar cells and ganglion cells were systematically counted in the two groups. Five mice, each of which received injections of 200 µmol/L simvastatin into one eye and vehicle solution into the other eye, were sacrificed. Three sections from each eye were imaged, for a sample size of 15 per group; the number of cells in each 200-µm region were counted, and the dimensions were measured.

RESULTS

Electroretinography Changes in the amplitudes of the scotopic a-wave and b-wave against an increasing stimulus intensity measured 1 and 7d after injection of 50 or 200 µmol/L simvastatin are shown in Figure 1. As shown in panels A-D, eyes injected with either 50 or 200 µmol/L simvastatin showed no significant differences in the a- or b-waves at 9 stimulus

intensities 1 or 7d after injection on ERG compared to the control eyes. Baseline a-wave and b-wave responses from another 7 pairs of eyes before injection are shown in panel E for reference.

To test whether simvastatin produced any toxicity to the retinal cone pathway, we employed paired flash ERG recordings to isolate the cone-driven responses from the rod-driven responses. As shown in Figure 2, there were no significant differences in the cone a-wave or b-wave between the simvastatin-injected eyes and the vehicle-injected control eyes based on ERG recordings performed 1 and 7d after injection (7 pairs of eyes). Figure 3 shows the changes in the positive STRs (pSTRs) and the negative STRs (nSTRs) in response to increasing stimulus intensities 1 and 7d after injection of 50 or 200 µmol/L simvastatin. There were no significant differences in the mean pSTR or nSTR between simvastatin-injected eyes and control eyes for either concentration at each time point. Given that the pSTR and the nSTR represent inner retinal signaling from third-order neurons such as RGCs and AII amacrine cells^[44-46], our results suggest that a vitreal simvastatin concentration of 200 µmol/L or less does not alter the electrophysiological functions of ganglion cells.

Pharmacokinetic Analysis The peak simvastatin concentration was calculated according to appropriate standard curves using LCsolution software. These standard curves, created using eight different concentrations of simvastatin, were linear from

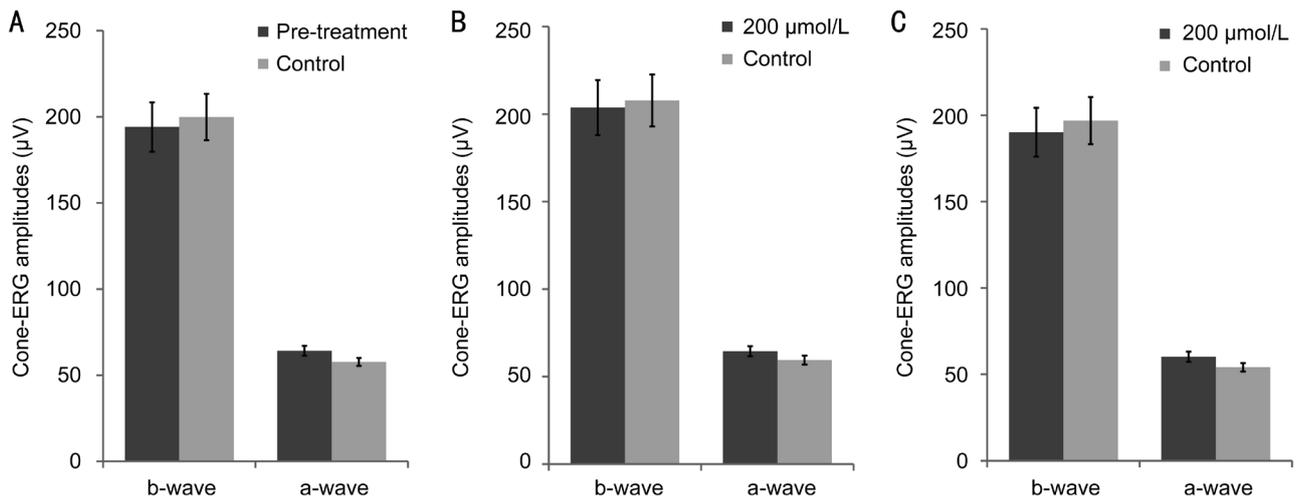


Figure 2 The differences in the cone a-wave or b-wave between the simvastatin-injected eyes and the vehicle-injected control eyes based on ERG recordings A: Pre-injection; B: Day 1 after injection of 200 µmol/L simvastatin; C: Day 7 after injection of 200 µmol/L simvastatin.

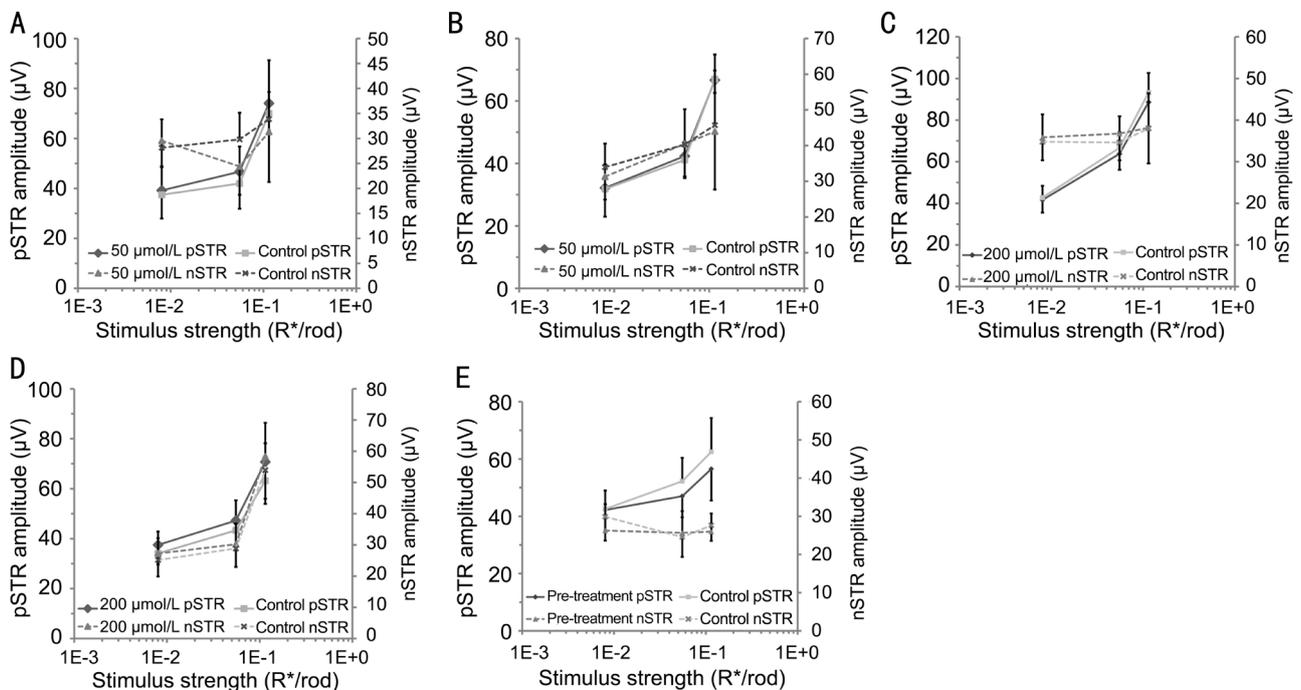


Figure 3 Changes in the positive STRs (pSTRs) and the negative STRs (nSTRs) in response to increasing stimulus intensities after injection of simvastatin A: Day 1 after injection of 50 µmol/L simvastatin; B: Day 7 after injection of 50 µmol/L simvastatin; C: Day 1 after injection of 200 µmol/L simvastatin; D: Day 7 after injection of 200 µmol/L simvastatin; E: Pre-injection.

0 to 640 pmol (correlation coefficient >0.99988). The detection limit was below 2 pmol.

Simvastatin was detectable in retinal samples collected 1h following the injection. Samples collected at the subsequent post-injection time points showed decreasing concentrations of simvastatin. These findings suggest that intravitreally injected simvastatin arrives at the retina shortly after injection; additionally, simvastatin reached its maximum concentration within 1h. The change in the mean simvastatin concentration in the retina over time as measured using HPLC was fitted using a one-compartment model (Figure 4). The half-lives of simvastatin for injected two concentrations of 200 and 50 µmol/L

were found to be 1.92 and 2.41h, respectively (Table 1). Projected C_{max} were 506.37 and 75.23 mol, respectively. Mean residence time were 2.77 and 3.48h, respectively.

Retinal Histology Gross examination of eye specimens revealed no evidence of retinal tearing, retinal detachment, or retinal hemorrhage or any signs of infection in any of the simvastatin-injected or control eyes. The mean thicknesses of the outer nuclear layer (ONL) were 62.5 ± 0.9 and 63.0 ± 1.3 µm in the simvastatin and control groups. The ONL thicknesses in terms of cell number (rods and cones) were 11.7 ± 0.2 and 11.9 ± 0.2 in the simvastatin and control groups. The mean numbers of cone soma were 14.5 ± 1.7 and 15.4 ± 1.0 per 200-µm

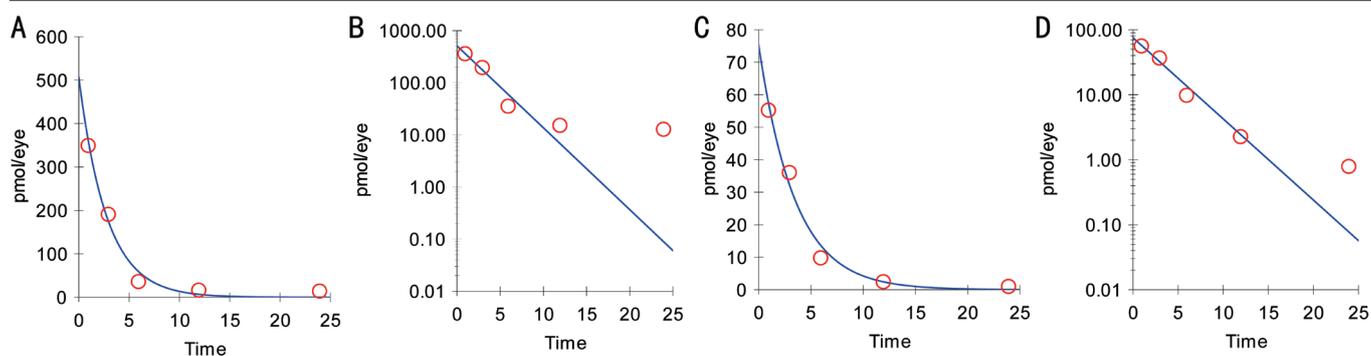


Figure 4 The change in the mean simvastatin concentration in the retina over time as measured using HPLC was fitted using a one-compartment model A, B: Injection of 200 µmol/L simvastatin; C, D: Injection of 50 µmol/L simvastatin.

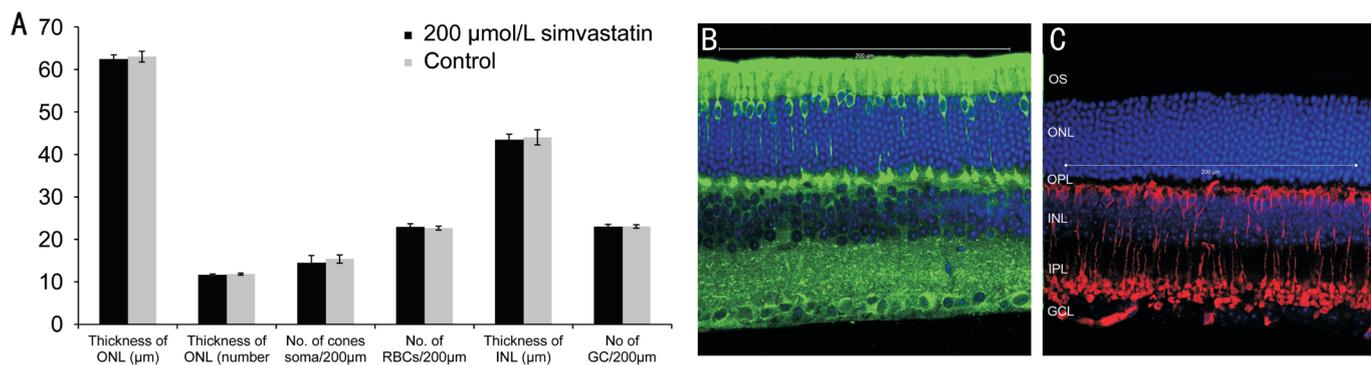


Figure 5 The cellular profiles of the Simvastatin-injected group with those of the control group A: A bar chart comparing the cellular profiles of the Simvastatin-injected group with those of the control group. Data points and error bars represent the mean values and standard errors, respectively. B: A representative fluorescent micrograph of a retina processed by staining with the nuclear dye TO-PRO3 (blue) and the antibody GNAT2 (green). A higher-than-normal exposure was used for the green channel to optimally show the cone soma and to facilitate cell counting. Ganglion cell soma were stained in blue in the inner retina. C: A representative fluorescent micrograph of a retina processed by staining with the antibody PKCα (red) and the nuclear dye TO-PRO3 (blue).

Table 1 Pharmacokinetic parameters of simvastatin in retina

Parameters	T _{1/2} (h)	C _{max} (pmol)	C _{max} (ng, per g of retina)	T _{max} (h)	AUC _{all} (h x µg/mL)	MRT (h)	CL (mL/h)
NCA 200 µmol/L	2.37	347.86	105.41	1	1429.3	2.38	0.14
1CM 200 µmol/L	1.92	506.37	153.45	0	1400.6	2.77	0.14
NCA 50 µmol/L	2.31	55.04	16.68	1	256.0	2.69	0.19
1CM 50 µmol/L	2.41	75.23	22.80	0	261.7	3.48	0.19

NCA: Non-compartmental analysis; 1CM: 1-compartment modeling; C_{max}: Observed/predicted max quantity; T_{max}: Time to C_{max}; AUC: Area under the curve; MRT: Mean residence time; CL: Clearance. Mean wet weight of mouse retina=3.3 mg; data taken from Cerani *et al*^[47].

Table 2 Comparing the retinal cellular profile of simvastatin injected eyes and vehicle injected control eyes

Groups	Thickness of ONL (µm)	Thickness of ONL (cell)	No. of cones soma/200 µm	No. of RBCs/200 µm	Thickness of INL (µm)	No. of GC/200 µm
Control	63.0±1.3	11.9±0.2	15.4±1.0	22.7±0.5	44.0±1.8	23.1±0.4
Simvastatin 200 µmol/L	62.5±0.9	11.7±0.2	14.5±1.7	22.9±0.7	43.4±1.3	23.0±0.6
Simvastatin/control	99.2	98.3	94.2	100.9	98.6	99.6
P	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05

horizontal ONL section in the simvastatin and control groups. Similarly, the mean numbers of rod bipolar cells (RBCs) in the inner nuclear layer (INL) were 22.9±0.7 and 22.7±0.5 per 200 µm in the simvastatin and control groups. The mean thicknesses of the INL were 43.4±1.3 and 44.0±1.8 µm in the simvastatin and control groups. Finally, the numbers of ganglion cells per 200-µm horizontal ganglion cell layer

section were 23±0.6 and 23.1±0.4 in the simvastatin and control groups (Figure 5). There was no significant difference (paired *t*-test, *P*>0.05) in the measured layer thicknesses or in the numbers of counted neurons between the 200 µmol/L simvastatin-injected group and the control group (Table 2). These findings suggested that intravitreally injecting 200 µmol/L simvastatin did not induce a loss of retinal neurons.

Low magnification histologic examination *via* light microscopy conducted 7d after injection revealed normal neurosensory retinae. No signs of inflammation- or toxicity-induced changes in any retinal layer were found. There were no observable differences in layer thicknesses or cell morphologies between the simvastatin-injected retinae and the control retinae (Figure 6). High magnification histologic examination of individual retinal layers *via* electron microscopy revealed very similar ultrastructure and cell morphologies between the simvastatin-injected retinae and the control retinae (Figure 7). No abnormalities in photoreceptors, bipolar cells or ganglion cells were identified in the simvastatin-injected eyes 7d after injection.

DISCUSSION

Simvastatin has been suggested to have a pleiotropic protective effect against a variety of ocular diseases, including diabetic retinopathy, AMD, retinal ischemia and glaucoma. More recently, its protective effect against glaucomatous neuronal damage has been hypothesized to be related to its anti-inflammatory properties^[11]. However, the ocular toxicity and pharmacokinetics of simvastatin had not been adequately studied previously. In the present study, we have shown that intravitreal injection of up to 200 $\mu\text{mol/L}$ simvastatin produced no apparent adverse effects in the mouse retina based on results from functional tests *via* ERG and histologic examinations *via* fluorescence and electron microscopy.

ERG is a useful tool for evaluating retinal functions both experimentally and clinically. In the present study, ERG was performed using a comprehensive range of stimuli to assess the functions of a comprehensive range of retinal neurons. Depending on the stimulus intensity, the a-wave and the b-wave are generated *via* the rod pathway, the cone pathway or a combination of the two. The scotopic a-wave and b-wave data suggest that simvastatin up to a vitreal concentration of 200 $\mu\text{mol/L}$ does not influence the functions of rod photoreceptors or rod-driven bipolar cells. There were no significant differences in the cone a-wave or b-wave between the simvastatin-injected eyes and the control eyes. This observation indicated that a vitreal simvastatin concentration of 200 $\mu\text{mol/L}$ did not produce any signs of toxicity to the retinal cone pathway. The pSTR and the nSTR are the most sensitive components of the ERG, and our results suggest that a vitreal simvastatin concentration of 200 $\mu\text{mol/L}$ or less does not alter the electrophysiological functions of ganglion cells.

Traditionally, statins are administered systemically *via* oral ingestion, subcutaneous injection, or intraperitoneal injection. In contrast, delivery *via* intravitreal injection has not been commonly used but has the advantage of enabling the application of a higher dose intraocularly and monocularly within a short interval. Intravitreal injection is also likely to be more efficient when the therapeutic target site is located at

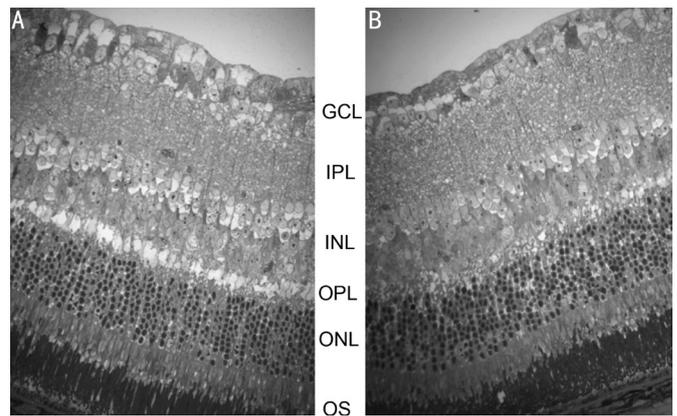


Figure 6 Light micrographs of vertical retinal sections of mouse eyes processed 7d after intravitreal injection of (A) the control vehicle solution or (B) 2.0 mmol/L Simvastatin GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; OPL: Outer plexiform layer; ONL: Outer nuclear layer; OS: Outer segment of photoreceptors.

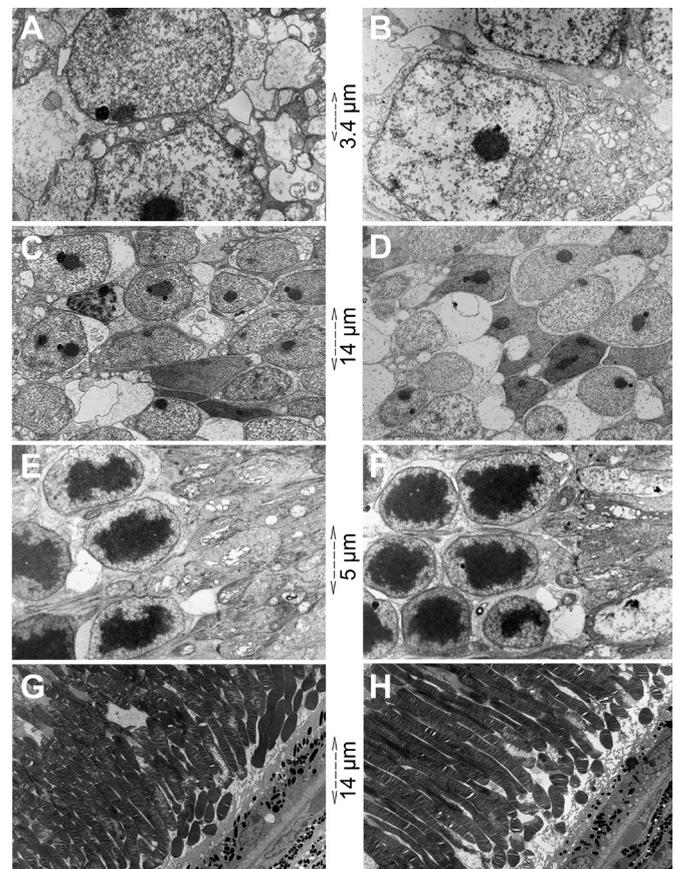


Figure 7 Electron micrographs of vertical retinal sections of mouse eyes processed 7d after intravitreal injection of (left column) the control vehicle solution or (right column) 200 $\mu\text{mol/L}$ Simvastatin A, B: Ganglion cells; C, D: Inner nuclear layer; E, F: Photoreceptor inner segment; G, H: Photoreceptor outer segment and retinal pigment epithelium.

the proximal retina, where drugs administered systemically moving from the choroid must overcome diffusion barriers to reach the retina. One successful application of simvastatin was that intravitreally administered 15 $\mu\text{mol/L}$ simvastatin

had no apparent adverse effects in rabbits and prevented the progression of induced proliferative vitreoretinopathy^[25].

We found that the ocular half-life of simvastatin in the retina following intravitreal administration was 1.92-2.41h; this half-life is relatively short compared with that of other drugs administered *via* intravitreal injection. For example, the half-lives of dexamethasone and triamcinolone were previously reported to be 3.48h and 1.57d, respectively^[37-38]. We cannot directly compare those findings to the present results because the previous studies performed measurements on the rabbit eye. We did not find any related data using mice. In the present study, the HPLC data suggested that intravitreally injected simvastatin rapidly reached the retina and had a short retinal half-life. Our findings indicate that a sustained release mechanism such as an intravitreal implant^[48] would be needed to extend the application of simvastatin to the treatment of chronic vitreal-retinal diseases.

Since the calculated half-life is about 2h and simvastatin is mostly cleared in 12h, the 1d time point is appropriate to evaluate acute toxicity and the 1wk time point was designed to evaluate delayed damages, for example, those resulted from inflammation or secondary degeneration. Our results have further shown that administering up to 200 $\mu\text{mol/L}$ simvastatin *via* intravitreal injection produced no apparent adverse effects on the retinal ultrastructure of mouse eyes, as the retinae showed no signs of anomalies one week after injection. Therefore, a vitreal simvastatin concentration between 50 and 200 $\mu\text{mol/L}$ would be a reasonable starting point for future therapeutic trials using mouse models of acute ocular diseases. Though the weakest point of this study is that the volume of the mouse vitreous is much smaller than that of the human vitreous, the present study is the first to assess the toxicity of simvastatin in mice, which represent a very useful model animal because of their potential for genetic modification and the wide availability of mouse models of disease. This study represents an important step in the exploration of the full potential benefits of simvastatin to patients with ocular disorders, including diabetic retinopathy, AMD, retinal ischemia, glaucoma and proliferative vitreoretinopathy. Intravitreal injection of simvastatin is a highly efficient route of delivery, but the half-life of intravitreally injected simvastatin is relatively short. To extend its application to the treatment of chronic ocular disorders, a slow-release drug delivery system or vehicle might be necessary to sustain a therapeutic simvastatin dosage in the retina.

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