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Effects of roscovitine, a cell cycling-dependent kinase inhibitor, on intraocular pressure of rabbit and retinal ganglion cell damage

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HIGHLIGHTS

- R-isomer and S-isomer of roscovitine showed IOP lowering effect in rabbit.
- R-isomer of roscovitine enhanced retinal ganglion cell death.
- S-isomer of roscovitine had a protective effect against retinal ganglion cell death.

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ABSTRACT

Glaucoma is characterized by increased intraocular pressure (IOP) and the death of retinal ganglion cells. Previously, we reported that roscovitine, a cell cyclin-dependent kinase (CDK) inhibitor, strongly induced relaxation of porcine trabecular meshwork cells, implicating an interaction with lowered IOP. In addition, the activity of CDKs is known to increase in response to high IOP, which is linked to retinal ganglion cell damage. However, the effects of roscovitine on IOP and retinal damage have not been investigated. Roscovitine has racemic isomers that differ in their inhibition of CDKs. Therefore, we investigated the effects of both the R-isomer and the S-isomer on the IOP of rabbits and on the death of cultured retinal ganglion cells. In the *in vivo* rabbit experiment, instillation of both isomers significantly lowered the IOP. In the *in vitro* cell experiment, the R-isomer amplified the effects of tunicamycin, an endoplasmic reticulum stress inducer, and increased oxygen–glucose deprivation-induced cell death, whereas S-isomer significantly inhibited this cell death. Therefore, both isomers of roscovitine can lower the IOP, but from the perspective of neuroprotective effects, the S-isomer was superior to the R-isomer. The S-isomer of roscovitine may be useful as an agent for lowering IOP and its neuroprotective effects.

Glaucoma is a high risk for irreversible blindness. Although an estimated 70 million individuals suffer from glaucoma, over half of these patients do not realize that their sight has been threatened due to the slow progression of the disease [22]. Intraocular pressure (IOP) is a main risk factor for progression and development of pathological conditions [7]. Every 1 mmHg increase in IOP during follow-up is related to a 10–19% increased risk of progression [4]; therefore, lowering IOP can suppress the progression of glaucoma [8,21].

The pathology of primary open-angle glaucoma (POAG) is characterized by morphological and biochemical changes in the trabecular meshwork (TM). Particularly well known factors include an increase in the extracellular matrix of the TM and acceleration of senescence in the cytoskeleton [16]. These changes might lead to increased outflow resistance [16]. Aqueous humor outflow and IOP can change, depending on the balance of generation and emission of aqueous humor. This is especially the case when emission through the trabecular meshwork is prevented, which often increases IOP [7,15]. In addition, the elevation of IOP induces substantial stress on retinal ganglion cells (RGCs), including oxidative stress, ischemia, excitotoxicity, and endoplasmic reticulum (ER) stress [18,25,29], which can lead to a failure of the RGCs. Therefore, treatments that protect against RGC death induced by various stresses and that lower the IOP may be useful as glaucoma therapies.

Previously, we reported that a cell cyclin-dependent kinase (CDK) inhibitor, roscovitine, modulates cell contraction-relaxation in TM [10], which suggested that roscovitine may lower IOP and promote aqueous humor outflow. In addition, CDK2, CDK4, and CDK5, which are inhibited by roscovitine, are activated and

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up-regulated by a number of various stresses [2,11,12,28], and have essential functions in apoptotic and excitotoxic pathways [14,23,27]. Therefore, roscovitine is expected to have a protective effect on RGC death.

Roscovitine is a racemic mixture, and these isomers differ in their reactivity to various substrates. Protective effects of each isomer differ in cerebral ischemia [19]; thus, the effects of each isomer on RGC death and lowering IOP may also differ. In the present study, we investigated the IOP lowering effect *in vivo* and the protective effects against RCG death of each roscovitine isomer *in vitro*.

Adult male New Zealand White (albino) rabbits (Kitayama Labes Co., Ltd., Nagano, Japan) weighing 2.5–3.4 kg were used. All animals were treated in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research that has been approved by the Association of Vision Research in Ophthalmology (ARVO).

IOP was measured with a Model 30 Classic Pneumatonometer (Medtronic Xomed Ophthalmics, Inc., Minneapolis, MN, USA). Before each measurement, the calibration was checked according to the manufacturer's instructions. Roscovitine, R-isomer (molecular quality >98%, Sigma–Aldrich St Louis, MO, USA) and S-isomer (molecular quality >98%, EMD Chemicals La Jolla, CA, USA) were suspended in 4% (w/v) polyethylene glycol (PEG) 4000 at a concentration of 0.1%. Each drug solution (50 μL) was topically administered in the left eyes. Vehicle (PEG4000) was topically administered in the contralateral eyes for use as a control. IOP was measured just before topical administration (13:00) and at 1–5 h after the administration. For IOP measurements, the eyes were anesthetized by topical instillation of 0.4% oxybuprocaine hydrochloride (Nitten Pharmaceutical Co., Ltd., Nagoya, Japan).

The cultured retinal ganglion cell (RGC-5) line, a transformed cell line, was generously provided by Dr. Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Valeant Pharmaceuticals, Costa Mesa, CA, USA), 100 U/mL penicillin (Meiji Seika Pharma Co, Ltd., Tokyo, Japan), and 100 μg/mL streptomycin (Meiji Seika Pharma Co, Ltd.). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The RGC-5 cells were passaged by trypsinization every 3–4 days.

The oxygen–glucose deprivation (OGD)-induced cell death assay involved seeding the cells at a density of 2×10^3 cells per well into 96-well plates, followed by incubation in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 h. The OGD stress was induced by washing the cells with glucose-free DMEM (Invitrogen, Carlsbad, CA, USA) and then placing them in the same medium in a hypoxic incubator (94% N_2, 5% CO_2, and 1% O_2) for 4 h. At the end of the OGD period, glucose solution and FBS were added at final concentrations of 4.5 mg/mL and 1%, respectively, and the cultures were returned to the incubator for an additional 16 h at the regular atmospheric oxygen level (reoxygenation). The roscovitine R-isomer or S-isomer was added to the culture immediately after replacement of the culture medium. At the end of this culture period, the cell viability was measured.

The tunicamycin-induced cell death assay involved seeding the cells at a density of 1×10^3 cells per well into 96-well plates, followed by incubation in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 h. The medium was then replaced with fresh medium containing 1% FBS, and the roscovitine R-isomer or S-isomer were added. After 1 h, tunicamycin was added (at a final concentration of 2 μg/mL) and incubated for 27 h. At the end of this culture period, the cell viability was measured.

Cell viability was determined using a combination staining with two fluorescent dyes, Hoechst 33342 (Invitrogen) and propidium iodide (PI; Invitrogen). The protective effects of the roscovitine isomers were examined after inducing retinal cell death by tunicamycin or OGD stress. After this induction, Hoechst 33342 (excitation/emission wavelengths, 360/490 nm) or PI (excitation/emission wavelength, 535/617 nm) was added to the culture medium for 15 min at final concentrations of 8 and 1.5 μM, respectively. Images were collected using an epifluorescence microscope (IX70; Olympus, Tokyo, Japan) fitted with a charge-coupled device camera (DP30BW; Olympus) and with fluorescence filters for Hoechst 33342 (U-MWU; Olympus) and PI (U-MWIG; Olympus)

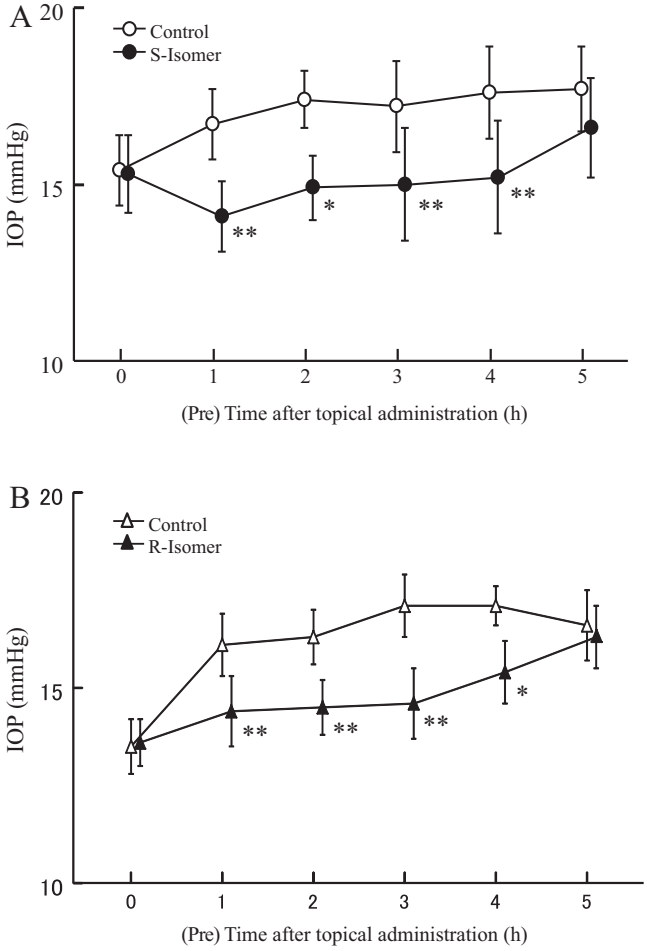

Fig. 1. Effects of the R-isomer and S-isomer of roscovitine on intraocular pressure. Roscovitine S-isomer (A), and R-isomer (B) significantly lowered IOP at 1, 2, 3, and 4 h after administration when compared to the control eyes. Data are shown as mean ± S.E.M. ($n = 6$). $^{*}p < 0.05$, and $^{**}p < 0.01$ *versus* control (Paired *t*-test).

Data are presented as the means ± S.E.M. Statistical comparisons were made by the Paired *t*-test using or one-way analysis of variance (ANOVA) followed by Dunnett's test using Stat view version 5.0 (SAS Institute Inc., Cary, NC, USA). A value of $p < 0.05$ was considered to indicate statistical significance.

In the present study, changes in IOP in normal male rabbits before topical administration (13:00) and at 1, 2, 3, 4, and 5 h after administration of 0.1% roscovitine R-isomer and S-isomer are shown in Fig. 1. Each roscovitine isomer showed IOP lowering effect.

In our previous study, roscovitine significantlly modulated the contraction of TM cells [10], suggesting that roscovitine promotes aqueous humor outflow in TM cells. The TM is the site of biosynthesis and secretion of various extracellular matrix (ECM) materials (type I, II, IV, and VI collagen) [30] and the ECM plays an important role in regulating IOP in both normal and glaucomatous eyes. In turn, CDKs regulate the ECM by regulating collagen production, such as the type I collagen expression in fibroblasts [20] and