Journal of Ophthalmology

Rho Kinase in Eye Disease

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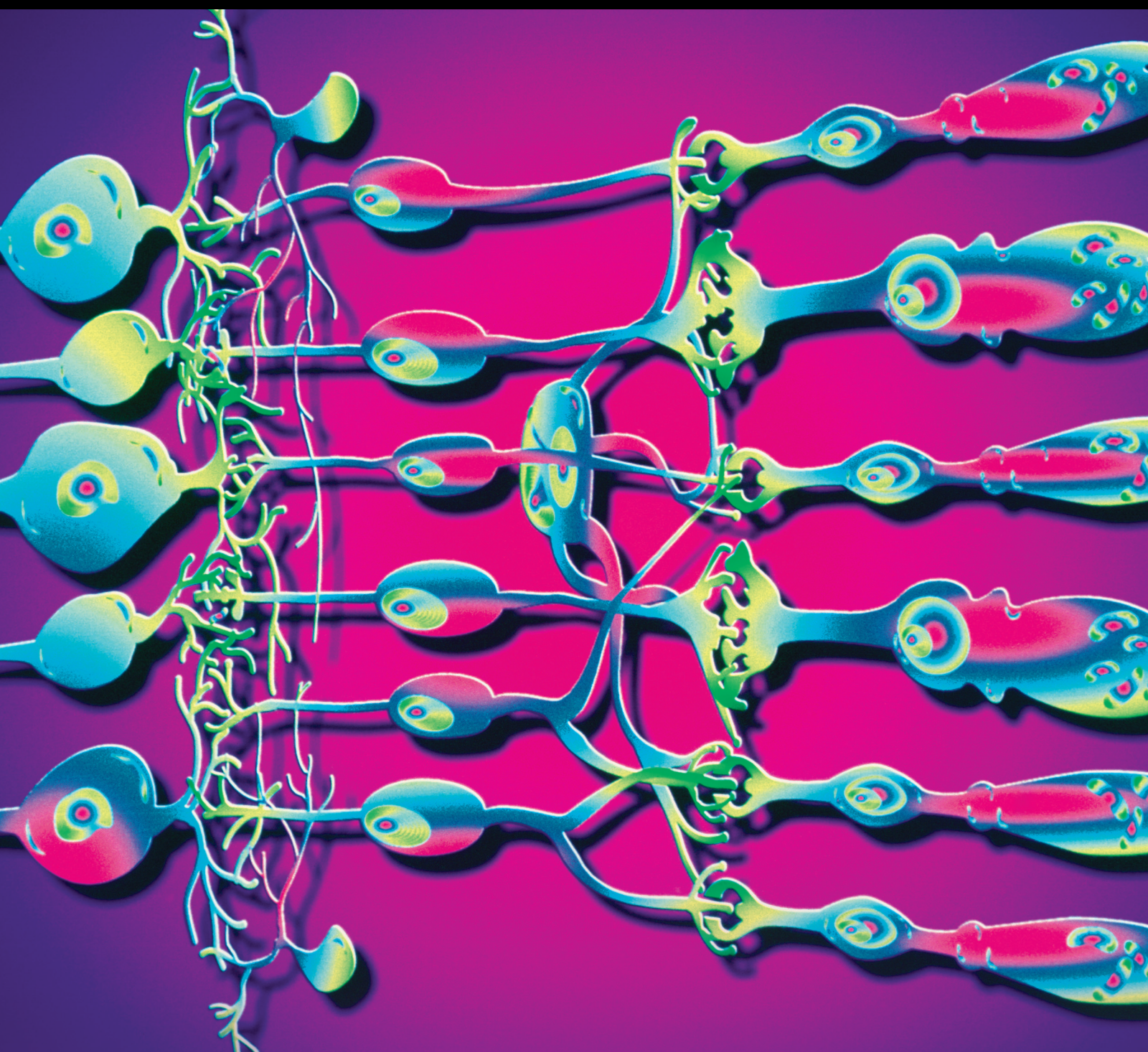

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Editorial Board

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Editorial

Rho Kinase in Eye Disease

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Rho-associated protein kinase (ROCK) is a well-characterized effector of Rho GTPase, a small GTP-binding protein. The Rho/ROCK signaling pathways contribute to a wide range of fundamental cellular events, such as cell adhesion, motility, proliferation, differentiation, and apoptosis. The role of ROCK in the control of a wide spectrum of biological events has made it a subject of intensive investigation as an important therapeutic target in a wide range of diseases, including vascular disease, cancer, neuronal degenerative disease, asthma, and glaucoma.

The ROCK inhibitor, fasudil, was approved in 1995 for the prevention of cerebral vasospasm in patients with subarachnoid hemorrhage. In 2014, another ROCK inhibitor, ripasudil, was approved in Japan for the treatment of glaucoma and ocular hypertension. More importantly, the research supporting the involvement of ROCK signaling in glaucoma has now extended to other eye diseases, such as corneal endothelial disease, cataract, age-related macular degeneration, and proliferative vitreous retinopathy.

This special issue focuses on the involvement of ROCK signaling in the pathobiology of eye-related diseases and the possible applications of ROCK inhibitors as drugs for treating these disorders.

Numerous researchers have devoted their efforts toward the development of ROCK inhibitors that can target glaucoma. ROCK inhibitors alter the distribution of actin stress fibers and modulate the cell-matrix interactions of cultured trabecular meshwork and Schlemm's canal cells. In addition, topical administration of ROCK inhibitor eye drops reduces the intraocular pressure (IOP) in rabbit and monkey models by increasing the outflow capacity. Several ROCK inhibitors also reduced IOP in healthy human volunteers, as well as in patients with glaucoma and ocular hypertension in clinical studies. As mentioned above, ripasudil was approved in Japan as a first-in-class ROCK inhibitor ophthalmic agent for the treatment of glaucoma and ocular hypertension. Y. Kaneko et al. showed that a specific ROCK inhibitor, ripasudil hydrochloride hydrate, had an additional IOP-lowering effect in rabbit or monkey models when used in combined regimens that included β-blockers, $\alpha\beta$-blockers, α2-agonists, carbonic anhydrase inhibitors, and prostaglandin analogs. Their study indicated that ripasudil has a particular efficacy when used in combination with conventional eye drops. Ripasudil is a first-in-class ROCK inhibitor eye drop, so further basic and clinical data regarding its safety and effectiveness will be beneficial, especially for clinicians.

The use of ROCK inhibitors has been proposed for the treatment of corneal endothelial decompensation. N. Okumura et al. reviewed the usefulness of ROCK inhibitors both as eye drops and as adjunct drugs in cell-based therapies. They showed that ROCK inhibitors, supplied in the form of eye drops, promoted cell proliferation in animal models, and pilot clinical research suggests the occurrence of a similar response in humans as well. They have predicted the future use of topically applied ROCK inhibitors for the treatment of (1) Fuchs endothelial corneal dystrophy, combined with central corneal endothelial removal, and (2) acute corneal endothelial damage induced by cataract surgery. Along these

lines, A. Akhbanbetova et al. designed a cryoprobe for corneal endothelial removal prior to ROCK inhibitor administration as part of a treatment for Fuchs endothelial corneal dystrophy. They demonstrated that their cryoprobe destroys the corneal endothelium in a consistent manner, without altering the collagen fibril structure of the corneal stroma. N. Okumura et al. have also reviewed the accumulating evidence supporting the usefulness of ROCK inhibitors as adjunct drugs in a cell-based therapy. The first-in-man clinical trial of the cell-based therapy combined with a ROCK inhibitor as an adjunct drug for the treatment of corneal endothelial dysfunction was initiated in Japan in 2013. Future clinical assessments are necessary, but the potential for ROCK inhibitors to target the corneal endothelium is very promising.

ROCK inhibitors have also been researched as potent pharmaceutical agents for targeting retinal diseases, such as wet age-related macular degeneration, diabetic retinopathy, diabetic macular edema, and proliferative vitreoretinopathy. In addition to the preclinical data obtained using animal models, several clinical studies have now demonstrated a positive effect of ROCK inhibitors on retinal diseases. M. Yamaguchi et al. reviewed the recent progress on the mechanisms of ROCK signaling in vitreoretinal diseases and the potency of their clinical application. Vitreoretinal diseases are strongly modulated by vascular endothelial growth factor (VEGF), so intravitreous injection of anti-VEGF agents has been extensively used in the clinical setting. M. Yamaguchi et al. have reviewed the potent effects of ROCK inhibitor administration on each biological process involved in eye disease and have carefully compared ROCK inhibitor effects with those of anti-VEGF agents.

J. S. Moon et al. demonstrated in a rabbit model that intraoperative subconjunctival injection of ROCK inhibitor suppressed the inflammation and fibrosis that typically occur after extraocular muscle surgery. ROCK inhibitors are known to trigger an antifibrotic effect in several cell types in the eye, such as Tenon fibroblasts, the trabecular meshwork, retinal pigment epithelial cells, hyalocytes, and corneal stroma cells. Human clinical trials have not yet recapitulated this antifibrotic effect of ROCK inhibitors, but this phenomenon is worth further investigation.

Thus, growing evidence supports the usefulness of ROCK inhibitors as promising therapeutic modalities for the treatment of various ophthalmological disorders. We expect that this special issue will provide a cross-sectional platform for readers to discover the importance and possible future applications of ROCK inhibitors in the treatment of eye diseases.

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Research Article

Development of Poly Lactic/Glycolic Acid (PLGA) Microspheres for Controlled Release of Rho-Associated Kinase Inhibitor

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Purpose. The purpose of this study was to investigate the feasibility of poly lactic/glycolic acid (PLGA) as a drug delivery carrier of Rho kinase (ROCK) inhibitor for the treatment of corneal endothelial disease. *Method*. ROCK inhibitor Y-27632 and PLGA were dissolved in water with or without gelatin (W1), and a double emulsion [(W1/O)/W2] was formed with dichloromethane (O) and polyvinyl alcohol (W2). Drug release curve was obtained by evaluating the released Y-27632 by using high performance liquid chromatography. PLGA was injected into the anterior chamber or subconjunctiva in rabbit eyes, and ocular complication was evaluated by slitlamp microscope and histological analysis. *Results*. Y-27632 incorporated PLGA microspheres with different molecular weights, and different composition ratios of lactic acid and glycolic acid were fabricated. A high molecular weight and low content of glycolic acid produced a slower and longer release. The Y-27632 released from PLGA microspheres significantly promoted the cell proliferation of cultured corneal endothelial cells. The injection of PLGA did not induce any evident eye complication. *Conclusions*. ROCK inhibitor-incorporated PLGA microspheres were fabricated, and the microspheres achieved the sustained release of ROCK inhibitor over 7–10 days in vitro. Our data should encourage researchers to use PLGA microspheres for treating corneal endothelial diseases.

1. Introduction

The corneal endothelium maintains corneal transparency by pump and leaky barrier functions [1]. The corneal endothelium is located at the anterior chamber side of the posterior cornea at a cell density of 2000–3000 cells/mm^2 in a healthy subject. A phenotypical feature of corneal endothelial cells (CECs) is a severely limited proliferative ability [2]. When the corneal endothelium is impaired by any pathological conditions, trauma, or aging, the residual remaining CECs migrate and spread out to cover the impaired area, resulting in a cell density drop and increased polymorphism. The remaining CECs compensate the function to maintain corneal transparency, but once cell density drops to critical level, which is usually less than 500–1000 mm^2/cells, the cornea loses its transparency [1].

Corneal transplantation using a donor cornea is the only therapeutic choice for treating corneal endothelial decompensation [3]. Researchers, including our group, have searched pharmaceutical agents such as EGF [4], PDGF [5], FGF-2 [6], lithium [7], and Rho kinase (ROCK) inhibitor [8], which can promote the proliferation of CECs, for the treatment of corneal endothelial diseases. Indeed, we performed pilot clinical research and showed that ROCK inhibitor eye drops have a potency for treating early stages of Fuchs endothelial corneal dystrophy and severe corneal endothelial damage induced by cataract surgery by enhancing the proliferation of residual CECs [9–11].

Poly lactic/glycolic acid (PLGA), a copolymer of poly lactic acid (PLA) and poly glycolic acid (PGA), has been intensively researched as a drug delivery carrier [12, 13]. During the degradation of PLGA for drug release, lactic acid and glycolic acid are coreleased and are both biologically removed by the surrounding cells through normal metabolic pathways [14]. The biocompatibility, low toxicity, and drug encapsulation capabilities of PLGA-based drug delivery

Table 1: Summary of poly lactic/glycolic acid (PLGA) or poly lactic acid (PLA) microspheres.

Type	Molecular weight	Composition ratio Lactic acid : glycolic acid	Drug incorporation rate (%)	Particle size (μm)
5005	5000	50 : 50	12.5	12.63 ± 5.76
5010	10000	50 : 50	1.38	11.83 ± 6.85
7505	5000	75 : 15	1.17	14.30 ± 5.13
0005	5000	100 : 0	9.33	16.44 ± 6.09
0020	20000	100 : 0	2.11	13.94 ± 7.32
5005 + gelatin	5000	50 : 50	18.1	11.80 ± 7.74

systems have attracted the attention of researchers [12, 13]. Indeed, several therapies using PLGA have recently entered preclinical development [13, 15].

In the current study, we designed the PLGA microspheres, which incorporate ROCK inhibitor Y-27632, to enable efficient drug delivery for targeting corneal endothelium. We also evaluated the safety and stability of ROCK inhibitor released from PLGA microspheres through in vitro experiments. In addition, the safety of PLGA microsphere injection into the anterior chamber or subconjunctiva was evaluated in a rabbit model.

2. Materials and Methods

2.1. Microsphere Preparation. A selective ROCK inhibitor, Y-27632 (1 mg, Wako Pure Chemical Industries Ltd., Osaka, Japan), was dissolved in double-distilled water (200 μL) with or without gelatin (20 μg, Nitta Gelatin, Osaka, Japan) (W1) and poured into dichloromethane (2 mL, DCM, Nacalai Tesque, Kyoto, Japan) in which PLGA or poly (L-lactic acid) (PLA) (90 mg) was dissolved (O). The first inner W1/O emulsion was prepared by agitating for 60 secs using a vortex mixer at room temperature, followed by sonication for 300 secs using a UD-21P ultrasonic generator (Tomy Seiko Co. Ltd., Tokyo, Japan). The first emulsion was then poured into 1% polyvinyl alcohol (2 mL, PVA, Japan Vam & Poval Co. Ltd., Osaka, Japan) solution (W2), followed by vigorous mixing using a vortex mixer for 60 sec. This procedure permitted the formation of the double emulsion [(W1/O)/W2], in which the W1 phase was homogeneously dispersed in the O phase. The resulting double emulsion was poured into 1 wt% PVA solution (300 mL) and continuously stirred for 3 hours at 4°C until DCM was completely evaporated. The microspheres were washed several times with double-distilled water by centrifugation and freeze-dried into powdered microspheres. The microspheres with different composition ratios of lactic acid/glycolic acid were prepared similarly (Table 1).

The shape and size of the microspheres was evaluated using light microscopy (CKX41, Olympus Corp., Tokyo, Japan) and a micro scale (OB1, 1 mm scale length, 100 divisions, 0.01 mm pitch, MeCan Imaging Inc., Saitama, Japan). For scanning electron microscopy analysis, microspheres were fixed on an aluminum support with carbon-adhesive glue and coated with a 10 nm thick coating of gold-palladium (30 mA, 40 sec) (JSM 6701F; JEOL, Tokyo, Japan). The samples were observed using a scanning electron microscope (S-2380N, Hitachi Ltd., Tokyo, Japan).

2.2. Release Study. The Y-27632 release profiles from the PLGA microspheres were determined in vitro. PLGA microspheres in which Y-27632 (10 mg) was incorporated were incubated in a tube containing 1.0 mL phosphate-buffered saline solution (PBS; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) (pH: 7.4) at 37°C in a heat block (ALB-221, Scinics Co., Tokyo, Japan). PBS including released Y-27632 was evaluated at 1, 3, and 6 hour(s) and 1 to 30 day(s) after incubation. The concentration of Y-27632 in the samples was determined by high performance liquid chromatography (HPLC; Prominence, Shimazu Corp., Kyoto, Japan) equipped with one pump, an auto sampler, and an ultra violet (UV) detector. Calibration curves were prepared for Y-27632 based on the UV absorbance peak area at 269 nm. These calibration curves were used for the estimation of in vitro drug release. The Y-27632 release profile was calculated as follows: (cumulative amount of Y-27632 released)/(total incorporated Y-27632) × 100.

2.3. Cell Cultures. Eight corneas from four cynomolgus monkeys (3–5 years of age; estimated equivalent human age, 5–20 years) housed at Nissei Bilis Co. Ltd. (Otsu, Japan) were used for the study. The corneas were harvested at the time of the euthanization of the monkeys for other research purposes. CECs were cultured according to a modified protocol reported previously [16]. Briefly, Descemet's membrane including CECs was stripped from the cornea and incubated with 0.6 U/mL of Dispase II (Roche Applied Science, Penzberg, Germany) for 60 minutes at 37°C. CECs were then seeded onto a culture plate (CECs from one cornea to one well of a 12-well culture plate) with a culture medium composed of Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL basic fibroblast growth factor (bFGF; Life Technologies Corp.). ROCK inhibitor was not added to the culture medium when preparing CECs for experiments. When the cells reached confluence in 10 to 14 days, they were trypsinized with 0.05% trypsin-EDTA (Life Technologies Corp.) for 5 minutes at 37°C and passaged at ratios of 1 : 2 to 4. CECs obtained after the 4–7th passage that still maintained a hexagonal and monolayer sheet-like structure were used for the experiments.

2.4. In Vitro Assessment of Y-27632 Released from Microspheres. To evaluate the stability and safety of Y-27632

released from the PLGA/PLA microspheres, the effect of released Y-27632 on cell growth of CECs was evaluated. Y-27632 incorporating PLGA microspheres (0020) were incubated in PBS, and PBS was recovered at 3 and 7 days after incubation. The concentration of Y-27632 was then evaluated by HPLC, and the recovered PBS including the released Y-27632 was added to the culture medium at a final concentration of Y-27632 of 10 μM. Cultured monkey CECs were seeded at a density of 5.0×10^3 cells/cm^2 per well on a 96-well plate for 24 hours and then subjected to serum starvation for an additional 24 hours in the presence or absence of fresh Y-27632 (10 μM) or Y-27632 released from PLGA microspheres after 3 or 7 days (10 μM). The number of viable cells was determined by use of the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Fitchburg, Wisconsin) performed in accordance with the manufacturer's protocol. The number of CECs at 24 hours after treatment with Y-27632 was measured using the Veritas™ Microplate Luminometer (Promega, Fitchburg, Wisconsin).

2.5. Safety Assessment of Microspheres in Rabbit. To evaluate the safety of PLGA/PLA microspheres, 1 or 10 mg PLGA microspheres (0020) suspended in 200 μL PBS were injected into the anterior chamber and 1 mg PLGA microspheres suspended in 200 μL PBS were injected into the subconjunctiva of the 9 right eyes of 9 rabbits ($n = 3$). The left eyes were used as a control. The corneal appearance of the 9 rabbits was examined at 1, 2, 3, 5, 7, 10, and 14 days using a slitlamp microscope, and corneal opacification and conjunctival hyperemia were evaluated according to the grading system (Supplemental Table 1 available online at https://doi.org/10.1155/2017/1598218) modified from the previous report [17]. Intraocular pressure was determined with a Tonovet® (Icare Finland, Vantaa, Finland) instrument. Corneal thickness was determined through the use of an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan).

2.6. Histological Examination of Rabbit Eyes after Poly Lactic/Glycolic Acid (PLGA) Microsphere Injection. After 14 days of PLGA microsphere injection, rabbits were euthanized, the eye balls were enucleated, and sclerocorneal specimens were prepared using ophthalmic surgical scissors. Samples were fixed in 4% formaldehyde and incubated for 30 minutes in 1% bovine serum albumin to block nonspecific binding. The samples were then incubated overnight at 4°C with antibodies against Na^+/K^+-ATPase (1 : 300, Upstate Biotechnology, Lake Placid, NY), ZO-1 (1 : 300, Life Technologies Corp.), and connexin 43 (1 : 300, Life Technologies Corp.). Alexa Fluor® 488-conjugated goat anti-mouse (Life Technologies Corp.) was used as a secondary antibody at a 1 : 1000 dilution. Cell morphology was evaluated after actin staining with a 1 : 400 dilution of Alexa Fluor 594-conjugated phalloidin (Life Technologies Corp.). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Dojindo Laboratories, Kumamoto, Japan). The samples were examined with a fluorescence microscope (TCS SP2 AOBS; Leica Microsystems, Wetzlar, Germany).

2.7. Statistical Analysis. The statistical significance (p value) of differences in the mean values of the two-sample comparison was determined with the Student's t-test. The statistical significance of comparisons of multiple sample sets was analyzed with Dunnett's multiple-comparisons test. $p < 0.05$ was considered as statistically significant. Results were expressed as mean ± standard error of the mean.

3. Results

3.1. Characteristics of Poly Lactic/Glycolic Acid (PLGA) or Poly Lactic Acid (PLA) Microspheres for Y-27632 Release. Y-27632-incorporated PLGA or PLA microspheres with different molecular weights and different composition ratios of lactic acid and glycolic acid were prepared (Table 1). All PLGA or PLA microspheres exhibited similar morphology and size. SEM showed that representative images of PLGA microspheres were spherical with a smooth surface (Figure 1(a), upper panel). The inside of the microspheres was porous core due to double emulsion, which is designed to incorporate Y-27632 (Figure 1(a), lower panel). We first evaluated the effect of molecular weight on the release profile in PLGA or PLA microspheres. PLGA5005 had released 65.5 ± 0.9% (approximately 8.22 μg) of the Y-27632 by 1 day and 98.7 ± 0.6% (approximately 12.4 μg) by 14 days, while PLGA5010, which has a higher molecular size than PLGA5005, released 40.8 ± 1.0% (approximately 0.56 μg) of Y-27632 by 1 day and 99.1 ± 1.7% (approximately 1.37 μg) by 14 days ($p < 0.01$ at 1 day). PLA0005 released 62.8 ± 0.8% (approximately 5.86 μg) of Y-27632 by 1 day and 77.7 ± 0.5% (approximately 7.25 μg) by 14 days, while PLA0020, which has a higher molecular size than PLA0005, released 30.2 ± 0.9% (approximately 0.64 μg) of Y-27632 by 1 day and 55.3 ± 0.6% (approximately 1.16 μg) by 14 days ($p < 0.01$ at both 1 day and 14 days) (Figure 1(b)). These data showed that a lower molecular weight increased the rate of drug release at a fixed composition ratio of lactic acid and glycolic acid. We next evaluated the effect of the composition of lactic acid and glycolic acid on the release profile. PLGA7505 released 58.9% of Y-27632 at 1 day and 97.4% at 14 days, and PLA0005 released 62.4% of Y-27632 at 1 day and 77.5% at 14 days. This data showed that a smaller amount of glycolic acid made the release slower and a smaller amount of lactic acid made the release of Y-27632 faster (Figure 1(c)).

The mean incorporation rate of Y-27632 was 8.74% in PLGA microspheres, though PLGA microspheres prepared with gelatin enabled a significantly higher incorporation of Y-27632 at 18.1%, showing that gelatin reduced the loss of ROCK inhibitor during preparation of the PLGA microspheres (Figure 1(d)). Characteristic release curves showed that both PLGA with or without gelatin have the potency to release Y-27632 and that the initial burst release of Y-27632 tended to decrease for microspheres with gelatin (Figure 1(e)).

3.2. In Vitro Evaluation of Y-27632 Released from Microspheres. The effect of Y-27632 released from PLGA microspheres on CEC proliferation was evaluated. Phase contrast images showed that CECs cultured with a culture medium supplemented with fresh Y-27632 (10 μM) showed

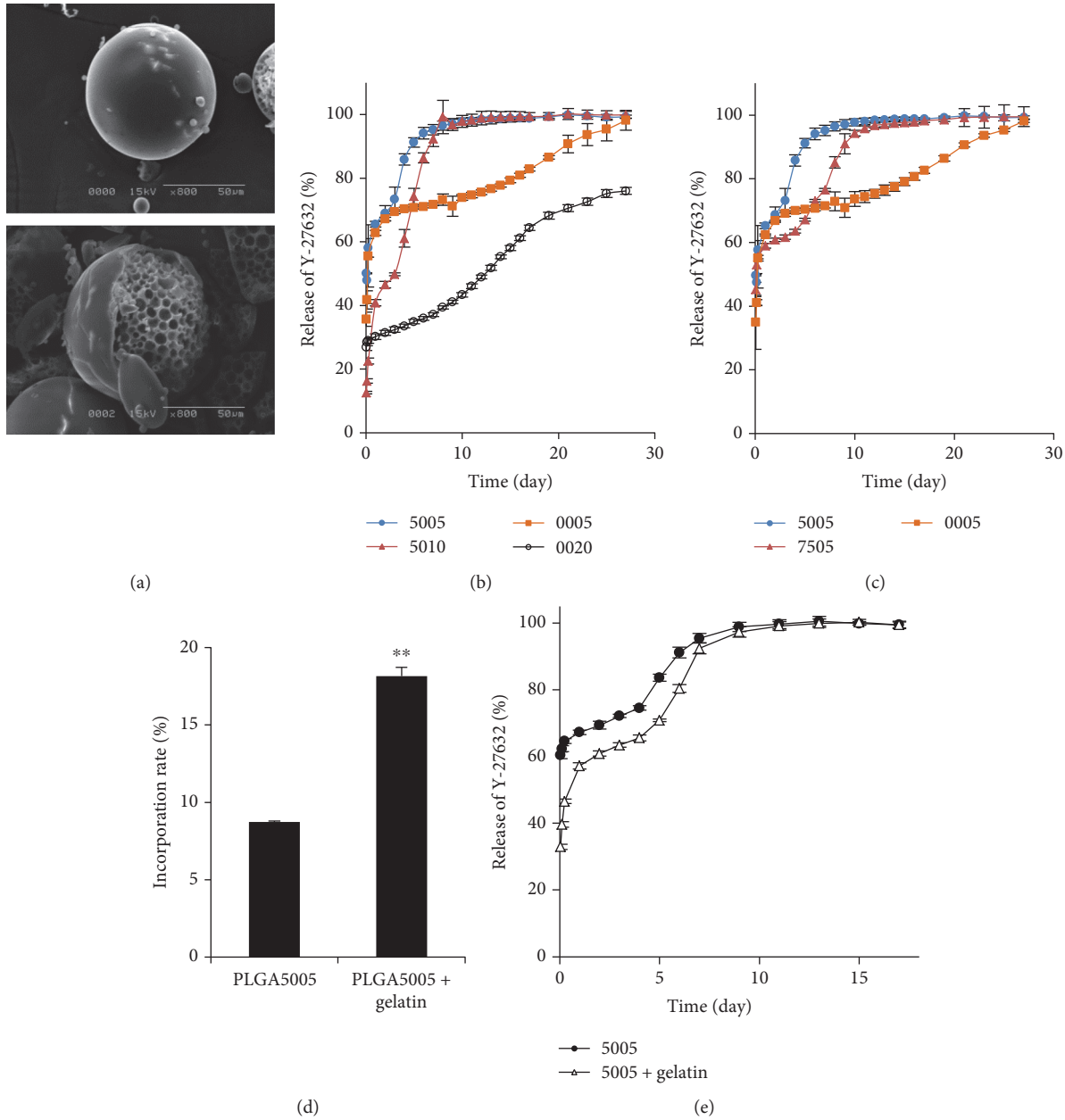

Figure 1: Characteristics of poly lactic/glycolic acid (PLGA) or poly lactic acid (PLA) microspheres for Y-27632 release. (a) PLGA microspheres were examined with a scanning electron microscope. The upper panel shows the surface of the microsphere, and the lower panel shows the internal structure of the microsphere. (b) PLGA or PLA microspheres were incubated in a PBS at 37°C for 14 days, and the amount of Y-27632 released was determined by HPLC. The effect of the molecular weight on the release profile for PLGA or PLA microspheres was evaluated. The cumulative amount of released Y-27632 was plotted in the graph ($n = 3$). (c) PLGA or PLA microspheres were incubated in PBS at 37°C, and the amount of Y-27632 released was determined by HPLC. The effect of the composition of lactic acid and glycolic acid on the release profile was evaluated ($n = 3$). The PLGA5005 release curve plotted is the same as that shown in Figure 1(b). (d) Y-27632 was dissolved in ultrapure water with or without gelatin, and PLGA5005 microspheres were prepared ($n = 3$). The incorporation rate was evaluated by HPLC. $^{**}p < 0.01$. (e) PLGA5005 with or without gelatin that incorporated Y-27632 was incubated at PBS at 37°C for 14 days, and the amount of Y-27632 released was determined by HPLC. Three independent experiments were performed.

higher numbers of cells than the control. The Y-27632 released from the PLGA microspheres was recovered after incubation and was added to a growth medium at a final concentration of 10 μM. Higher numbers of CECs were observed than those in the control after 24 hours of cultivation with those growth media that were supplemented with Y-27632 released from PLGA microspheres (Figure 2(a)). The cell numbers of CECs were significantly increased by the supplementation of fresh Y-27632 or the Y-27632 recovered from PLGA microspheres after an incubation of 3 and 7 days (118.8%, 110.6%, and 111.4%, resp.) (Figure 2(b)). These results showed that Y-27632 incorporated in PLGA

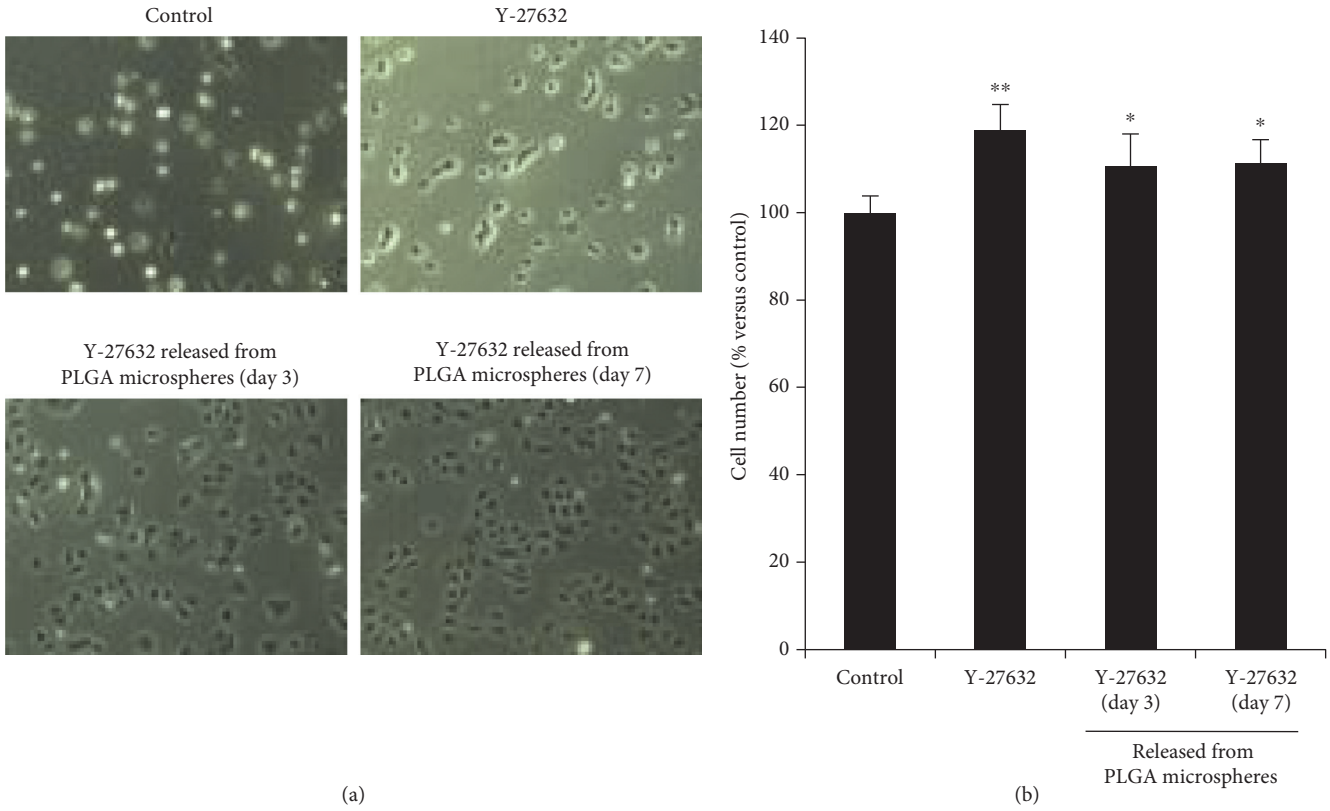

Figure 2: Stability and safety assessment of Y-27632 released from PLGA microspheres. (a) Y-27632 incorporating poly lactic/glycolic acid (PLGA) microspheres was incubated in PBS, and PBS was recovered after 3 and 7 days. The amount of Y-27632 released in the PBS was added to a culture medium as final concentration of Y-27632 as 10 μM. Phase contrast images of corneal endothelial cells (CECs) cultured for 24 hours are shown. (b) CECs were seeded at a density of 5.0×10^3 cells/cm^2 per well on a 96-well plate for 24 hours and subjected to serum starvation for an additional 24 hours supplemented with fresh Y-27632 (10 μM) or Y-27632 released from PLGA microspheres after 3 or 7 days (10 μM). Control cells were CECs cultured for an additional 24 hours without supplementation with Y-27632. The data represent average ± SE ($n = 5$). $^{**}p < 0.01$, $^{*}p < 0.05$. These experiments were performed in triplicate.

microspheres was released and its biological features were maintained.

3.3. In Vivo Safety Evaluation of Microspheres. We evaluated the feasibility of using PLGA microspheres in the eye by conducting a study using healthy rabbit eyes. Slitlamp microscopy showed that no eye complications, such as conjunctival injection, corneal opacity, cataract formation, or severe inflammation, occurred in any of the groups (Figure 3(a)). Corneal opacification and conjunctival hyperemia scores were zero in all of the eyes for all of the groups in which PLGA microspheres were injected into the anterior chamber (1 mg or 10 mg) and into the subconjunctiva (1 mg) (data not shown). Some PLGA microspheres were visible by slitlamp microscopy examination, especially in the iris, but no aggregation of microspheres was observed in the anterior chamber. Corneal thickness and intraocular pressure were not altered to abnormal levels during the 14-day observation period (Figures 3(b) and 3(c)).

Corneal endothelium of eyes in which PLGA microspheres were injected into the anterior chamber and the subconjunctiva expressed Na^+/K^+-ATPase (a marker of pump function), ZO-1 (a marker of tight junction), and connexin 43 (a marker of gap junctions) as did the control. Phalloidin staining showed that the cell morphology was hexagonal and monolayer in PLGA microsphere-treated eyes as with the control, and no morphological change was observed (Figure 4).

4. Discussion

Bullous keratopathy caused by cataract surgery is one of the leading causes of corneal transplantation due to corneal endothelial decompensation [3]. The Eye Bank Association of America reported that of the 28,394 corneal transplantations performed in 2015, 15,707 (55.3%) were for corneal endothelial decompensation [18]. In terms of bullous keratopathy due to severe corneal endothelial damage by cataract surgery, 8290 (29.2%) corneal transplantations were performed in the United States [18]. Likewise, 20–40% of corneal transplantations are performed to treat bullous keratopathy caused by cataract surgery in Asian countries [19–21].

In 2009, we reported that the ROCK inhibitor Y-27632 promoted cell proliferation of cultured CECs [8]. We subsequently demonstrated that the topical application of ROCK inhibitor such as Y-26732 and ripasudil as a form of eye drops enhances wound healing of the cornea endothelium by promoting cell proliferation of CECs in rabbit and monkey models [10, 11, 22, 23]. Based on these findings,

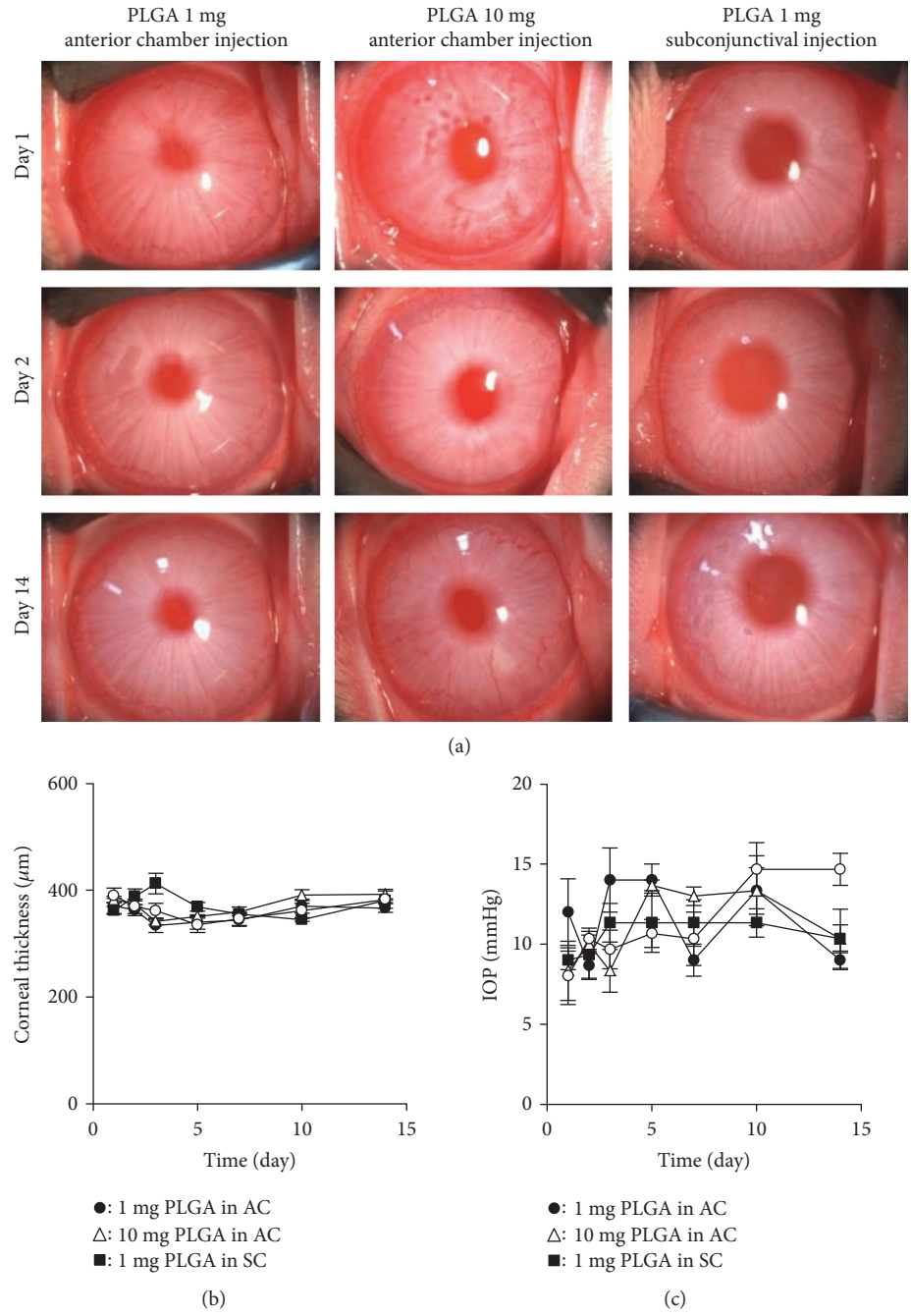

Figure 3: Safety assessment of poly lactic/glycolic acid (PLGA) microspheres in a rabbit eye. (a) One or 10 mg PLGA microspheres suspended in 200 μL phosphate buffer solution (PBS) was injected into the anterior chamber, and 1 mg PLGA microspheres suspended in 200 μL PBS was injected into the subconjunctiva. Nine right eyes of 9 rabbits were used for the experiments ($n = 3$). Anterior segments were evaluated by slitlamp microscopy for 14 days. (b, c) Corneal thickness and intraocular pressure were evaluated for 14 days and are shown in the graph. The central corneal thickness was evaluated by ultrasound pachymetry. Intraocular pressure was determined with a Tonovet.

we performed pilot clinical research in which patients with corneal decompensation were treated with Y-27632 eye drops following to a transcorneal freezing procedure to partially remove the damaged cornea endothelium. Y-27632 eye drops showed effectiveness in reducing the central corneal thickness in patients with early stage Fuchs endothelial corneal dystrophy [9, 10]. In addition, we demonstrated that ROCK inhibitor eye drops can enhance the wound healing of damaged corneal endothelium caused by cataract surgery, thus successfully avoiding the need for corneal transplantation [11]. Although further randomized clinical trials are necessary, ROCK inhibitor currently might be one of the most promising pharmaceutical agents that can be applied for corneal decompensation.

Possible complications of the injection of PLGA into the anterior chamber are direct mechanical damage to the

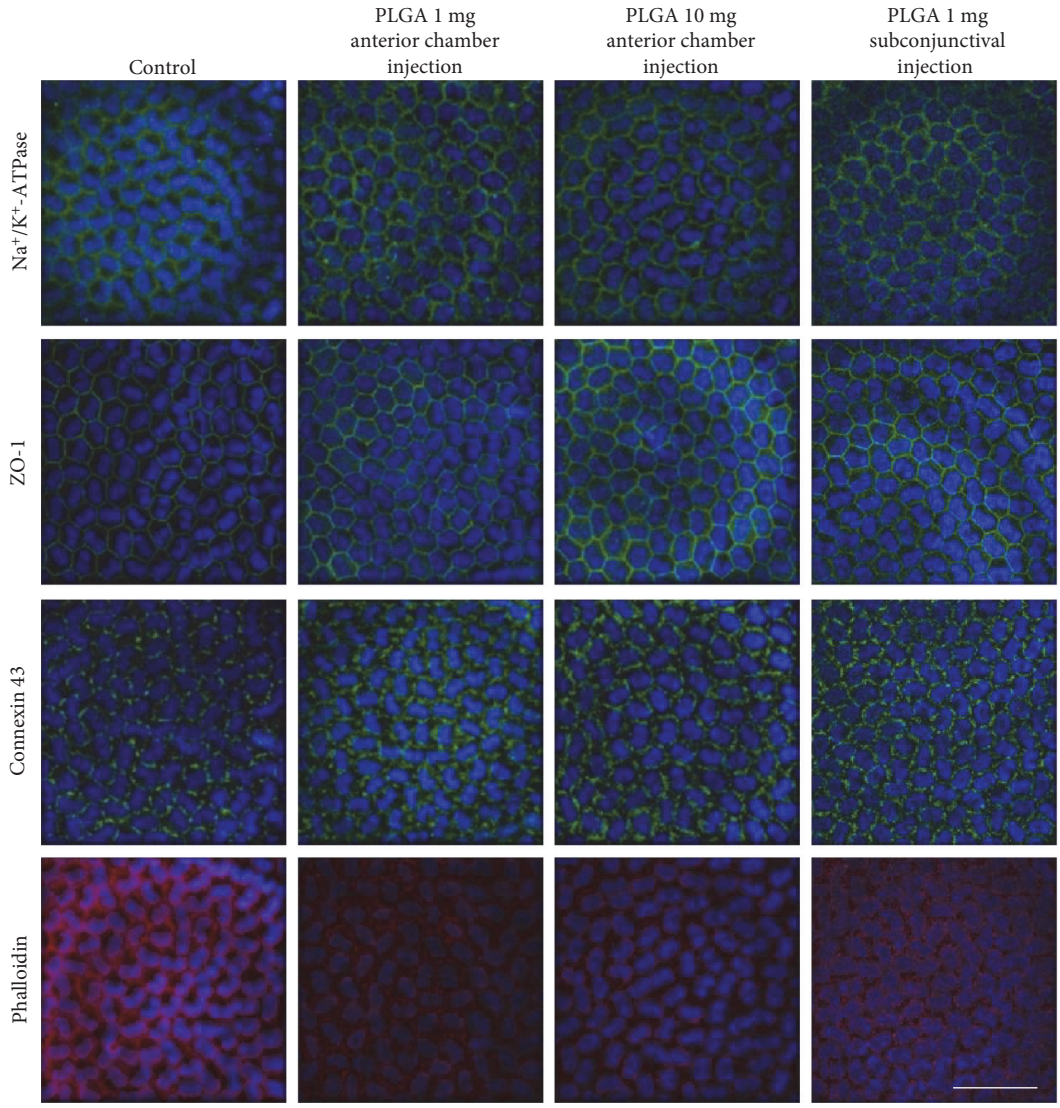

Figure 4: Histological evaluation of corneal endothelium in rabbit eyes injected with poly lactic/glycolic acid (PLGA) microspheres. One or 10 mg PLGA microspheres was injected into the anterior chamber, and 1 mg PLGA microspheres was injected into the subconjunctiva. After 14 days, corneal endothelium was evaluated by immunofluorescence staining and phalloidin staining. Na^+/K^+-ATPase (a marker of pump function), ZO-1 (a marker of tight junction), and connexin 43 (a marker of gap junctions) were used to evaluate the functional property of corneal endothelium. Phalloidin staining was used for morphological analysis. Nuclei were stained with DAPI. Scale bar: 50 μm.

corneal endothelium or lens, indirect damage to the corneal endothelium by biodegradation products from PLGA, and intraocular pressure elevation by inhibiting aqueous humor outflow. We therefore conducted a safety assessment using healthy rabbit eyes to evaluate the feasibility of applying PLGA microspheres for intracameral injection. Here, we showed that the injection of PLGA into the anterior chamber or the subconjunctiva did not induce any evident complications. No inflammatory response was observed, in agreement with the reported biocompatibility safety profile of PLGA. PLGA is capable of encapsulating a wide range of drugs of almost any molecular size [12]. Our in vitro study consistently showed that ROCK inhibitor released from PLGA promoted the proliferation of CECs in the same manner as unencapsulated ROCK inhibitor, suggesting that ROCK inhibitor was incorporated into and released from PLGA without damage to the molecular properties of the inhibitor. Further study is needed, but it is suggested that PLGA might be applicable as a drug carrier that is injected into the anterior chamber.

In our clinical research, we suggested that a ROCK inhibitor has the potency to promote the cell proliferation of residual relatively healthy CECs [9–11] and that there is a "golden time" for a ROCK inhibitor, when the wounded space has not yet been covered by the migration and spreading of residual CECs associated with a cell density drop [24]. We showed that ROCK inhibitor eye drops restored corneal clarity within one week in patients with Fuchs endothelial corneal dystrophy following a transcorneal freezing procedure and within 1-2 months in patients with severe corneal endothelial damage due to cataract surgery. Therefore, in this study, we aimed to fabricate a PLGA system that would

release Y-27632 for 7 to 10 days to improve the therapeutic efficacy during this window of opportunity; however, the release time should be optimized by further studies. The drug release from PLGA with degradation is influenced by various factors such as the initial molecular weight, the monomer composition rate of PLA and PGA, the drug type, and the pH of the surrounding circumstances [12, 25]. Among these factors, the molecular weight and the composition rate of PLA and PGA are recognized as important and are also easily controlled during fabrication [12]. Here, we showed that PLGA5010 exhibited a slower release than PLGA5005 and that PLA0020 exhibited slower release than PLA 0005, showing that PLGA or PLA microspheres with a high molecular weight produce slower and more prolonged Y-27632 release. This may be because polymers with lower molecular weights are less hydrophobic, which increases the rate of water absorption, consequent hydrolysis, and erosion [26]. The composition of PLA and PGA in PLGA is also an important factor influencing polymer degradation. In this study, PLGA7505 showed a slower release of Y-27632 than PLGA5005, and PLA0005 showed the slowest and longest release, although it exhibited an initial burst in release. This suggests that a lower content of GA in PLGA results in a slower and longer-term drug release from the PLGA microspheres. It has been demonstrated that the PGA : PLA ratio of 50 : 50 make PLGA the most hydrophilic and amorphous when compared to other ratios, resulting in faster degradation and drug release [12].

Although the further optimization of the release profile of the ROCK inhibitor is needed before clinical application, our results offers fundamental information for modifying the molecular weights and composition rates of PLA and PGA. Various mathematical, computational, and theoretical models may be applicable for the optimization of the release profile [27–29]. However, drug release from microspheres differs in the in vitro versus in vivo conditions, in part due to immunological responses and the plasticizing effects of biological substances [12]. For instance, the release of thymosin alpha 1 from PLGA was slightly faster in an in vivo assay than in an in vitro assay [30]. Conversely, other researchers have shown that PLGA degradation was slower in vivo than in vitro [31]. Therefore, pharmacokinetics studies are needed on the release of ROCK inhibitor in the anterior chamber. The drawback of this study is the lack of in vivo experiments that show an enhancement of corneal endothelial wound healing by ROCK inhibitor incorporated into PLGA. The fate of the PLGA injected into the anterior chamber is also not well characterized, and further in vivo experiments are needed prior to initiating clinical applications. In the clinical setting, however, the treatment of corneal endothelial damage induced by cataract surgery would seem to be an important target for this PLGA therapy.

In conclusion, we fabricated ROCK inhibitor-incorporated PLGA microspheres, and those microspheres act as carrier for the sustained release of the ROCK inhibitor over 7–10 days. In addition, the PLGA microspheres did not exhibit any evident complication in the eyes of a rabbit model. Although further optimization of the release profile of PLGA and in vivo experiments for safety and effectiveness assessment are required, PLGA microspheres would appear to represent a promising drug delivery carrier for ROCK inhibitor.

Ethical Approval

In all experiments, animals were housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit experiments were performed at Doshisha University (Kyoto, Japan) according to the protocol approved by the University's Animal Care and Use Committee (Approval no. 1315).

Conflicts of Interest

Noriko Koizumi is listed as inventor of the patent regarding the application of the ROCK inhibitor for corneal endothelium (Registration no. 5657252).

Acknowledgments

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Research Article

Effects of the Rho-Kinase Inhibitor Y-27632 on Extraocular Muscle Surgery in Rabbits

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Purpose. To evaluate the effect of the Rho-kinase inhibitor Y-27632 on postoperative inflammation and adhesion following extraocular muscle surgery in rabbits. *Methods*. The superior rectus muscle reinsertion was performed on both eyes of 8 New Zealand white rabbits. After reinsertion, the rabbits received subconjunctival injections of the Rho-kinase inhibitor and saline on each eye. To assess acute and late inflammatory changes, Ki-67, CD11β+, and F4/80 were evaluated and the sites of muscle reattachment were evaluated for a postoperative adhesion score and histopathologically for collagen formation. *Results*. F4/80 antibody expression was significantly different in the Rho-kinase inhibitor-injected group at both postoperative day 3 and week 4 ($p = 0.038$, 0.031). However, Ki-67 and CD11β+ were not different the between two groups. The difference in the SRM/conjunctiva adhesion score between the two groups was also significant ($p = 0.034$). *Conclusion*. Intraoperative subconjunctival injection of the Rho-kinase inhibitor may be effective for adjunctive management of inflammation and fibrosis in rabbit eyes following extraocular muscle surgery.

1. Introduction

During the surgical wound healing after extraocular muscle surgery, the damaged tissue must restore its original function and structural integrity. Therefore, it is important to prevent inflammation and fibrosis, as the perfect restoration of previous tissue architecture without scar is the purpose of wound healing. The major complications of strabismus surgery are postoperative adhesions and fibrosis, which can cause limitation of ocular movement and adversely affect the surgical outcome. These adhesions can occur with various tissues, extraocular muscles, sclera, orbital fat, intermuscular membrane, Tenon's capsule, and conjunctiva.

Numerous studies by strabismologists have been devoted to reducing postoperative adhesions using a variety of materials, including polyglactin 910 mesh [1], mitomycin C [2, 3], and Seprafilm (Genzyme, Cambridge, MA) [4]. However, these methods have problems with their associated complications, unavailability, or inconsistent results. There is also a debate as to whether mitomycin C reduces inflammatory response after strabismus surgery significantly [2, 3].

Small guanosine triphosphatase (GTPase) Rho-kinase, a member of the Rho-kinase subfamily of the Ras superfamily of monomeric GTPases, constitutes an important modulator of vascular smooth muscle contraction [5–8]. Rho-kinase and its downstream effector are important mediators of not only vascular contraction but also actin cytoskeleton reorganization [9, 10], cellular morphology [11], motility [12], adhesion, and proliferation [5–8]. Due to its effects on various cellular functions, Rho-kinase has attracted significant interest as a potential target for the treatment of a wide range of pathological conditions including cancer, neuronal degeneration, kidney failure, asthma, glaucoma, osteoporosis, erectile dysfunction, and surgical adhesion [13–15]. Recent studies have shown that the Rho/Rho-kinase pathway is associated with tissue fibrosis and inflammation. It has also been

demonstrated that the Rho/Rho-kinase pathway is involved in the tissue fibrosis process through regulation of TGF-β activation [16–18].

Therefore, we hypothesized that Y-27632 could be used as an adjuvant to suppress inflammation and fibrosis after strabismus surgery. The present study was conducted to investigate the effect of Y-27632 on postoperative inflammation and fibrosis following extraocular muscle surgery in a rabbit model.

2. Materials and Methods

2.1. Rabbits. In total, 16 eyes from 8 New Zealand white rabbits (each weighing 2.0–3.0 kg, 20 weeks old) were used for this study. All rabbits had anatomically normal eyes. All experiments were conducted in accordance with the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The protocol followed the guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of The Catholic University of Korea, College of Medicine.

2.2. Surgical Procedures. Each rabbit was anesthetized with intramuscular xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (40 mg/kg) and received a topical anesthetic containing 0.5% proparacaine hydrochloride (Alcaine 0.5%; Alcon, USA).

In both eyes of each rabbit, the superior conjunctiva and Tenon's capsule were opened, and the superior rectus muscle (SRM) was exposed using cotton swabs and a muscle hook. The SRM was then placed on a double-armed 6-0 Vicryl (polyglactin) suture that was located near the insertion site and detached from the globe. The SRM was sutured back to its original insertion after detachment, and the conjunctival suturing was performed with an interrupted 8-0 Vicryl suture.

In the right eye (treated eye) of each rabbit, 0.2 mL of 10 μM Y-27632 was injected; 0.2 mL saline was injected into the left eye (control eye) subconjunctivally. To prevent infection, ofloxacin eye drops (Tarivid, Santen, Osaka, Japan) were applied once on each eye after surgery.

Rabbits were randomly selected for sacrifice using barbiturate anesthesia in a group of 4 (8 eyes) at postoperative day 3 and week 4. All eyes were enucleated and examined.

2.3. Evaluation of Inflammation. After animal sacrifice at postoperative day 3 and week 4, the sites of muscle reattachment were fixed in 10% formalin for 24 h, stored in 70% alcohol, and embedded in paraffin. Sequential 5 μm sections of the wound site were prepared. Sections were examined microscopically using hematoxylin and eosin (H&E) staining and immunofluorescence. The sections were examined using rabbit polyclonal antibodies for Ki-67, CD11β+, and F4/80. The antibody-positive cell staining level was graded by a masked observer based on a histologic grading scale of 1 to 4 (1 = 0–10%, 2 = 10–30%, 3 = 30–50%, and 4 = >50% antibody-positive cell density).

2.4. Assessment of Late Adhesion and Fibrosis. Both eyes from 4 rabbits were examined regarding their gross adhesion score to evaluate postoperative fibrosis 4 weeks after the operation. Adhesion score severities were from 0 to 3 according to the criteria in a previous report, where 0 indicated no adhesion, 1 indicated adhesion easily separated with blunt dissection, 2 indicated mild-to-moderate adhesion with the freely dissectible plane, and 3 indicated moderate-to-dense adhesion with difficult dissection or a nondissectable plane [19].

Sections were also stained using Masson's trichrome staining kit (HT15, Sigma-Aldrich, St. Louis, MO, USA) and a fibronectin staining kit for quantification of collagen. Fibrosis was graded according to the amount of collagen as follows: 0, no fibrosis; 1, mild perimuscular fibrotic reaction (stained collagen was detectable only in thin bands immediately adjacent to muscle); 2, easily detected thick bands; 3, well-developed, dense bands of collagen; and 4, severe fibrotic response replacing large areas [20].

2.5. Statistical Analysis. All statistical analyses were performed using the SPSS software (ver. 17.0; SPSS, Chicago, IL, USA). Wilcoxon signed-rank tests were applied to make the comparison between the two postoperative groups. Values of $p < 0.05$ were considered statistically significant.

3. Results

During the follow-up period, all rabbits survived and remained in good health until sacrifice. No severe systemic complications were observed in any of the animals.

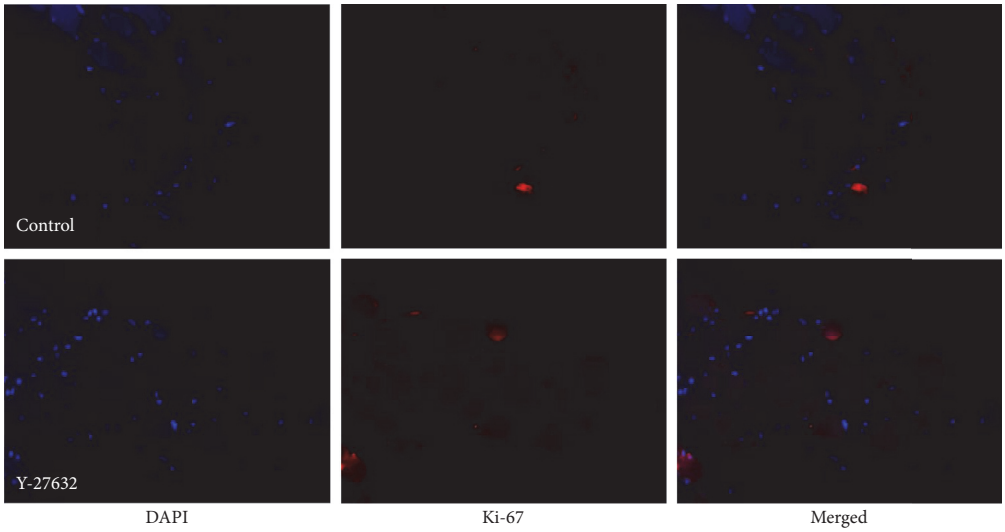
3.1. Acute Inflammation. Histologic examination by H&E staining showed inflammatory cell infiltration around the SRM and surrounding tissue in both groups.

Immunofluorescence of Ki-67 and CD11β+ was not significantly different in the Y-27632 injection group compared to the control at 3 days ($p = 0.500$, 0.054) and 4 weeks ($p = 0.235$, 0.692), respectively, postoperatively. However, F4/80 antibody expression was significantly different in the Y-27632 injection group at both 3 days and 4 weeks postoperatively ($p = 0.038$, 0.031) (Figures 1 and 2).

3.2. Late Fibrosis. The SRM/conjunctiva adhesion score differed significantly between the groups ($p = 0.034$). However, the SRM/sclera adhesion score did not differ significantly between the groups ($p = 0.178$) 4 weeks after extraocular muscle surgery.

H&E staining revealed some degree of granulation tissue with fibrosis around the SRM in both groups. Masson's trichrome stain in the SRM at postoperative 4 weeks presented some degree of blue-colored collagen in the endomysium in both groups. In addition, fibronectin stain in the SRM at postoperative 4 weeks presented some degree of red-colored tissues. The degrees of fibrosis in both stainings were lower in the Y-27632 injection group compared to the control, but not so significant (Figures 3(a) and 3(b)).

In addition, the muscle fibers were more highly separated by collagen deposition in the control group. With regard to postoperative changes, some muscle fibers were stained red faintly in the control group, whereas most were stained red distinctly in the Y-27632 injection group.



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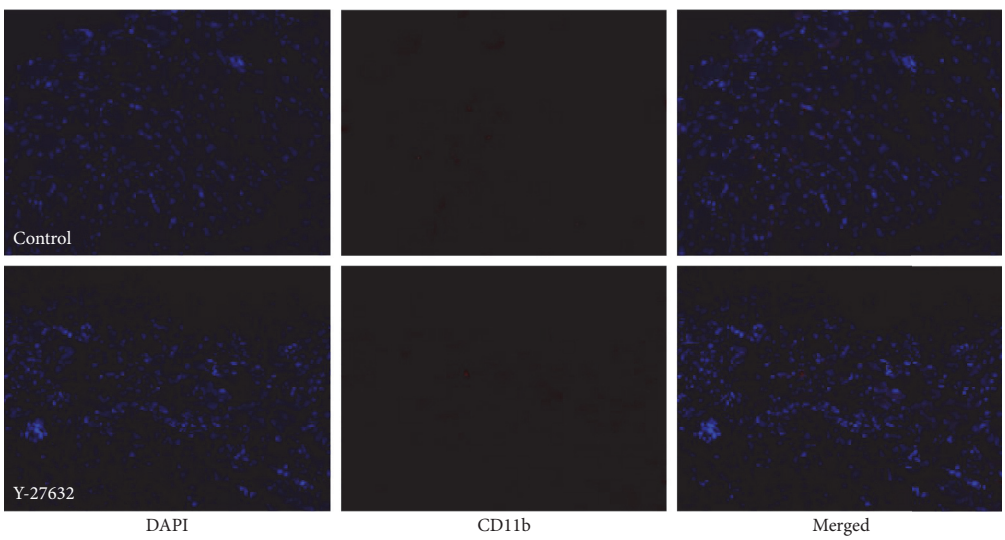

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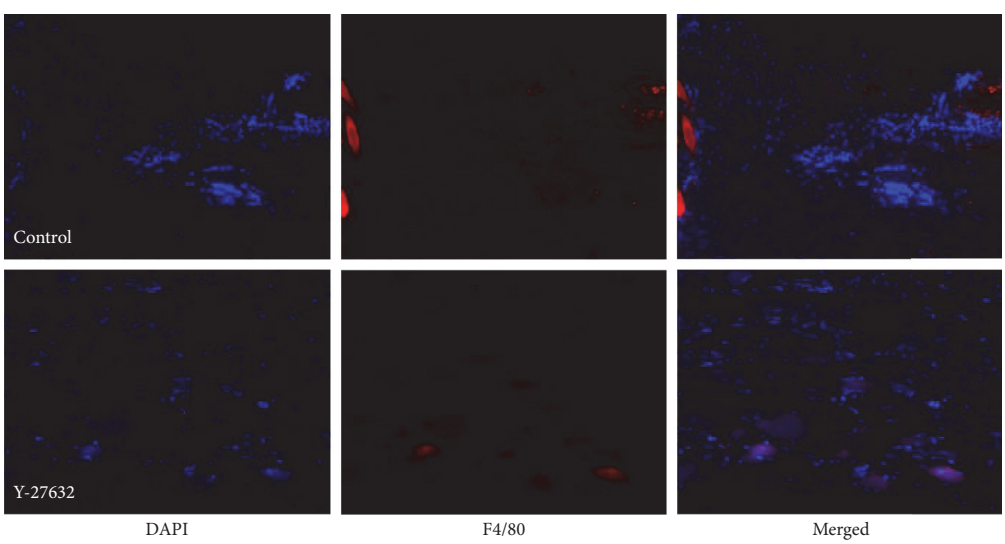

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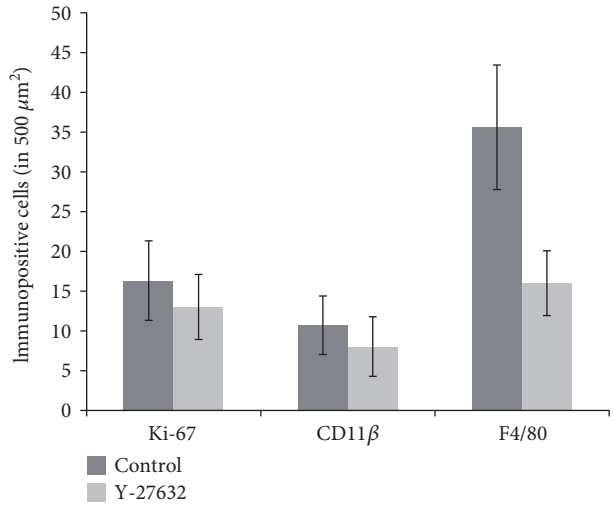


(d)

Figure 1: Immunohistochemical staining for Ki-67 (a), CD11 beta (b), and F4/80 (c) of the superior rectus muscle (SRM) 3 days after reinsertion of the SRM in a rabbit model: the normal saline injection group (above) and the Y-27632 injection group (below). Compared to the control group in which saline was injected into the SRM, only F4/80-positive areas were less present in the Y-27632 injection group (magnification, ×100). Immunepositive cells of Ki-67, CD11 beta, and F4/80 at 3 days after reinsertion of the SRM in a rabbit model (d).

4. Discussion

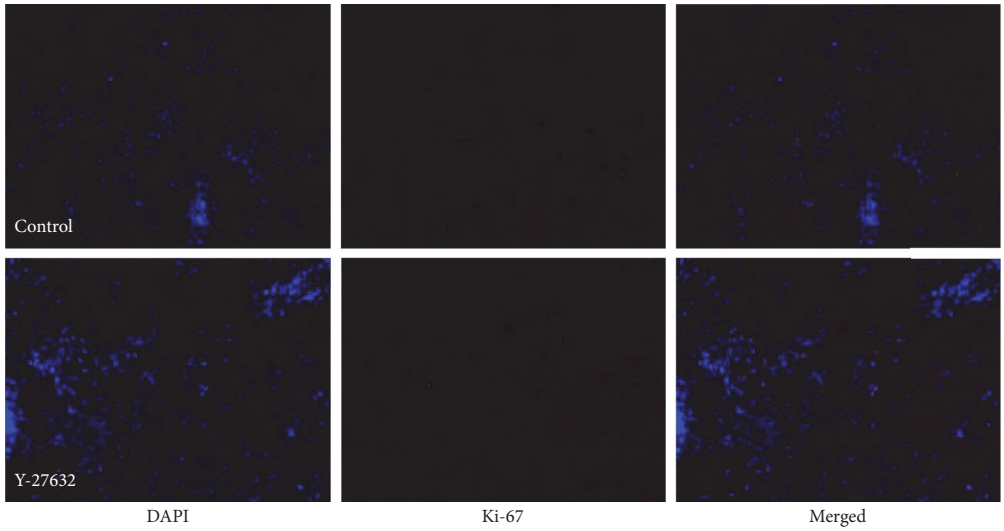
In the present study, we demonstrated that the inhibitor of Rho-kinase, Y-27632, was able to reduce inflammation and SRM/conjunctiva adhesion in rabbit eyes following extraocular muscle surgery. Our results show that immunofluorescence staining of F4/80 decreased at postoperative day 3 and week 4. F4/80, a macrophage marker [21], exhibited lower positivity in the Y-27632 injection group compared to the control, implying that Y-27632 decreased the number of macrophages recruited to the wound site; that is, Y-27632 may suppress the macrophage-mediated inflammatory response. The protein serine/threonine kinase ROCK1 is the main effector of Rho GTPase RhoA, and the RhoA/ROCK pathway is the main regulator of actin cytoskeleton and actin-related functions in the cells. During the immune response, the movement of macrophages toward the target relies on proper organization of actin cytoskeleton and focal adhesions in the front and back of the cell. Y-27632 is a recently identified specific inhibitor of the ROCK-ROK family of protein kinases. Inhibition of ROCK1 by Y-27632 abolishes macrophage polarity and reduces their podosomes, motility, and phagocytosis and increases matrix degradation [22–25].

One of the predominant inflammatory cells within a wound is the macrophage at 48 to 96 h after wounding. Macrophages act as a major source of various cytokines and growth factors and are required to support cellular recruitment and activation, matrix synthesis, angiogenesis, and remodeling. Unlike neutrophils, macrophages remain within a wound until healing is complete [26].

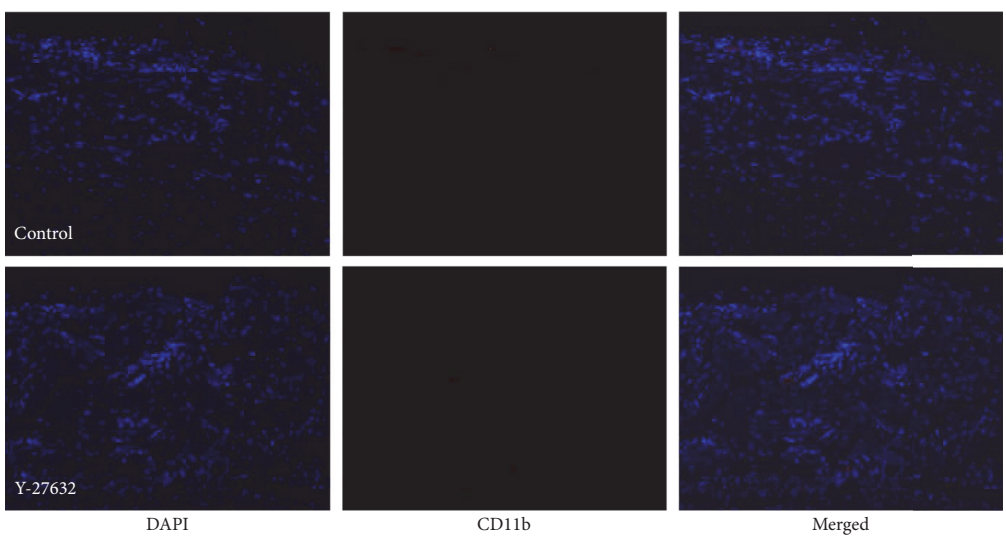
Immunofluorescence staining of F4/80 was decreased in the Y-27632 injection group, but an expression of CD11β and Ki-67 at postoperative day 3 and week 4 was not different between the groups. CD11β is expressed on the surface of many leukocytes involved in the innate immune system, including monocytes, granulocytes, macrophages, and natural killer cells [27]. Additionally, Ki-67 is a cellular marker of proliferation [28] with which it is strictly associated. Our results imply that Y-27632 injection may decrease the inflammatory response mediated by macrophages, but this does not occur by either inhibition of leukocyte recruitment or inflammatory cell proliferation. Our study shows only the results of postoperative day 3 and week 4. Therefore, further studies are needed to demonstrate the intermediate process in order to confirm that the results of three days last up to four weeks.

Macrophages and fibroblasts release numerous growth factors and cytokines that contribute to fibroblast migration: fibroblast growth factor (FGF), IGF-1, vascular endothelial growth factor (VEGF), IL-1, IL-2, IL-8, platelet-derived growth factor (PDGF), TGF-α, TGF-β, and TNF-α. Fibroblasts are activated to proliferate and begin synthesizing collagen. These activated fibroblasts are related to adhesion and fibrosis [26].

In this study, the SRM/conjunctiva adhesion score differed between the groups; this might be interpreted as a reduction in the macrophage-mediated inflammatory response, thus reducing adhesion and fibrosis. However, the SRM/sclera adhesion score and Masson's trichrome/fibronectin staining did not differ significantly. Therefore, we believe that other factors in addition to macrophages might be involved in the inflammatory process and the activation of fibroblasts in the extraocular muscle and surrounding connective tissue. Analysis of the tissue should consider the possibility of biases due to lengthened scars, consisting of amorphous connective tissue. Therefore, all procedures of this study were conducted by a single ophthalmologist who had experiences of strabismus operation more than ten years. In addition, factors that distinguish stretched scars from



(a)



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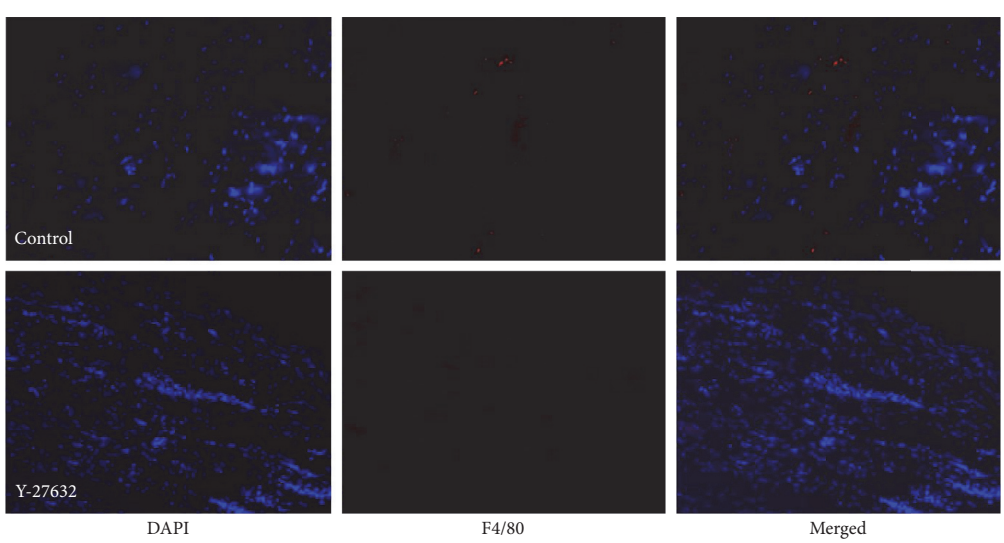

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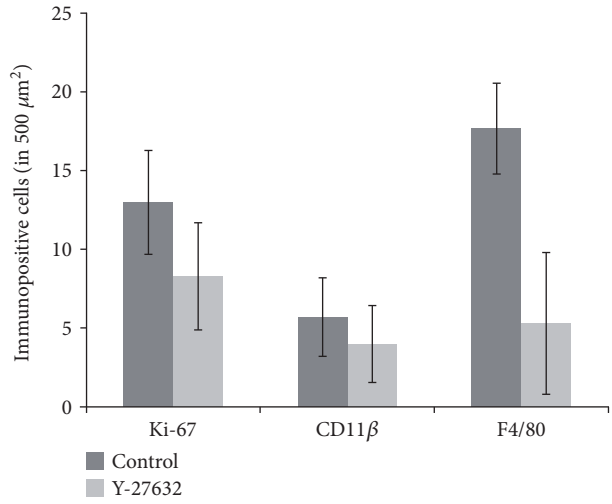
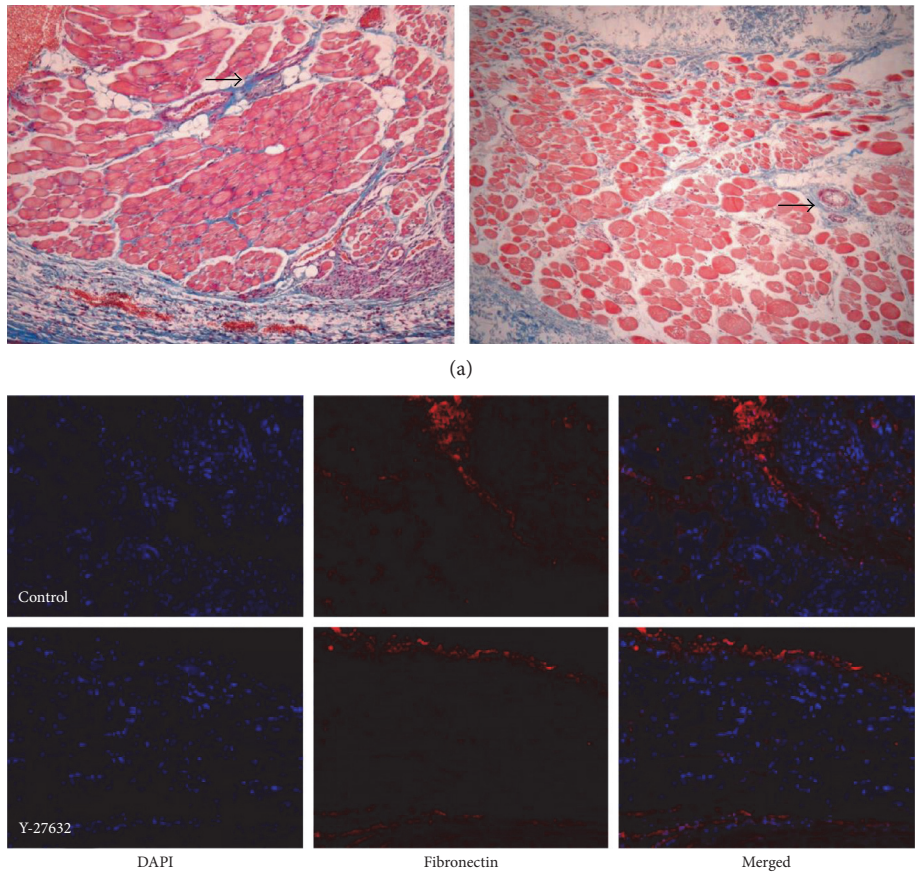


(d)

Figure 2: Immunohistochemical staining for Ki-67 (a), CD11 beta (b), and F4/80 (c) of the SRM 4 weeks after reinsertion of the SRM in a rabbit model: the normal saline injection group (above) and the Y-27632 injection group (below). Compared to the control group in which saline was injected into the SRM, only F4/80-positive areas were less present in the Y-27632 injection group (magnification, ×100). Immunepositive cells of Ki-67, CD11 beta, and F4/80 at 4 weeks after reinsertion of the SRM in a rabbit model (d).



(a)

(b)

Figure 3: Light microscopy findings (Masson trichrome; original magnification, ×100) of the SRM 4 weeks after surgery (a): the normal saline injection group (left) and the Y-27632 injection group (right). Noted fibrosis within the muscle bundles was observed in the control group. Some muscle fiber was stained red in addition to the blue color (arrow). Mild fibrosis was observed in the Y-27632 group. Light microscopy findings (fibronectin; original magnification, ×100) of the SRM 4 weeks after surgery (b): the normal saline injection group (above) and the Y-27632 injection group (below).

classic slipped muscles included minimal or no limitation of versions, less separation of the tendons from the sclera, and thicker appearance of the scar segments [29].

Rho-GTPase participates in signaling pathways leading to the formation of actin stress fibers and focal adhesions. Rho is also involved in diverse physiological functions associated with cytoskeletal rearrangements, such as cell morphology, cell motility, cytokinetics, and smooth muscle contraction. Rho-associated protein kinases (ROCKs) play a key role in focal adhesions and stress fiber formation in cultured fibroblasts and epithelial cells and in Ca^{+2} sensitization of smooth muscle cells [30]. Y-27632 is a recently identified specific inhibitor of the ROCK-ROK family of protein kinases. Y-27632 inhibits Ca^{+2}-sensitive smooth muscle contraction and has been suggested as a useful therapeutic for hypertension [13].

Recent studies have shown that the Rho/Rho-kinase pathway is associated with tissue fibrosis and inflammation [31–34]. The Rho/ROCK-mediated pathway plays a role in the infiltration of inflammatory cells both *in vitro* and *in vivo* [16, 17]. Y-27632 also prevented the upregulation of α-smooth muscle actin (α-SMA), a marker of tissue fibrosis, and inhibited tubulointerstitial fibrosis in mouse kidney with unilateral ureteral obstruction [14]. Another study reported that the selective ROCK inhibitor, fasudil, had beneficial effects on bleomycin-induced pulmonary fibrosis in mice. The number of infiltrated inflammatory cells in bronchoalveolar lavage fluid (BALF) was attenuated by fasudil [32]. The present results suggest that Y-27632 inhibits the macrophage-mediated inflammatory response and reduces adhesion and fibrosis.

In conclusion, our data suggest that injection of Y-27632 inhibited inflammation and adhesion during the wound healing process. Y-27632 may thus have significant potential as prophylaxis for postoperative adhesion syndrome in extraocular muscle surgery.

Disclosure

This paper was presented as a conference paper in the 10th ISOPT (International Symposium on Ocular Pharmacology and Therapeutics) Clinical.

Conflicts of Interest

All authors have completed and submitted the ICMJE form for disclosure of potential conflicts of interest.

Authors' Contributions

Sun Young Shin designed the study. Hyun Kyung Kim and Sun Young Shin conducted the study. Hyun Kyung Kim and Sun Young Shin contributed to the collection, management, analysis, and interpretation of the data. Ji-Sun Moon and Sun Young Shin prepared the manuscript. Ji-Sun Moon and Sun Young Shin reviewed and approved the manuscript.

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Review Article

Rho-Kinase/ROCK as a Potential Drug Target for Vitreoretinal Diseases

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Rho-associated kinase (Rho-kinase/ROCK) was originally identified as an effector protein of the G protein Rho. Its involvement in various diseases, particularly cancer and cardiovascular disease, has been elucidated, and ROCK inhibitors have already been applied clinically for cerebral vasospasm and glaucoma. Vitreoretinal diseases including diabetic retinopathy, age-related macular degeneration, and proliferative vitreoretinoapthy are still a major cause of blindness. While anti-VEGF therapy has recently been widely used for vitreoretinal disorders due to its efficacy, attention has been drawn to new unmet needs. The importance of ROCK in pathological vitreoretinal conditions has also been elucidated and is attracting attention as a potential therapeutic target. ROCK is involved in angiogenesis and hyperpermeability and also in the pathogenesis of various pathologies such as inflammation and fibrosis. It has been expected that ROCK inhibitors will become new molecular target drugs for vitreoretinal diseases. This review summarizes the recent progress on the mechanisms of action of ROCK and their applications in disease treatment.

1. Introduction

Rho-associated kinase (Rho kinase/ROCK), identified as a Rho GTP-binding protein, is a downstream effector of the small GTP-binding protein Rho [1–5]. Two isoforms, ROCK1 (also known as ROKβ or p160ROCK) and ROCK2 (known as ROKα), were isolated as Rho-A-GTP interacting proteins [6]. The Rho/ROCK signaling pathway is implicated in various cellular functions, such as cell proliferation, migration, and contraction [7], and has been reported to be crucial for cardiovascular diseases, central nervous disorders, and cancer. Therefore, ROCK has attracted attention as a therapeutic target for various diseases [8, 9]. Recent studies have implicated Rho/ROCK signaling in both physiological and pathological ophthalmology [10] (Table 1). This review summarizes the recent progress on the role of Rho kinase/ROCK and its therapeutic potential in vitreoretinal diseases (Figure 1).

2. Clinical Application of a ROCK Inhibitor

Pre-clinical research has indicated that ROCK is an important molecule in the pathogenesis of cardiovascular diseases [11]. Based on these accumulated data, a selective ROCK inhibitor, fasudil, has been used in the clinical setting for cerebral vasospasm and ischemic stroke in Japan and China [12]. In ophthalmology, Honjo et al. have demonstrated lowering of intraocular pressure by ROCK inhibition in rabbits. This is the first report to show a therapeutic potential of ROCK inhibitor in eye disease [13]. In another study, a novel, potent, and selective ROCK inhibitor, ripasudil hydrochloride hydrate (K-115), could undergo a structural change and enhance the stearic affinity of the enzyme for ROCK [14]. The enzyme inhibitory effect of ripasudil is about five to ten times higher than that of the previous ROCK inhibitors such as fasudil

Table 1: Comparison between VEGF and Rho/ROCK in disease pathogenesis.

Biological process	VEGF inhibition	Rho/ROCK inhibition
Ischemia	Possible induction of ischemia [24]	Vascular normalization via pericyte coverage [45]; vessel dilation [58]; increased blood velocity and retinal blood flow [59]
Angiogenesis	Antiangiogenesis [81, 82]	Antiendothelial proliferation in vitro [44, 45]; antiendothelial migration in vitro [44, 45]; antiangiogenesis in vivo retina [43, 45]; antiangiogenesis in vivo choroid [63, 66]
Hyperpermeability	Antipermeability [83, 84]	Antipermeability in choroidal neovascularization [63, 66]
Inflammation	Antileukocyte trafficking [81]; antileukostasis [84]	Antileukostasis [38]; anti-M2 macrophage [63]
Membrane contraction	Possible induction of membrane contraction and tractional retinal detachment [23]; vessel contraction [25]	Inhibition of membrane contraction in vivo [50, 73]; reduced collagen synthesis in RPE [66]; inhibition of gel contraction by RPE [72, 73]; anti-RPE proliferation [72]; actin depolymerization in RPE [74]
Neuronal damage	Possible induction of photoreceptor damage [85, 86]	Neuroprotection of RGC [78, 87, 88]
Fibrosis	Risk of inducible fibrosis [22, 65]	Antifibrosis in choroidal neovascularization [66]

and ripasudil which were clinically approved in 2014 as an eye drop for glaucoma in Japan [15].

3. Unmet Needs in Vitreoretinal Diseases

Vitreoretinal diseases are a common cause of blindness among working age adults [16, 17]. Anti-VEGF administration is currently the most commonly used treatment option for wet age-related macular degeneration (AMD), macular edema secondary to retinal vein occlusion (RVO), and diabetic macular edema (DME) [18–20]. However, in addition to a need for repeated administration and the possibility of local or systemic adverse complications [21], its wide use is implicated in conditions beyond VEGF inhibition such as fibrosis in AMD, retinal ischemia, and fibrovascular membrane contraction in DR [22–25] (Table 1). Chronic anti-VEGF therapy may also increase medical expenses [26]. Therefore, novel therapies aside from VEGF are needed in the treatment of vitreoretinal diseases [27].

4. ROCK as a Therapeutic Target for Diabetic Retinopathy

4.1. Role of ROCK in Microvascular Complications in DR. While visual acuity is not always affected in nonproliferative stages of diabetic retinopathy (DR) without DME, DR progression can cause neovascularization, vitreous hemorrhages, preretinal fibrovascular proliferation, and tractional retinal detachment, which can lead to severe vision loss [28]. DR pathogenesis is accompanied by microvascular complications such as hyperpermeability, angiogenesis, microthrombosis, and inflammation [29, 30]. Diabetic retinal capillary disorder may be associated with retinal leukocyte stasis (leukostasis) at early nonproliferative stages of DR [31–34]. Leukostasis is mediated by adhesion molecules, intercellular adhesion molecule-1 (ICAM-1), and leukocyte β2 integrins (CD18/CD11a and CD18/CD11b) [31, 35]. ROCK pathway has been reported to regulate the expression and function of ICAM-1 in endothelial cells [36] and could be activated in vascular cells by serum from diabetic retinopathy patients [37]. This observation suggested that endothelial cells in diabetic retinopathy patients could be in a "ROCK-activated status" at the systemic level. Furthermore, a study with streptozotocin-induced diabetic model confirmed activation of the Rho/ROCK pathway in retinal microvessels [38]. Moreover, intravitreal fasudil significantly reduced ICAM-1 expression, leukocyte adhesion, and the number of damaged endothelial cells in retinas of diabetic rats [38] (Table 2). These data indicate that ROCK signaling plays important roles in the pathogenesis of microvascular complications in diabetic retinopathy, and its inhibition may represent a new strategy for managing early stage diabetic retinopathy, which is an observation period with no ophthalmic treatment.

4.2. Controversial Role of ROCK in Hyperpermeability and Angiogenesis. VEGF plays a critical role in the pathogenesis of DR-related hyperpermeability and angiogenesis [39]. While ROCK inhibition by Y27632 could block VEGF-induced endothelial hyperpermeability [40], the role of ROCK in TNF-α-induced endothelial permeability is still controversial [41, 42]. The effect of ROCK inhibitors on hyperpermeability in diabetic retinopathy may be different for each case. A ROCK inhibitor, Y27632, blocked VEGF-induced angiogenesis in an oxygen-induced retinopathy (OIR) model [43], while fasudil inhibited angiogenesis in corneal and OIR models [44, 45] (Table 2). In vitro, ROCK inhibition by fasudil significantly inhibited VEGF-induced retinal endothelial cell proliferation and migration in human and bovine retinal endothelial cells [44, 45]. These previous data suggest that a mechanism of ROCK inhibition on VEGF-induced angiogenesis could be via blockade of endothelial migration and proliferation. In contrast, a study with a ROCK inhibitor H-1152 showed increased VEGF-induced angiogenesis in an OIR model and an in vitro sprouting model via ERK1/2 activation [46]. This discrepancy might be due to different drug affinities against the two ROCK isoforms or an unexpected nonspecific effect [47]. It has also

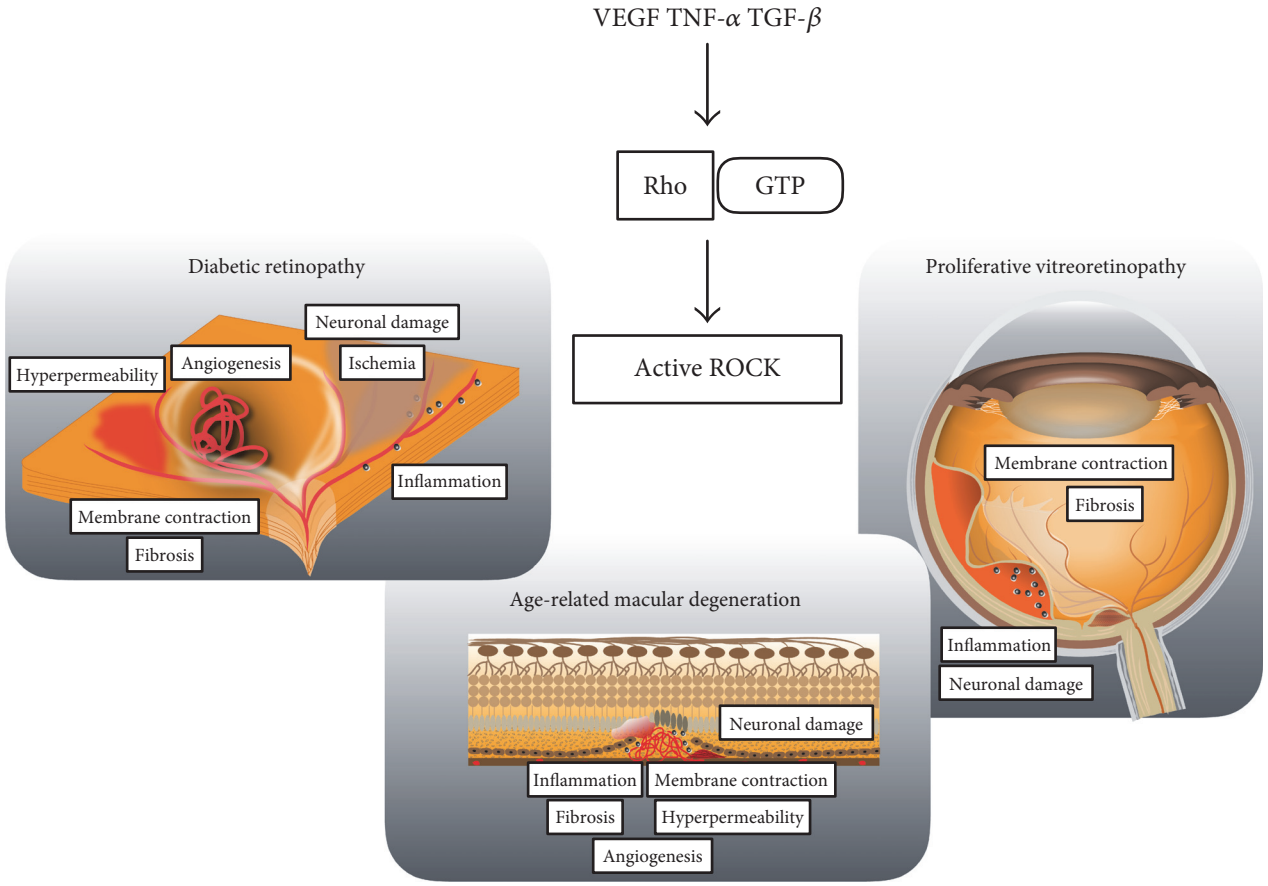

Figure 1: ROCK-activated vitreoretinal diseases. ROCK activation is involved in the pathology of retinal vitreous diseases such as diabetic retinopathy, age-related macular degeneration, and PVR, termed as "ROCK-activated diseases." In each disease, the Rho-ROCK signaling pathway is activated by various cytokines, implicating ROCK in various pathology. Therefore, ROCK is a potential therapeutic target for these vitreoretinal diseases.

been reported that ROCK signaling could upregulate VEGF in diabetic retina [48].

4.3. ROCK as a Therapeutic Target in Proliferative Membrane. In the later stages of DR, epiretinal fibrovascular membranes that form along with retinal neovascularization contract and result in traction retinal detachment (TRD) [49]. ROCK inhibition effectively disrupted α-SMA organization and blocked contraction of the proliferative membrane in an in vivo experimental rabbit model [50] (Table 2). In hyalocyte-containing collagen gel assays, ROCK inhibition almost completely abolished PDR vitreous-induced collagen gel contraction mediated through MLC phosphorylation suppression [50, 51].

4.4. Involvement of ROCK Different from VEGF in Diabetic Macular Edema. Diabetes reduces occludin quantity at tight junctions in retinal endothelial cells and causes tight junction protein disorganization in retinal arterioles and capillaries [52], presumably leading to vascular hyperpermeability and DME. The Rho/ROCK pathway has been associated with tight junction protein degradation and blood-brain barrier disruption [53]. Furthermore, recent clinical observations suggested that combination therapy of bevacizumab and fasudil intravitreal injection was effective based on structural and functional outcomes in eyes with severe DME that were resistant to current anti-VEGF therapy [54, 55], indicating that ROCK inhibition is mechanistically different from anti-VEGF therapy.

4.5. ROCK Inhibition for Retinal Ischemia. Currently, there is no effective treatment for microthrombosis and retinal ischemia. Although laser photocoagulation has been used to treat diabetic retinopathy patients with ischemic retinal tissue, this treatment could cause several adverse events including night blindness. A recent paper showed ROCK inhibition by ripasudil could cause intraretinal vascularization while inhibiting preretinal angiogenesis, leading to reduced hypoxic area in an OIR model [45]. Furthermore, the ripasudil treatment could improve retinal vascular perfusion and induce pericyte coverage [45] (Table 2). This phenomenon could be the vascular normalization that has been proposed in cancer research [56]. However, further investigation using other ROCK inhibitors would be necessary to validate the induction of vascular normalization. Fasudil has already been shown to improve ischemia in patients with acute ischemic stroke [57]. It has been previously reported that ROCK inhibition could cause retinal vessel dilation,

Table 2: ROCK inhibitors in animal models of vitreoretinal diseases.

Animal model	OIR model (oxygen-induced retinopathy)	STZ model (streptozotocin-induced diabetes model)	CNV model (choroidal neovascularization model)	PVR (proliferative vitreoretinopathy model)
Fasudil	Antiangiogenesis [45]	Antileukostasis [38]	Antipermeability [63]; anti-M2 macrophage [63]; antiangiogenesis [63]	Inhibition of membrane contraction [50]
Ripasudil (K115)	Vascular normalization via pericyte coverage [45]; antiangiogenesis [45]	No report	No report	No report
Y27632	Antiangiogenesis [43]	No report	No report	Inhibition of membrane contraction [73]
AMA0428	Antiangiogenesis [89]; inhibition neuronal cell death [89]	Antileukostasis [89]; antipermeability [89]; neuroprotection of RGC [89]	Antiangiogenesis [66]; antifibrosis [66]; antiinflamation [66]	No report

and this in turn could contribute to ischemia improvement [58]. A recent study in cats showed that intravitreal ripasudil injection could significantly increase retinal blood velocity and flow [59]. ROCK inhibition may therefore be a new therapeutic strategy for retinal ischemia in retinal vascular disorders.

5. ROCK as a Therapeutic Target for Age-Related Macular Degeneration

5.1. ROCK2-Mediated Macrophage Polarization in Aging. There are two types of AMD, a dry form that ultimately leads to macular atrophy and a wet and exudative form characterized by choroidal neovascularization (CNV) and leakage [60]. The pathogenesis of AMD remains incompletely understood. Macrophages are found in CNV lesions and have been reported to promote and inhibit CNV [61, 62]. This phenotype-associated mechanism was unknown. Furthermore, it was also unclear how aging promotes the pathogenesis. A recent paper by Zandi et al. showed that macrophage polarization was triggered by ROCK2 signaling, which is increased with age, and a shift of the fundus microenvironment through selective ROCK2 inhibition improved the pathology [63] (Table 2).

5.2. ROCK as a Possible Target in Subretinal Fibrosis. Wet AMD-related CNV eventually causes fibrosis that could lead to irreversible vision loss [64, 65], and there is currently no effective treatment for this fibrosis. A ROCK inhibitor, AMA0428, was recently reported to be effective in reducing fibrosis in a mouse CNV model [66] (Table 2). As the Rho/ROCK pathway is a downstream signaling of fibrotic disease drivers, such as TGF-β [67, 68], ROCK inhibition might block TGF-β-related subretinal fibrosis although the detailed mechanism is still unknown. ROCK inhibition may therefore be a new therapy for fibrosis and neovascularization in AMD.

6. ROCK as a Therapeutic Target for Proliferative Vitreoretinopathy

Proliferative vitreoretinopathy (PVR) is the leading cause of failure after retinal detachment surgery. PVR is characterized by the growth and contraction of cellular membranes within the hyaloid and retina and on both retinal surfaces following retinal reattachment surgery [69]. Retinal detachment allows macrophages, retinal pigment epithelial (RPE) cells, glial cells, and fibroblasts to migrate to the vitreous, where they proliferate, survive, form extracellular matrix proteins, and assemble into a membrane [70]. Some studies suggest that cytokines such as TGF-β2 and PDGF contribute to PVR pathogenesis [71]. However, there is currently no effective treatment other than surgery. Various recent papers have shown that the ROCK pathway is involved in PVR pathogenesis. The importance of ROCK for TGF-β-induced gel contraction by retinal pigment epithelium has been reported [67, 72–74]. Furthermore, in vivo studies suggest that ROCK inhibition could block TRD development [50, 73] and that ROCK inhibitors might aid in PVR prevention and development apart from vitrectomy surgery [75] (Table 2).

7. Future Directions of ROCK Inhibitors: Neuroprotection

Microvascular changes underlie DR and AMD, while histological studies have characterized the loss of neurons [76]. The roles of neural retinal alterations in the pathogenesis of early retinopathy and the mechanisms of vision loss have been emphasized [77]. A recent report has demonstrated that administration of an oral ROCK inhibitor, K115, delayed RGC death [78]. Fasudil also resulted in ischemia-related apoptosis of retinal cells by inhibiting Bax/Bcl-2, caspase-3, and iNOS in rats [79]. However, the importance of ROCK for neural degeneration in vitreoretinal diseases including DR and AMD is unknown. Future investigations are expected to demonstrate a therapeutic potential of ROCK inhibitors in vitreoretinal disorders.

8. Future Directions of ROCK Inhibitors: Beyond VEGF

In summary, some disease states extend beyond VEGF inhibition, including fibrosis in AMD, retinal ischemia, retinal neuropathy, and fibrovascular membrane contraction in

DR (Table 1). ROCK inhibition may be effective in these pathological conditions. A previous study using radiolabeled drug revealed that ripasudil could reach the retina and choroid after eye drop administration in rabbits [14]. If proven effective, topical ophthalmic treatment would be beneficial for patients with vitreoretinal diseases. Furthermore, the role of ROCK isoforms in vitreoretinal diseases is unclear. In a recent paper, a ROCK2 inhibitor, but not the pan-ROCK inhibitor fasudil, was beneficial in age-related immune changes in AMD [63]. Intensive investigation is needed to elucidate the role of ROCK isoforms in the pathogenesis of these vitreoretinal diseases.

9. Conclusion

The clinical application of anti-VEGF therapy and its success constitutes the beginning of the era of molecular targeting drugs in ophthalmology. Currently, various molecular targeting drugs are under clinical trials for vitreoretinal diseases [80]. Several will be clinically applied in the near future, and these are expected to impact the therapeutic strategy of vitreoretinal diseases. ROCK could be one of these potential drug targets. An optimal administration method/administration protocol is expected to emerge based on both clinical and nonclinical investigations.

Conflicts of Interest

Shintaro Nakao and Tatsuro Ishibashi have conflicting interests of Kowa (Patent). The other authors declare that there is no conflict of interest regarding the publication of this paper.

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Research Article

A Surgical Cryoprobe for Targeted Transcorneal Freezing and Endothelial Cell Removal

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Purpose. To examine the effects of transcorneal freezing using a new cryoprobe designed for corneal endothelial surgery. *Methods*. A freezing console employing nitrous oxide as a cryogen was used to cool a series of different cryoprobe tip designs made of silver for high thermal conductivity. In vitro studies were conducted on 426 porcine corneas, followed by preliminary in vivo investigations on three rabbit corneas. *Results*. The corneal epithelium was destroyed by transcorneal freezing, as expected; however, the epithelial basement membrane remained intact. Reproducible endothelial damage was optimally achieved using a 3.4 mm diameter cryoprobe with a concave tip profile. Stromal edema was seen in the pre-Descemet's area 24 hrs postfreeze injury, but this had been resolved by 10 days postfreeze. A normal collagen fibril structure was seen 1 month postfreeze, concurrent with endothelial cell repopulation. *Conclusions*. Transcorneal freezing induces transient posterior stromal edema and some residual deep stromal haze but leaves the epithelial basement membrane intact, which is likely to be important for corneal re-epithelialization. Localized destruction of the endothelial monolayer was achieved in a consistent manner with a 3.4 mm diameter/concave profile cryoprobe and represents a potentially useful approach to remove dysfunctional corneal endothelial cells from corneas with endothelial dysfunction.

1. Introduction

Corneal transparency is maintained in the healthy eye by a monolayer of endothelial cells on the inner surface of the cornea. Even though human corneal endothelial cells do not possess the capacity for proliferation in vivo, the endothelium as a whole has a functional reserve to cope with cell loss via the spreading and enlargement of cells adjacent to those lost [1, 2]. Excessive endothelial loss and deterioration caused by eye pathologies such as Fuchs' endothelial corneal dystrophy (FECD), however, lead to corneal edema, clouding, and eventually loss of vision. FECD is a progressive degenerative disorder that is a major indication for corneal transplant surgery. Surgical intervention in the form of a full-thickness-penetrating keratoplasty—or more commonly nowadays a posterior lamellar graft—is the main treatment option. But, despite the success of corneal graft surgery, some questions about the long-term survival of the donor tissue [3]

and the recurring problem of sufficient tissue availability remains. These limitations have led researchers to seek potential alternatives to corneal transplantation to treat corneal endothelial dysfunction.

One promising route involves the use of selective inhibitors of the Rho kinase pathway. The so-called ROCK inhibitors regulate the actin cytoskeleton and influence vital cell activities such as motility, proliferation, and apoptosis [4]. Owing to their demonstrable value, numerous studies have been conducted in recent years which focus on the effect of ROCK inhibitors on corneal endothelial cells both in vivo and ex vivo [5–9]. One approach involves transcorneal freezing to damage corneal endothelial cells in the central portion of the cornea in patients with FECD followed by the topical delivery of a ROCK inhibitor, Y27632, in the form of eye drops to encourage peripheral endothelial cells that had been unaffected by the freeze injury to repopulate the central zone of the corneal endothelium [10–12]. Freeze damage is achieved by application of a cold probe to the corneal surface. Another approach involves cell injection therapy whereby cultivated human corneal endothelial cells are injected into the anterior chamber of the eyes with FECD in a suspension that includes Y27632 ROCK inhibitor [13]. This agent has also been tested in the form of eye drops as a long-term pharmacological treatment for bullous keratopathy [14].

A small series of first-in-man surgeries to test the concept of transcorneal freezing followed by short-term ROCK inhibitor eye drop application for the treatment of FECD was conducted a few years ago and showed promise [10–12]. In this approach, the tip of a stainless steel rod, 2 mm in diameter, was immersed in liquid nitrogen at −196°C before being applied to the surface of the central cornea for an arbitrarily chosen time of 15 sec. The assumption was that central corneal endothelial cells located underneath the cold-rod applicator would be destroyed by freeze injury, although this could not be directly confirmed in the human subjects because the cloudy FECD corneas did not allow a view of the endothelium by specular microscopy. The freezing of corneal tissue has also been used as a modality to induce an injury to facilitate basic research into corneal wound healing [15–28]. If corneal freezing is to be used in a clinical setting, however, (either for the destruction of diseased cells in the central endothelium prior to ROCK inhibitor eye drop application for FECD as described above or to pretreat the cornea prior to targeted drug delivery to combat conditions such as fungal keratitis) we contend that it needs to be achieved in a more sophisticated, reliable, and reproducible manner than that achieved with an immersion-cooled steel rod. Here, we report the development and validation of a new cryoprobe based on the expansion of nitrous oxide as a cryogen and its effect, in vitro and in vivo, on the corneal epithelium, stroma, and endothelium.

2. Materials and Methods

2.1. Cryoprobe Development. A console that uses nitrous oxide as a cryogen was manufactured in conjunction with a series of cryoprobes with newly designed tips, some of which matched the cornea's curvature (Figure 1). This prototype project was carried out by Coronet Medical Technologies Ltd., the ophthalmic arm of Network Medical Products Ltd. Enclosed gas expanded within the tip and was recycled therein, achieving a low temperature based on the Joule/Thomson effect. The tip of the cryoprobe, used to contact the corneal surface, was circular around the probe's main axis and a number of designs were tested. Probe tips were 1.8 mm, 2.4 mm, or 3.4 mm in diameter and were manufactured from silver for high thermal conductivity. Larger diameter probe tips were not considered because of the option of multiple surface freeze placements should a wider area of the cornea need to be treated. Probes had either a flat surface profile or a concave one with a radius of curvature of 8 mm. For ease of use, a foot switch was incorporated into the design, which initiates cooling at the cryoprobe tip and maintains the reduced temperature throughout the whole time it is depressed. The foot switch is linked to a timer on the main console that provides a visual output of freezing time plus an audible signal (with a mute option) at 1 sec intervals when the foot switch is depressed. Freezing temperature at the probe tip (−50°C) is reached within 2 sec of depressing of the foot switch; after release, ambient room temperature is achieved within seconds. The hand-held cryoprobe has an ergonomic-angled design to allow easy application to the corneal surface (Figure 1). Probes should be thoroughly cleaned, inspected, and autoclaved prior to use.

2.2. Transcorneal Freezing In Vitro. The porcine cornea is comparable to that of the human cornea in terms of its structure and its overall dimensions and the pig eye is thus often used for practice by trainee corneal surgeons. The central corneal thickness in adult pigs is usually around 660 μm [29, 30], which approximates a representative measurement of edematous corneal thickness in individuals with FECD [31, 32]. The porcine eyes, therefore, were well suited for our investigations, and the intact eyeballs, including extraocular muscles, were obtained soon after slaughter at a local abattoir (W. T. Maddock, Kembery Meats, Maesteg, Wales, UK). These were brought to the laboratory on ice and experiments were begun within 2-3 h of death. When the eyes arrived at the home laboratory, ultrasound measurements of central corneal thickness were made with a Tomey SP-100 pachymeter (Erlangen, Germany), which revealed that the corneas had thickened (~1000 μm) postmortem compared to those of published values [31, 32]. Consequently, the eyes were placed in a humidified incubator (Brinsea Octagon 100, Egg Incubator, Sandford, UK) at 45°C for 30–45 min to reverse the postmortem swelling and attain a thickness similar to what might be expected in humans with FECD. In total, 426 porcine eyes were used for the in vitro experiments in which the corneal thickness ranged from 483 μm to 831 μm owing to differences in eye size and likely differential postmortem swelling and deswelling.

Immediately after an eye was removed from the humidified incubator, its central corneal thickness was recorded as an average of eight measurements. The cryoprobe tip was then applied to the corneal surface and cooling was activated by pressing the foot switch to control the flow of nitrous

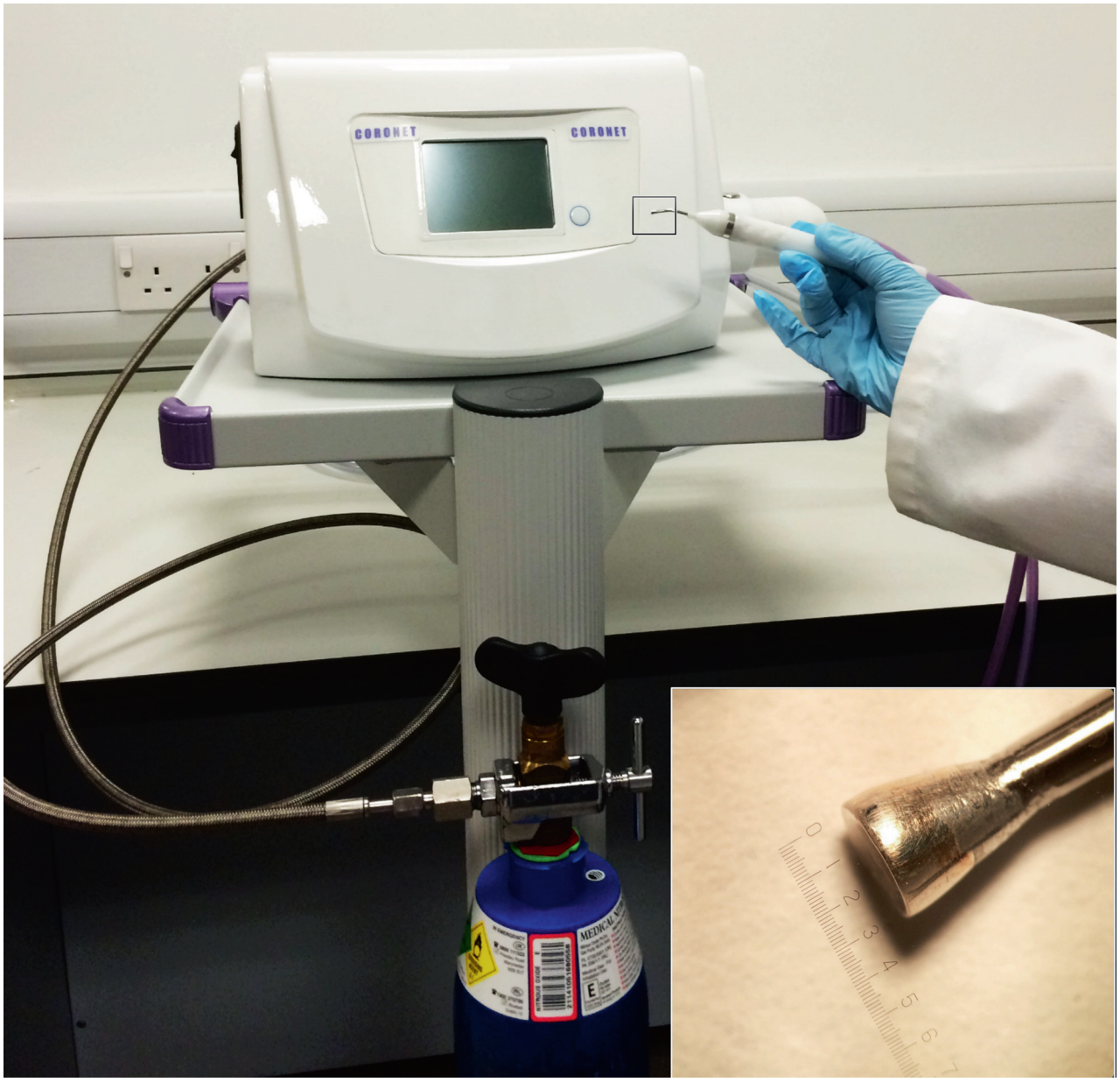

Figure 1: The transcorneal freezing machine attached to a cylinder of medical grade nitrous oxide (the cryogen), which comprises a main console with a monitor screen that illustrates freeze time, cryogen levels, and readiness for freeze plus interchangeable probe tips and a footswitch (not shown). Inset: the 3.4 mm diameter concave probe tip manufactured from silver for high thermal conductivity.

oxide gas. Freezing times of 3, 5, 9, and 13 sec were tested, allowing an additional 2 sec for the freezing temperature to be reached in all cases (thus, a 3 sec freeze required for a 5 sec application and foot-switch depression). The cornea was then carefully excised at the limbus after which staining solutions of 0.2% alizarin red (~1 ml) and 0.25% trypan blue were applied sequentially to the endothelial surface for 60–90 sec each to identify dead cells. The stains were then gently washed off using approximately 5 ml of 0.9% sodium chloride buffer solution, after which digital images of the corneal endothelial surface were captured on a Zeiss Stemi 1000 light microscope (Carl Zeiss, Jena, Germany). A successful freeze injury was deemed to have occurred when a clear circular wound area was seen. The endothelial wound area in each corneal image was manually traced and calculated using Image J software (http://imagej.nih.gov/ij/). Magnification was calibrated for each set of experiments using the image of an eyepiece graticule. Averages of three measurements were calculated and data were further collated using Microsoft Excel software (Microsoft Corp., Redmond, WA, USA).

2.3. Statistical Analysis. Statistical analyses were carried out operating IBM SPSS Statistics software (Version 23.0, IBM Corporation, New York, USA). Spearman's rank order correlation test was run to determine the relationship between central corneal thickness and endothelial wound areas induced by the freezing.

2.4. Scanning Electron Microscopy. To investigate the endothelial damage morphologically, four treated corneas were examined by scanning electron microscopy (SEM). Immediately after excision, the corneas were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for at least 2 h. Samples were then washed in PBS followed by the gradual dehydration through a graded ethanol series (from 50% to 100% in 30 min steps), after which the ethanol was replaced by hexamethyldisilazane for 20 min to minimize the shrinkage of the specimens. After drying, the corneas were placed on stubs (Agar Scientific, Stansted, UK) and sputter coated with gold (Edwards S150 sputter coater, Edwards High Vacuum Co. International, Wilmington, USA) to allow imaging in a JEOL JSM 5600 scanning electron microscope (JEOL Company, Tokyo, Japan) operating with a beam acceleration voltage of 15.0 kV.

2.5. Transcorneal Freezing In Vivo. Three adult New Zealand White rabbits were used to investigate corneal recovery after transcorneal freezing. At all stages, animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the research was approved by the IRB of Doshisha University. As reported later, the 3.4 mm concave cryoprobe was found to be the most effective in inducing endothelial damage in the porcine eye in vitro, but owing to the thinner cornea of the rabbit, the 2.4 mm concave cryoprobe was chosen for the in vivo transcorneal freezing experiments.

Under general anesthesia, a 2.4 mm probe was applied to the corneal surface of the rabbit eyes for a total of 5 sec. The contralateral eye was used as a control. Transcorneal freezing did not induce any severe general adverse effects. After 24 h, 10 days, and 1 month of treatment, the anterior segment of each eye was assessed by the use of a slit-lamp microscope and the rabbits were then euthanized. Corneal thickness was determined by the use of an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan), and the mean of eight measured values was calculated. Intraocular pressure was measured by the use of a Tonovet tonometer (292000; KRUUSE, Langeskov, Denmark). Transmission electron microscopy (TEM) was conducted, as described below, on the corneas of the rabbits that were euthanized at 24 h- and 1-month time points after transcorneal freezing.

2.6. Transmission Electron Microscopy. The rabbit corneas were examined by TEM 24 h and 1 month postfreeze was conducted in Doshisha University, Japan. Briefly, after animals were euthanized, corneas were excised at the limbus and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen buffer (pH 7.2–7.4) overnight at 4°C. Samples in fresh fixative were then express shipped to the UK. Full-thickness-dissected portions of the corneas were then subjected to alcohol dehydration and resin infiltration, after which they were embedded in epoxy resin (Araldyte CY212 resin, TAAB Laboratories, England, UK). Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a JEOL 1010 microscope operating at 80 kV (JEOL Company, Tokyo, Japan).

3. Results

3.1. In Vitro Transcorneal Freezing. Initial experiments into the degree of endothelial damage caused by transcorneal freezing induced by four different types of cryoprobe tip—that is, 1.8 mm diameter/flat profile, 2.4 mm diameter/flat profile, 2.4 mm diameter/concave profile, and 3.4 mm diameter/concave profile—revealed that the time of contact with the corneal surface did not affect the area of endothelial damage, with freezing times of 3, 5, 9, and 13 sec tested (data not shown). Based on this outcome, a freezing time of 3 sec was chosen for all the in vitro experiments described herein. It also became apparent during the initial investigations that the probe tip had to be in contact with the corneal surface before the foot switch was depressed to initiate cooling. If the probe tip was cooled in air prior to being brought into contact with the cornea, no appreciable endothelial damage was seen; no matter how long, up to 15 sec, the probe remained in contact with the cornea.

Light microscopy of the endothelial surfaces of the post-freeze, trypan blue-stained corneas indicated the area of cell damage caused by the four different probes used in these experiments. Representative images of 426 technical replicates denoting the typical extent of endothelial damage are shown in Figure 2, with examples of what were considered to be successful or unsuccessful freeze injuries. A successful freeze injury was defined as one that resulted in a well-delineated circular area of cell damage. An unsuccessful freeze injury, on the other hand, was considered to have occurred either when there was no evidence of any cell damage or when the area of damage was irregular. Based on these criteria, only 14 of 102 eyes (14%) treated with the 1.8 mm diameter/flat profile probe were judged to have been successfully wounded, whereas 78 of 108 (72%) treated with the larger (i.e., 2.4 mm diameter) flat profile probe contained successful endothelial injuries. A similar number of the eyes exhibited corneal endothelial freeze damage—that is, 76 eyes of 108 (70%)—when the 2.4 mm diameter cryoprobe with a concave profile was used. Our data clearly indicated, however, that the 3.4 mm diameter/concave profile cryoprobe induced the most consistent endothelial damage with 90 eyes of 108 (83%) being successfully wounded in a reproducible manner (Table 1).

To quantify the extent of endothelial cell damage, we used Image J to manually trace around each wound deemed to have been successfully created (i.e., $n = 258$ of 426 technical replicates). The area of each wound was calculated, which, unsurprisingly, disclosed that larger probe tips led to more extensive endothelial damage (Table 1). All of the 426 corneas examined were subjected to multiple pachymetry measurements immediately prior to transcorneal freezing. This revealed that the average corneal thickness was 649 μm ($\pm$61 μm SD), which is a fair representative value for corneal edema in humans with endothelial dysfunction [31, 32]. Moreover, when the corneal thickness of the individual corneas was taken into account, a statistical Spearman's rank order correlation test identified that there was a weak relationship between central corneal thickness and endothelial damage when treated with the smallest and largest probes

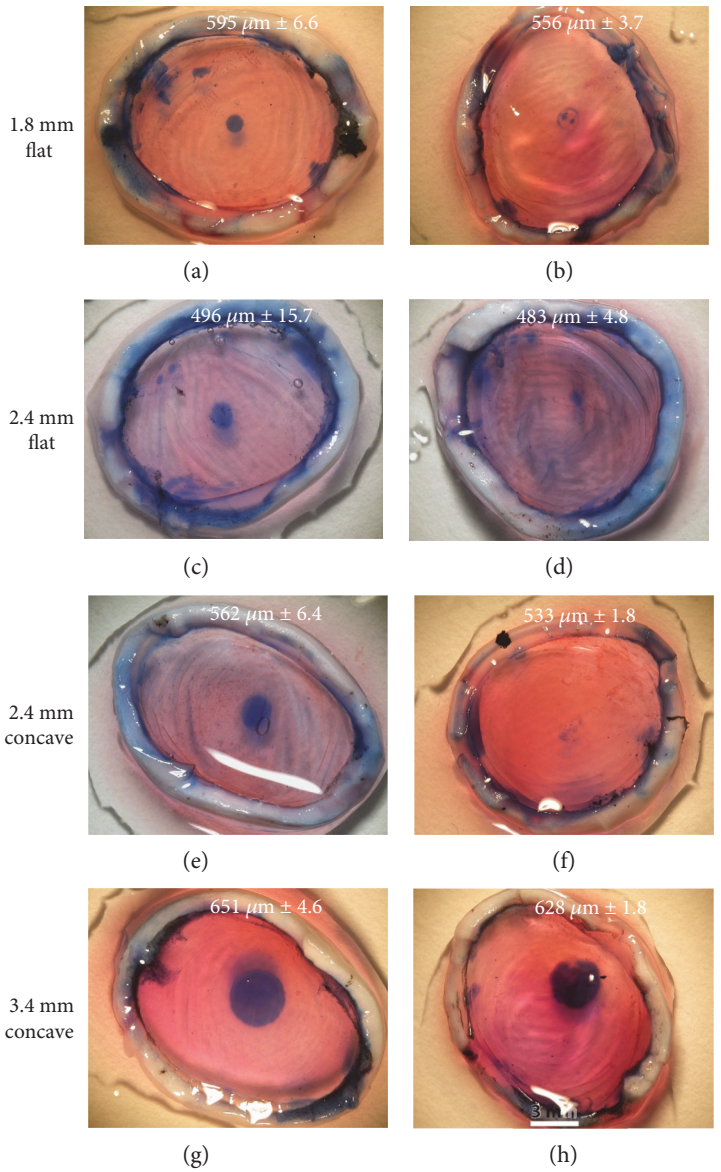

Figure 2: Representative images of corneal endothelial freeze injury on the pig eyes ex vivo induced by 3 sec freeze with four different cryoprobe tips and assessed by trypan blue staining. The thickness of each cornea is indicated on each panel (±SD) based on eight pachymetry readings. The area of cell damage is seen via the blue stain, and successful and non/less successful freeze injuries are shown in the left and right columns, respectively. Freezing with the 1.8 mm diameter/flat profile probe only rarely resulted in a reproducible wound (a and b). Endothelial freeze injury was more reliably achieved with 2.4 mm diameter probe tips with flat or concave profiles (c–f), but the optimal result and best consistency was achieved using the 3.4 mm diameter/concave profile cryoprobe (g and h). Scale bar, 3 mm. See Table 1 also.

Table 1: Data summary of transcorneal freezing for 3 sec on porcine eyes.

Probe tip (mm)/profile	Number of eyes	Number of eyes with successful freeze (%)	Mean/SD damaged area (mm^2)	Mean diameter (mm)	Mean/SD corneal thickness (nm)
1.8/flat	102	14 (14)	0.79/0.4	1.0	642/64
2.4/flat	108	78 (72)	2.12/1.0	1.6	650/71
2.4/concave	108	76 (70)	2.29/1.0	1.6	645/87
3.4/concave	108	90 (83)	6.91/1.9	2.9	654/59

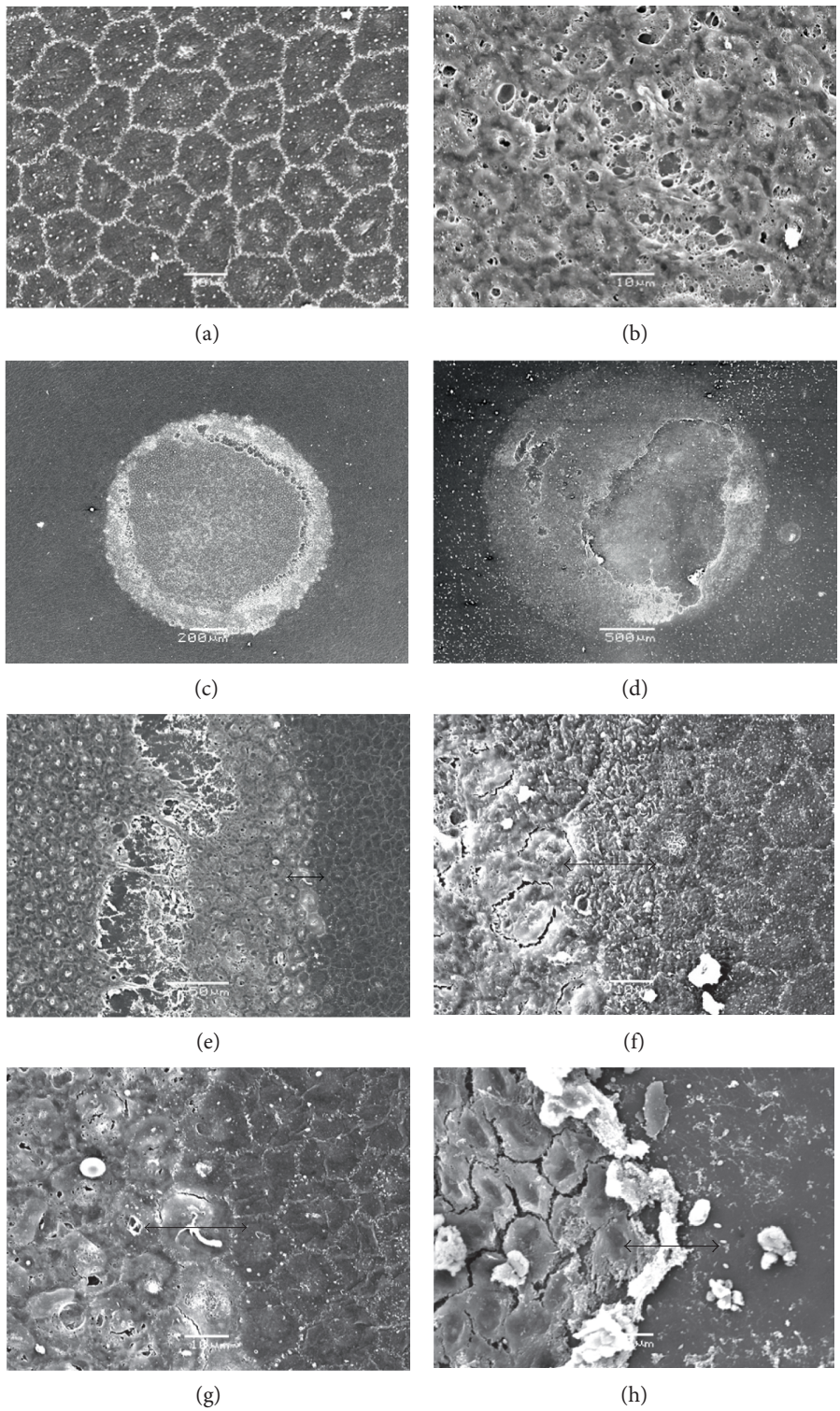

Figure 3: SEM of the endothelium of transcorneally frozen pig corneas. (a) An untreated pig cornea with cell boarders in white showing a characteristic hexagonal mosaic. The outline if the cell nucleus is evident as a slightly lighter area within each cell. Scale bar, 10 μm. (b) A representative image taken at the same magnification of the freeze-damaged area after treatment with a 2.4 mm diameter/concave profile tip, illustrating severe damage to endothelial cells by freezing. Scale bar, 10 μm. (c and d) Lower magnification images of endothelial freeze-injured wounds showing circular areas of endothelial cell damage including some endothelial debridement, exposing Descemet's membrane (c): 2.4 mm diameter/concave profile cryoprobe (scale bar, 200 μm); (d): 3.4 mm diameter/concave profile cryoprobe (scale bar, 500 μm). As expected, the larger probe induces more widespread damage (see Table 1 also). (e–h) Transition zones between unfrozen endothelial cells and those that were destroyed by freeze injury are often sharp (e) and (g); same area but different magnification (scale bars,10 μm, apart from (g) which is 50 μm).

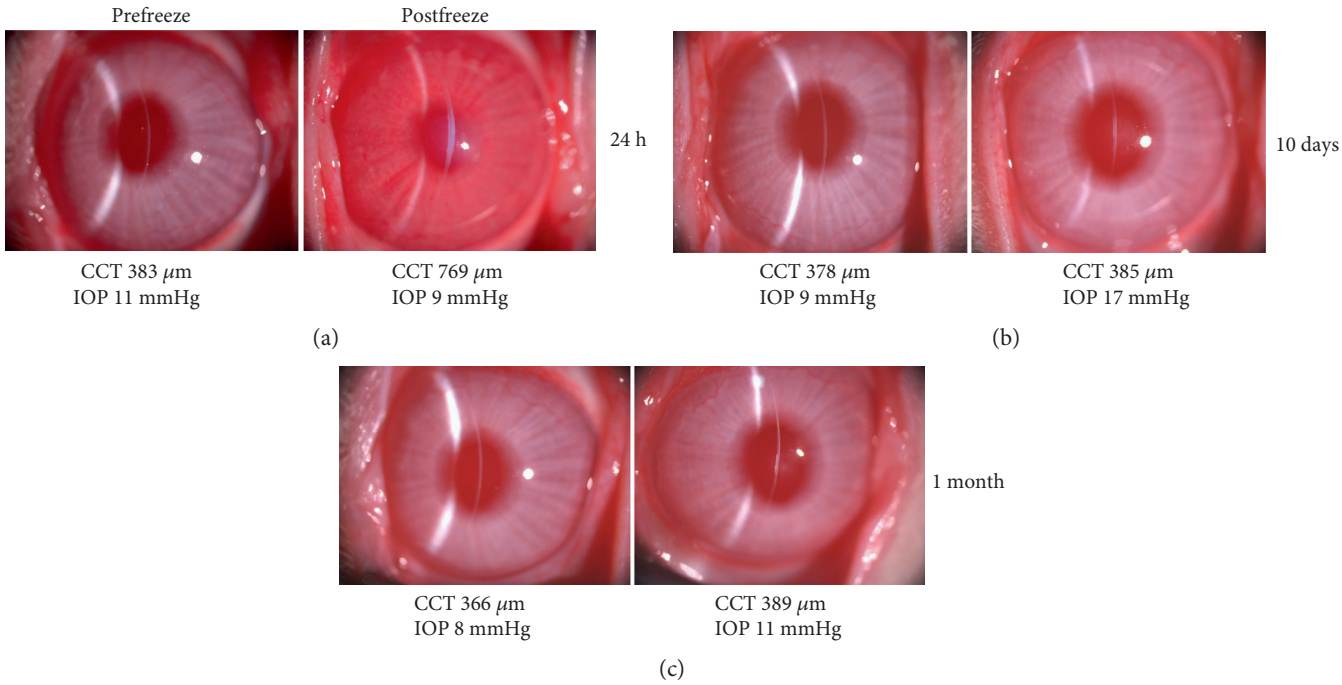

Figure 4: Effects of a 3 sec freeze on the rabbit cornea in vivo using the 2.4 mm diameter/concave profile cryoprobe tip. (a) 24 hrs after freeze, central corneal thickness (CCT) is increased considerably (769 μm) compared to that before freeze (383 μm), and the cornea is hazy, indicative of corneal endothelial damage, as well as epithelial and stromal cell damage. (b) 10 days after a freeze injury (in a different animal), CCT was at normal levels. (c) This was the case also, 1 month after treatment. (b and c) Some corneal haziness at the level of the posterior stroma or Descemet's membrane is evident at 10 days and 1 month.

(1.8 mm diameter ($r = -0.208$, $n = 102$, $p = 0.408$) and 3.4 mm diameter ($r = -0.258$, $n = 108$, $p = 0.007$) but a stronger relationship using the 2.4 mm diameter probe tips with concave and flat profiles ($r = -0.433$, $n = 108$, $p < 0.001$; 2.4 mm ($r = -0.466$, $n = 108$, $p < 0.001$, respectively) (Table 1).

To provide higher resolution information as to the status of the cells after freeze injury in the in vitro pig eye model, we conducted a series of SEM studies (Figure 3). This indicated that the corneal endothelial monolayer was severely disrupted in the central portion of the cornea beneath the site of the cryoprobe injury. Injured cells tended to become damaged and/or disassociated from each other, whereas noninjured cells adjacent to the area of damage exhibited classic hexagonal endothelial cell morphology. Interestingly, there appeared to be two transition zones between healthy nonfrozen cells and the more centrally damaged ones. The immediate transition at the inner edge of the morphologically normal cells was fairly abrupt; and in some cases, this was on a μm scale (Figures 3(e), 3(f), and 3(g)). More centrally, there was evidence of cellular dissociation (Figure 3(e)) and also total removal of large areas of frozen endothelial cells, exposing a bare Descemet's membrane (Figures 3(d) and 3(h)).

To investigate endothelial healing after transcorneal freeze, a small number of rabbit corneas were studied. For these investigations, a 2.4 mm diameter/concave profile cryoprobe was used rather than the 3.4 mm concave one owing to the relative thinness (approximately 350–400 μm) of the rabbit cornea compared to that of the pig (approximately 660 μm [29, 30]). This revealed that one day after a 3 sec surface freeze, the rabbit cornea had become significantly edematous, with its thickness approximately twice the normal value (Figure 4(a)). The central corneal thickness returned to normal values by day 7, and this was maintained up to one month post-freeze (Figure 4(c)). Slit-lamp images showed some evidence of corneal haze at the level of the posterior stroma or Descemet's membrane at 10 days and 1 month (Figures 4(b) and 4(c), resp.).

TEM examinations of rabbit corneas 24 hrs after freeze indicated that the corneal epithelium peripheral to the wound area was structurally normal, with typical epithelial stratification, cell-cell contact and surface microvilli (Figure 5(a)). As expected, the corneal epithelium was severely damaged in the central freeze-injured region of the cornea, with considerable cellular vacuolation and membrane destruction (Figure 5(b)). However, it was clear that the epithelial basement membrane remained intact, which presumably is important to aid subsequent epithelial resurfacing of the wound area. In the deep stroma, increased collagen fibril spacing accompanied by disorder in the fibril arrangement was sometimes observed focally 24 hrs post-freeze (Figure 5(c)), but this had been resolved by the 1-month timepoint at which time the stromal architecture appeared normal throughout the cornea (Figure 5(d)). These structural matrix changes likely contribute to the increased corneal thickness and opacity seen at 24 hrs (Figure 4(a)).

Just as with the corneal epithelium, the corneal endothelium in the periphery of the cornea away from the region of the tissue under the surface wound zone remained unaffected

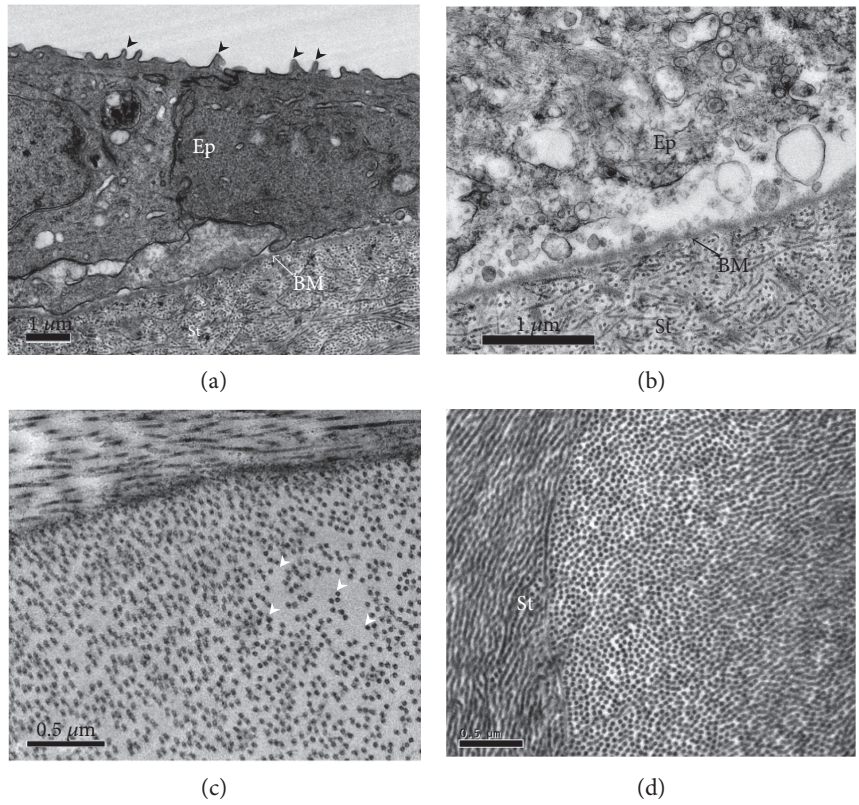

Figure 5: TEM of the corneal epithelium (Ep), epithelial basement membrane (BM), and stroma (St) following a 3 sec transcorneal freeze injury using a 2.4 mm diameter/concave profile cryoprobe on rabbit cornea in vivo. (a) The peripheral epithelium away from the wound zone, 24 hrs after the cryoprobe was applied and appeared morphologically normal. Arrowheads indicate microvilli on apical surface of epithelial cells. (b) Intact basement membrane is observed in the central freeze-injured area 24 hrs postfreeze. (c) After 24 hrs freeze injury, occasional focal regions of stromal matrix disruption were evident in the cornea, manifesting as tissue regions with increased spacing between collagen fibrils. (d) One month after the freezing, throughout the cornea, the spacing between collagen fibril appeared normal. Scale bars, 1 μm (a and b) and 0.5 μm (c and d).

24 hrs after transcorneal freeze of the central rabbit cornea (Figure 6(a)). The endothelium more centrally, however, began to exhibit clear signs of damage (Figure 6(b)). The central endothelium was often fully debrided with a bare Descemet's membrane that showed no apparent structural changes (Figure 6(c)). One month after freeze, the central corneal endothelium had reattained its normal character, although occasionally fibrous tissue deposition between Descemet's membrane and the recovered endothelium was observed (Figure 6(d)), which perhaps contributes to the deep stromal haze seen at this time (Figure 4(c)).

4. Discussion

A number of investigative surgical procedures, which utilize the selective ROCK inhibitor, Y27632, to combat FECD and bullous keratopathy are under investigation, including cell-injection therapy [10–14]. However, one alternative approach for FECD, especially in its early stage, involves freezing the central cornea using a cold probe to damage corneal endothelial cells beneath the surface contact area; this is then followed by the short-term delivery, for one week, of Y27632 in eye drop formulation [10–12]. In these surgeries, freezing was achieved by touching the corneal surface with a stainless steel rod, which had been immersed in liquid nitrogen. An arbitrary freezing time of 15 sec was chosen for these experiments, along with a 2 mm diameter for the steel rod. Encouragingly, the outcomes of these surgeries showed some promise, especially if the extent of the corneal endothelial dysfunction was not widespread, but if the approach is to be adopted more widely by the ophthalmic community, the corneal freeze would probably need to be achieved in a more reliable manner and with more knowledge of the nature and extent of the freeze damage.

Historically, and up to the present day, corneal freezing has been carried out by a variety of methods, most of which use it to induce an experimental injury for research into corneal wound healing [15–17, 28]. Freezing studies tend to employ either a brass rod or dowel, which had been immersed in liquid nitrogen [17–21] or similarly cooled steel ones [22, 23]. Retinal cryoprobes have also been used in investigational studies of transcorneal freezing [24, 33]. Here, we report the design and manufacture of a corneal cryoprobe that uses circulating nitrous oxide as a cryogen and report the type of freeze damage it induces.

Typically, the tips of cryoprobes that use high-pressure gas as a cryogen are made of stainless steel owing to the need to contain high-pressure gas safely. In our design, however,

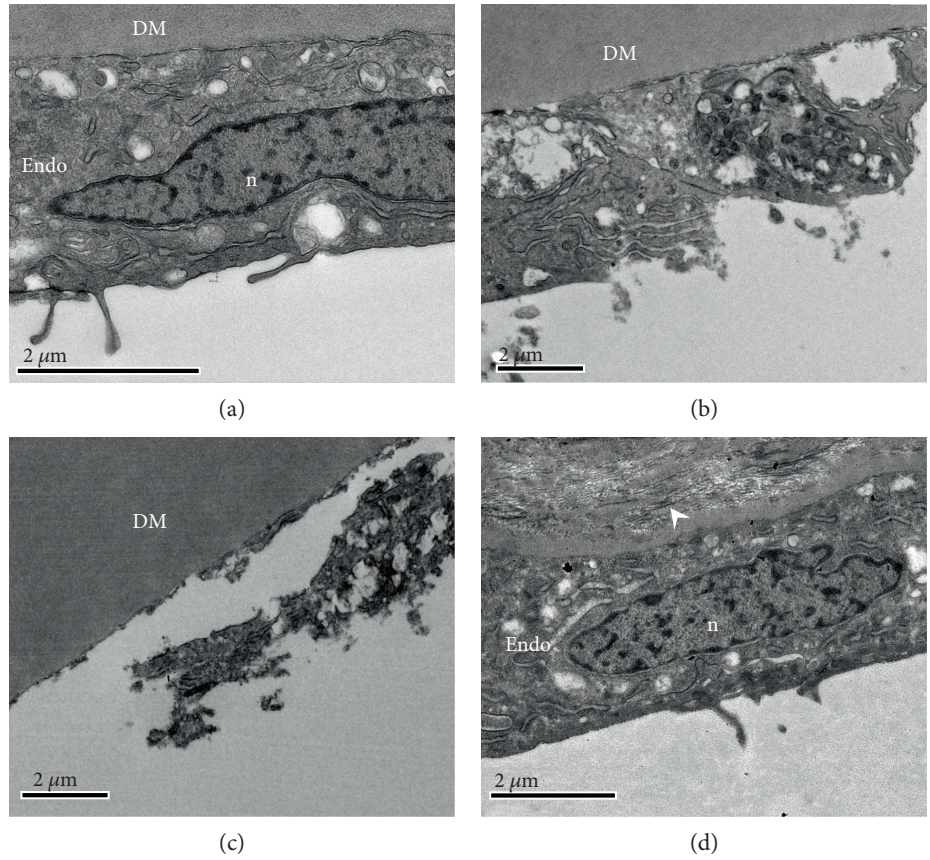

Figure 6: TEM of corneal epithelium following a 3 sec transcorneal freeze injury using a 2.4 mm diameter/concave profile cryoprobe on the rabbit cornea in vivo. (a) An endothelial cell (Endo) at 24 hrs postfreeze in a region peripheral to the freeze-injured area appeared morphologically normal, with normal organelles and nucleus (n). It adhered to Descemet's membrane (DM). (b) Closer to the region below the cryoprobe surface application, there were clear signs of cell damage including the destruction of the cell membrane, while even more centrally, (c) the cell damage was more extreme revealing a bare Descemet's membrane, consistent with the SEM analysis (Figure 3). (d) One month after the transcorneal freezing was performed, the central region of the inner cornea contained fairly normal endothelial cells that were sometimes accompanied by extracellular matrix material (white arrowheads) in the area posterior to Descemet's membrane. Scale bars, 2 μm.

the use of stainless steel would have resulted in a freeze that started in the center of the tip and thereafter spread, albeit quickly, to its outer circumference at a speed which relies on the thermal conductivity of the metal. To achieve a more uniform cooling across the probe tip, we manufactured a cryoprobe tip out of silver, which has a higher thermal conductivity than that of steel. The benefit of this design feature is that the diameter of the freeze in the cornea is decoupled from the depth of the freeze. Cooling in this design is based on the expansion and internal recycling of nitrous oxide inside the cryoprobe tip. At the point of its transition from liquid to gas, nitrous oxide exists at a temperature of −88.5°C, and this transition, which occurs inside our probe tip, of course, rapidly cools it. Owing to thermal conductivity within the whole probe and thermal loss at ambient room temperature, an equilibrium is reached, which in our design means that the temperature at the outer surface of the probe tip reaches −50°C.

The response of a cell to freezing is explained by Fraunfelder in his comprehensive review of corneal cryotherapy, the main mechanisms of cell damage being a piercing of the cell membrane by ice crystals or the creation of a sizeable osmotic imbalance between the inside and outside of the cell because of the removal of liquid water into the ice crystals [34]. Armitage also describes, from the other side of the coin, how careful freezing using time-mediated freeze-thaw protocols accompanied by the use of cryoprotectants can lead to cell survival [35]. The freezing we achieve here can probably be thought of as being fairly conservative in terms of the rate of endothelial cooling, but our current observations clearly indicate that sufficient levels of endothelial damage are achieved after a single freeze treatment. Experiments that applied additional treatments to the same surface location did not enhance the extent of the freeze injury (data not shown). We also found that if the foot switch was depressed to initiate cooling of the probe tip before it was brought into contact with the corneal surface, then no appreciable endothelial freeze damage was seen, even if the cryoprobe was kept in contact with the cornea for periods up to 15 sec. It is not immediately clear why this is the case, but perhaps, frosting of the probe tip when it is cooled in the moist air could contribute to this. This likely lack of frosting also might help facilitate the easy

release of the probe tip from the corneal surface after the foot switch is released, which we found to happen within a second or two of the cryogen circulation being suspended.

In the experiments described here, the central corneal epithelium was destroyed by the application of the cryoprobe, as we would expect. But, it is of potential importance that the corneal epithelial basement membrane remains intact as seen by TEM. The lack of epithelial basement membrane damage will presumably aid the epithelial resurfacing of the debrided epithelial area. Fibril arrangement changes are also apparent focally in the stromal matrix after the in vivo rabbit freeze injury, but these are transient, and the increased collagen fibril separation and disorganization which are seen 24 hrs after the treatment subsequently decrease as corneal thickness reduces. The obvious conclusion is that the recovering endothelium is mostly responsible. Of course, the in vivo healing studies reported here do not reflect the situation in the human cornea because of the different behavior of the endothelial cells and their limited replicative ability in humans. TEM also discloses the presence of fibrotic extracellular matrix tissue in the region of Descemet's membrane one month after freeze injury (Figure 6(d)). This might contribute to the deep stromal haze seen at this time, although a potential contribution to light scatter by freeze-damaged keratocytes cannot be discounted.

As mentioned, the use of ROCK inhibitors has aided the recovery of the corneal endothelium in situations where diseased corneal endothelial cells have been scraped away surgically [13] or frozen with a cooled steel rod applied to the corneal surface [10–12]. A report by Balachandran et al. [36] and Shah and associates also suggests that corneal endothelial cells can repopulate in FECD patients after damage alone [37–39]. The data presented here show that endothelial cells can be functionally damaged and/or removed by the application of a cryogenic cold probe and that this can be done in a targeted and reproducible manner with cell damage restricted to the area below the surface contact. Of course, the transcorneal freezing technique is unlikely to induce any significant change to the guttae which exist in FECD and their continued presence will conceivably hinder the reformation of a normal endothelial layer. Nevertheless, cell damage can be achieved through use of a silver 3.4 mm diameter cryoprobe with a concave profile, which was discovered to be the optimal design of the cryoprobes tested in the experiments described here. It thus has the potential to rapidly and reliably induce damage to the human corneal endothelium via transcorneal freezing, leaving the epithelial basement membrane intact. This has the potential to be used prior to the application of ROCK inhibitors to the eye in the form of eye drops [10–12] or as slow-release chemicals from thin films [40] to aid the recovery of corneal endothelial function.

Disclosure

The technology, the use of our freezing device coupled with the application of a Rho-kinase inhibitor for the nonevasive treatment of endothelial dysfunction, is the subject of patent (WO2013034907) currently under examination in Europe, Japan, and the United States of America. Preliminary results of this study were presented at the ARVO Annual Meeting, Seattle, USA, 1–5 May 2016.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Additive Intraocular Pressure-Lowering Effects of Ripasudil with Glaucoma Therapeutic Agents in Rabbits and Monkeys

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Ripasudil hydrochloride hydrate (K-115), a specific Rho-associated coiled-coil containing protein kinase (ROCK) inhibitor, is developed for the treatment of glaucoma and ocular hypertension. Topical administration of ripasudil decreases intraocular pressure (IOP) by increasing conventional outflow through the trabeculae to Schlemm's canal, which is different from existing agents that suppress aqueous humor production or promote uveoscleral outflow. In this study, we demonstrated that ripasudil significantly lowered IOP in combined regimens with other glaucoma therapeutic agents in rabbits and monkeys. Ripasudil showed additional effects on maximum IOP lowering or prolonged the duration of IOP-lowering effects with combined administration of timolol, nipradilol, brimonidine, brinzolamide, latanoprost, latanoprost/timolol fixed combination, and dorzolamide/timolol fixed combination. These results indicate that facilitation of conventional outflow by ripasudil provides additive IOP-lowering effect with other classes of antiglaucoma agents. Ripasudil is expected to have substantial utility in combined regimens with existing agents for glaucoma treatment.

1. Introduction

Rho-kinase (Rho-associated coiled-coil containing protein kinase; ROCK), a member of the serine-threonine protein kinases, is an effector protein of low-molecular-weight G-protein, Rho [1]. ROCK has two isoforms, ROCK-1 and ROCK-2, which are extensively distributed throughout the various organs, including the ocular tissues [2, 3]. ROCK binds with Rho to form a Rho/ROCK complex and regulates various physiological functions, such as smooth muscle contraction, chemotaxis, neural growth, and gene expression [1, 4–8]. However, aberrant regulation of ROCK levels in the eyes has been shown to be involved in the pathogenesis of diabetic retinopathy, age-related macular edema, cataract, corneal dysfunction, retinal disorders, and glaucoma [9–20].

Glaucoma is primarily a disease affecting the optic nerve head that characteristically leads to visual field loss and ultimately blindness. Primary open-angle glaucoma (POAG), the commonest form of glaucoma, often observed chronic elevation of intraocular pressure (IOP). These were developed as a result of pathologically increased resistance to the drainage of the aqueous humor through outflow pathways [21]. IOP reduction is currently the only reliable and evidence-based management approach for the treatment of glaucoma [22]. The strategies of glaucoma treatment are decided according to the stages of glaucoma, types, and different conditions, with pharmacological agents considering the first-line therapy in most types of glaucoma [23]. The ocular hypotensive mechanisms of currently available antiglaucoma agents are categorized into two types. One is to promote uveoscleral outflow, such as prostaglandin (PG) analogs, $\alpha\beta$-adrenergic receptor blockers, α1-adrenergic receptor blockers, and α2-adrenergic receptor agonists, and the other is to suppress aqueous humor production, such as β-adrenergic receptor blockers, carbonic anhydrase inhibitors (CAI), and $\alpha\beta$-adrenergic receptor blockers [23]. However, reduction of IOP below the target level is often challenging with monotherapy [24]. Consequently, there is a great clinical need for a novel class of agents, which possesses potent IOP-lowering effects, and can be used with other agents for combination therapy.

Ripasudil is the first-in-class ROCK inhibitor ophthalmic agent developed for the treatment of glaucoma and ocular

hypertension [25–32]. In the previous study, we showed that ripasudil decreased IOP by potentiation of the outflow facility from the conventional outflow route [26, 27]. The mechanism of actions of ripasudil is different from that of other agents, such as promotion of uveoscleral outflow and suppression of aqueous humor production. In this study, we demonstrated that topical instillation of ripasudil ophthalmic solution with other glaucoma therapeutic agents, such as β-blocker, $\alpha\beta$-blocker, α2-agonist, CAI, and PG analogs, further reduced IOP and for a longer duration.

2. Materials and Methods

2.1. Animals. Male Japanese white rabbits weighing 2.0–3.0 kg and male cynomolgus monkeys weighing 5.0–8.5 kg (5 years or older) were used in this study. The rabbits were housed in an air-conditioned room (22–25°C, 50%–70% humidity) lit from 7:00 to 19:00, and they were allowed food and water ad libitum throughout the experiments. The monkeys were housed in an air-conditioned room (22–28°C, 40%–80% humidity) lit from 7:00 to 19:00, and they were provided 100 g of feed daily between 9:00 and 12:00, with the exception that feed were provided after the last measurement on each day of IOP measurement. All studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee of Kowa Tokyo New Drug Research Laboratories.

2.2. Chemicals and Drug Preparation. Ripasudil was synthesized at Tokyo New Drug Research Laboratories, Kowa Co. Ltd. (Tokyo, Japan) and was dissolved in a vehicle containing preservative for clinical use as an ophthalmic agent. 0.25% nipradilol (HYPADIL Kowa ophthalmic solution 0.25%) was purchased from Kowa Pharmaceutical Co. Ltd. (Tokyo, Japan); 1% brinzolamide (Azopt 1% ophthalmic suspension) was purchased from Alcon Japan Ltd. (Tokyo, Japan); 0.1% brimonidine (Aiphagan ophthalmic solution 0.1%) was purchased from Senju Pharmaceutical Co. Ltd. (Osaka, Japan); 0.005% latanoprost (Xalatan eye drops 0.005%) and 0.005% latanoprost/0.5% timolol fixed combination (Xalacom combination eye drops) were purchased from Pfizer Inc. (Tokyo, Japan); and 0.5% timolol (Timoptol Ophthalmic Solution 0.5%) and 1% dorzolamide/0.5% timolol fixed combination (COSOPT ophthalmic solution) were purchased from Santen Pharmaceutical Co. Ltd. (Osaka, Japan).

2.3. Method of Topical Administration. In rabbit experiments, 50 μL of agents were instilled into one eye. For combined regimens with other agents, 0.5% timolol, 0.25% nipradilol, 0.1% brimonidine, or 1% brinzolamide was administrated 5 min after the instillation of 0.4% ripasudil. The contralateral eye was not treated. In monkey experiments, 20 μL of agents was instilled into one eye. For combined regimens with other agents, 0.005% latanoprost (alone) and 0.005% latanoprost/0.5% timolol or 1% dorzolamide/0.5% timolol (in combination) were administrated 5 min after the instillation of 0.4% ripasudil or vehicle. The contralateral eye was not treated.

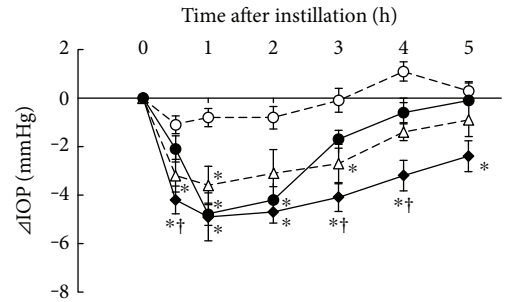

Figure 1: Additive IOP-lowering effect of ripasudil with timolol. Male albino rabbits were administered 50 μL of vehicle (○), 0.4% ripasudil (●), 0.5% timolol (△), or 0.4% ripasudil + 0.5% timolol (◆) into one eye ($n = 9$). The contralateral eye was not treated. IOP were measured using pneumotonometers prior to the experiments and 0.5, 1, 2, 3, 4, and 5 h after instillation. For combined use of ophthalmic agents, 0.5% timolol was administered 5 min after instillation of 0.4% ripasudil. All data are presented as means ± SEs. $^{*,\dagger}P < 0.05$, compared with vehicle and 0.4% ripasudil, respectively (Tukey's multiple comparison test).

2.4. Measurement of Intraocular Pressure. Pneumotonometers (Model 30 Classic Pneumotonometer; Medtronic Solan Ophthalmic Products Inc., Jacksonville, FL) were used to monitor IOP. For IOP measurements, the eyes were anesthetized by topical instillation of 0.4% oxybuprocaine (0.4% Benoxil ophthalmic solution; Santen Pharmaceutical Co. Ltd., Osaka, Japan). IOP was measured in both eyes prior to the instillation of agents and 0.5, 1, 2, 3, 4, and 5 h after instillation in the albino rabbits. For the experiments with monkeys, IOP was measured before and 1, 2, 4, and 6 h or 1, 2, 3, 4, 6, and 8 h after instillation of agents.

2.5. Statistical Analyses. Difference in IOP (ΔIOP) between pretreatment (0 h) and at each time point after the instillation of agents was calculated. All data are expressed as means ± SEs. For ΔIOP, Tukey's multiple comparison test (comparing means of more than two groups) or Student's t-test (comparing means of two groups) was performed at each time point for each treatment group. $P < 0.05$ was predetermined as the criterion of statistical significance for all the analyses.

3. Results

3.1. Additive IOP-Lowering Effect of Ripasudil with Timolol. IOP-lowering effects of 0.4% ripasudil, 0.5% timolol, and combined treatment of 0.4% ripasudil with 0.5% timolol were demonstrated in rabbits (Figure 1). Compared with vehicle, ripasudil significantly lowered the IOP 1 and 2 h after instillation, and timolol significantly lowered 0.5, 1, and 3 h after instillation. Combined treatment of ripasudil and timolol significantly lowered IOP at 0.5, 1, 2, 3, 4, and 5 h after instillation compared with vehicle and at 0.5, 3, and 4 h after instillation compared with ripasudil.

3.2. Additive IOP-Lowering Effect of Ripasudil with Nipradilol. IOP-lowering effects of 0.4% ripasudil, 0.25% nipradilol, and combined treatment of 0.4% ripasudil with

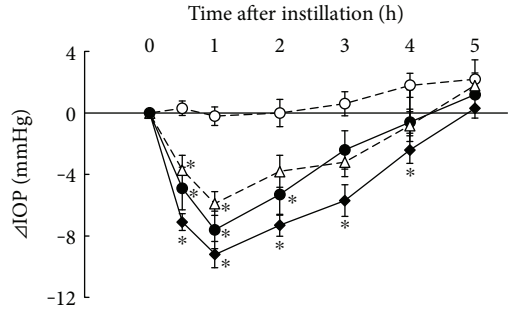

Figure 2: Additive IOP-lowering effect of ripasudil with nipradilol. Rabbits were administered vehicle (○), 0.4% ripasudil (●), 0.25% nipradilol (△), or 0.4% ripasudil + 0.25% nipradilol (◆) into one eye ($n = 10$). IOP were measured 0.5, 1, 2, 3, 4, and 5 h after instillation. For combined use of ophthalmic agents, 0.25% nipradilol was administered 5 min after instillation of 0.4% ripasudil. All data are presented as means ± SEs. $^{*}P < 0.05$, compared with vehicle (Tukey's multiple comparison test).

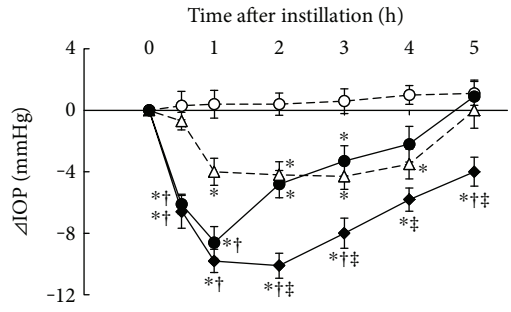

Figure 3: Additive IOP-lowering effect of ripasudil with brinzolamide. Rabbits were administered vehicle (○), 0.4% ripasudil (●), 1% brinzolamide (△), or 0.4% ripasudil + 1% brinzolamide (◆) into one eye ($n = 10$). IOP were measured 0.5, 1, 2, 3, 4, and 5 h after instillation. For combined use of ophthalmic agents, 1% brinzolamide was administered 5 min after instillation of 0.4% ripasudil. All data are presented as means ± SEs. $^{*,\dagger,\ddagger}P < 0.05$, compared with vehicle, 0.4% ripasudil, and 1% brinzolamide, respectively (Tukey's multiple comparison test).

0.25% nipradilol were demonstrated in rabbits (Figure 2). Compared with vehicle, a significant IOP-lowering effect was observed at 0.5, 1, and 2 h after instillation of ripasudil; 0.5 and 1 h after instillation of nipradilol; and 0.5, 1, 2, 3, and 4 h after instillation of combined treatment of ripasudil and nipradilol.

3.3. Additive IOP-Lowering Effect of Ripasudil with Brinzolamide. IOP-lowering effects of 0.4% ripasudil, 1% brinzolamide, and combined treatment of 0.4% ripasudil with 1% brinzolamide were demonstrated in rabbits (Figure 3). Compared with vehicle, a significant IOP-lowering effect was observed at 0.5, 1, 2, and 3 h after instillation of 0.4% ripasudil; 1, 2, 3, and 4 h after instillation of brinzolamide; and 0.5, 1, 2, 3, 4, and 5 h after instillation of combined treatment of ripasudil and brinzolamide. Moreover, combination of ripasudil and brinzolamide showed significant IOP-lowering effect at 2, 3, and 5 h against both single instillation of 0.4% ripasudil and 1% brinzolamide.

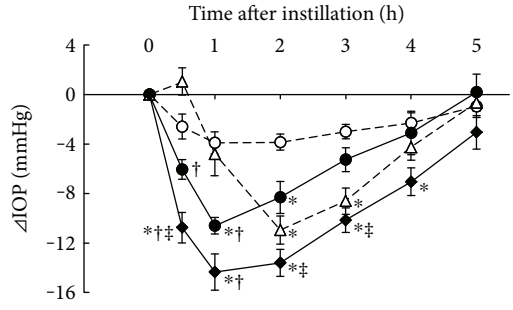

Figure 4: Additive IOP-lowering effect of ripasudil with brimonidine. Rabbits were administered vehicle (○), 0.4% ripasudil (●), 0.1% brimonidine (△), or 0.4% ripasudil + 0.1% brimonidine (◆) into one eye ($n = 10$). IOP were measured 0.5, 1, 2, 3, 4, and 5 h after instillation. For combined use of ophthalmic agents, 0.1% brimonidine was administered 5 min after instillation of 0.4% ripasudil. All data are presented as means ± SEs. $^{*,\dagger,\ddagger}P < 0.05$, compared with vehicle, 0.4% ripasudil, and 0.1% brimonidine, respectively (Tukey's multiple comparison test).

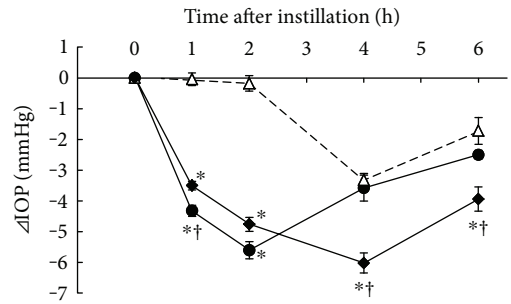

Figure 5: Additive IOP-lowering effect of ripasudil with latanoprost. Male cynomolgus monkeys were administered 20 μL of 0.4% ripasudil (●), 0.005% latanoprost (△), or 0.4% ripasudil + 0.005% latanoprost (◆) into one eye ($n = 4$). The contralateral eye was not treated. IOP were measured using pneumotonometers prior to the experiments and 1, 2, 4, and 6 h after instillation. For combined use of ophthalmic agents, 0.005% latanoprost was administered 5 min after instillation of 0.4% ripasudil. All data are presented as means ± SEs. $^{*,\dagger}P < 0.05$, compared with 0.005% latanoprost and ripasudil, respectively (Tukey's multiple comparison test).

3.4. Additive IOP-Lowering Effect of Ripasudil with Brimonidine. IOP-lowering effects of 0.4% ripasudil, 0.1% brimonidine, and combined treatment of 0.4% ripasudil with 0.1% brimonidine were demonstrated in rabbits (Figure 4). Compared with vehicle, ripasudil significantly lowered the IOP at 1 and 2 h after instillation; 0.1% brimonidine at 2 and 3 h after instillation; and combined treatment of ripasudil and brimonidine at 0.5, 1, 2, 3, and 4 h after instillation. Additionally, combined treatment of ripasudil and brimonidine significantly lowered IOP compared with ripasudil, and brimonidine alone, at 0.5 h after instillation.

3.5. Additive IOP-Lowering Effect of Ripasudil with Latanoprost in Cynomolgus Monkeys. IOP-lowering effects of 0.4% ripasudil, 0.005% latanoprost, and combined treatment of 0.4% ripasudil with 0.005% latanoprost were demonstrated in monkeys (Figure 5). Compared with both single

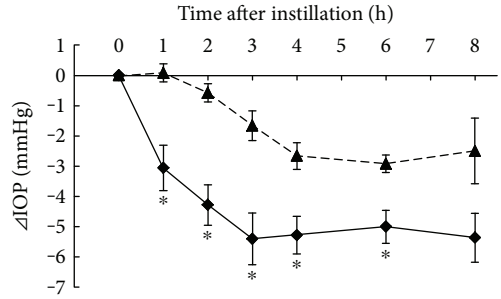

Figure 6: Additive IOP-lowering effect of ripasudil with latanoprost/timolol fixed combination. Monkeys were administered vehicle + 0.005% latanoprost/0.5% timolol fixed combination (▲), or 0.4% ripasudil + 0.005% latanoprost/0.5% timolol fixed combination (◆) into one eye ($n = 5$). IOP were measured before and 1, 2, 3, 4, 6, and 8 h after instillation. 0.005% latanoprost/0.5% timolol fixed combination was administered 5 min after instillation of vehicle or 0.4% ripasudil. All data are presented as means ± SEs. $^*P < 0.05$, compared with vehicle + 0.005% latanoprost/0.5% timolol fixed combination (Student's t-test).

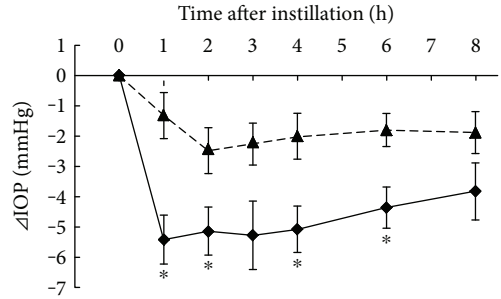

Figure 7: Additive IOP-lowering effect of ripasudil with dorzolamide/timolol fixed combination. Monkeys were administered vehicle + 1% dorzolamide/0.5% timolol fixed combination (▲) or 0.4% ripasudil + 1% dorzolamide/0.5% timolol fixed combination (◆) into one eye ($n = 5$). IOP were measured before and 1, 2, 3, 4, 6, and 8 h after instillation. 1% dorzolamide/0.5% timolol fixed combination was administered 5 min after instillation of vehicle or 0.4% ripasudil. All data are presented as means ± SEs. $^*P < 0.05$, compared with vehicle + 1% dorzolamide/0.5% timolol (Student's t-test).

instillation of ripasudil and latanoprost, a significant IOP-lowering effect was observed 4 and 6 h after instillation of the combined treatment of ripasudil and latanoprost.

3.6. Additive IOP-Lowering Effect of Ripasudil with Fixed Combination Agents in Cynomolgus Monkeys. Additive IOP-lowering effects of 0.4% ripasudil with fixed combination (0.005% latanoprost/0.5% timolol or 1% dorzolamide/0.5% timolol) were demonstrated in monkeys. Compared with latanoprost/timolol and vehicle, combination of latanoprost/timolol and ripasudil significantly lowered the IOP 1, 2, 3, 4, and 6 h after administration (Figure 6). Compared with dorzolamide/timolol and vehicle, combination of dorzolamide/timolol and ripasudil significantly lowered the IOP 1, 2, 4, and 6 h after administration (Figure 7).

4. Discussion

In this study, we demonstrated the additive IOP-lowering effects of ripasudil topical instillation with other glaucoma therapeutic agents, β-blocker, $\alpha\beta$-blocker, α2-agonist, CAI, PG analogs, and fixed combination. A lot of therapeutic agents are used to manage IOP for glaucoma treatment. For example, PG analogs, $\alpha\beta$-blockers, α1-blockers, and α2-agonists are currently used to promote uveoscleral outflow, and β-blockers, CAI, and $\alpha\beta$-blockers are used to suppress aqueous humor production. In addition, these agents are used in different ways for glaucoma treatment, such as combined administration of agents, fixed-dose combination formulations, and appropriate agents are selected according to the target IOP of each patient. However, there are unmet medical needs in the market for developing novel class of ocular hypotensive agents, as present antiglaucoma agents are insufficient for obtaining the required reduction of IOP. In this study, we aimed to evaluate the additive IOP-lowering effects of combined regimens of ripasudil and other antiglaucoma agents in rabbits and monkeys. We believe that the mechanism of facilitation via conventional outflow by ripasudil differs from those of other agents.

The pharmacological features of ripasudil have previously been investigated. Ripasudil inhibited both human ROCK-1 and ROCK-2 with IC_{50} values of 0.051 and 0.019 μmol/L, respectively. The inhibitory effect of ripasudil was more potent than that of Y-27632 or fasudil [26]. Inhibitory activities (as in IC_{50} values) of ripasudil on other serine/threonine kinases are approximately 1000-fold less potent than ROCK inhibition. Moreover, ripasudil does not inhibit carbonic anhydrase and has no binding affinity for α-, β-, and prostanoid receptors. These results indicate that ripasudil is a selective ROCK inhibitor.

In in vivo studies using rabbits and monkeys with normal IOP, a clinical dose of 0.4% ripasudil showed a significant IOP-lowering effect, which was comparable with existing glaucoma therapeutic agents [26, 27]. In a study of aqueous humor dynamics in rabbits, instillation of 0.4% ripasudil significantly increased outflow facility; however, it had no effect on uveoscleral outflow or aqueous flow rate [26]. In in vitro studies, ripasudil induced retraction and rounding as well as reduced actin bundles in monkey trabecular meshwork (TM) cells [27]. In addition, ripasudil reduced transendothelial electrical resistance (TEER), increased FITC-dextran permeability, and decreased ZO-1 immunostaining areas in monkey Schlemm's canal endothelial (SCE) cells [27]. These findings corroborate previous studies of other ROCK inhibitors in rabbits or monkeys [19, 20, 33–35]. Therefore, promotion of aqueous outflow by ripasudil is likely due to TM cytoskeletal changes, reduced outflow resistance, and increased SCE permeability as a result of ROCK inhibition. These results strongly indicate that the ocular hypotensive effect of ripasudil is associated with its potentiation of outflow facility from the conventional outflow route.

In this study, the IOP-lowering effect of ripasudil was enhanced by instillation with brimonidine, brinzolamide, latanoprost, latanoprost/timolol fixed combination, and

Table 1: Additive effect by ripasudil for categories of glaucoma therapeutic agents.

Categories	Target	Additive effect with ripasudil
β-Blockers	Suppression of aqueous humor production	IOP reduction Prolonged duration
CAI		IOP reduction Prolonged duration
Combination of β-blockers and CAI		IOP reduction Prolonged duration
PG analogs	Promotion of uveoscleral outflow	IOP reduction Prolonged duration
α2-Agonists	Suppression of aqueous humor production and promotion of uveoscleral outflow	IOP reduction Prolonged duration
$\alpha\beta$-Blockers		Prolonged duration
Combination of PG analogs and β-blockers		IOP reduction Prolonged duration

dorzolamide/timolol fixed combination. Furthermore, combined instillation of ripasudil with latanoprost/timolol fixed combination showed more additive IOP-lowering effect compared with combined instillation of ripasudil with timolol or latanoprost. Therefore, additive IOP-lowering effect by ripasudil was able to show with two or more agents. These results suggest that increment of conventional outflow is effective for lowering the IOP under the increase in uveoscleral outflow or increment of uveoscleral flow with suppressing the aqueous humor production. However, combined instillation of ripasudil with nipradilol did not show additive effect on IOP compared with their single instillations. The maximum IOP-lowering effect of nipradilol was observed at 1 h after instillation, and the IOP value was 14.1 mmHg, which is similar to the episcleral venous pressure in rabbits [36]. Ripasudil and nipradilol have similar IOP-lowering effect, that is, both agents show maximum IOP reduction at 1 h after instillation and disappear rapidly in rabbits. This might be the reason why we could not take more additive IOP reduction by combination of ripasudil with nipradilol. On the other hand, combination of ripasudil with other agents prolonged the duration of IOP-lowering effects compared with single instillation of each agent. The ocular hypotensive mechanism of ripasudil, facilitation of conventional outflow, provides additive ocular hypotensive effect with combination use of other types of ocular hypotensive agents (Table 1). Furthermore, there was no adverse event regarding the topical instillation of ripasudil of coadministration of ripasudil with other antiglaucoma drugs in this study. Our results in this study agree with clinical studies, in that the administration of ripasudil with timolol or latanoprost showed additive IOP-lowering effect in glaucoma patients [31]. Therefore, we believe that the additive IOP-lowering effect of ripasudil with other glaucoma therapeutic agents in this study would provide beneficial clinical effects.

There are many reports that not only an elevation of IOP but also an impairment of ocular circulation are the etiology of glaucoma and evaluated the effect of antiglaucoma drugs on ocular blood flow in experimental animals and humans [37–42]. Nakabayashi et al. reported that ripasudil increased retinal blood flow in cats [43]. Similar results were reported by other ROCK inhibitor reagents [44, 45], and this effect might be due to direct vasodilating action of ROCK inhibitors in the posterior side of the eye.

Glaucoma is a condition that involves distinctive change in the optic nerve and visual field [23], and neuroprotective effect might be a beneficial effect on suppressing the progression of glaucomatous neural damage. There are many reports for the neuroprotective effect of antiglaucoma agents. Brimonidine showed neuroprotective effect in rats [46] and prevented the progression of visual field loss in humans [47]. Yamamoto et al. reported the neuroprotective effect of ripasudil in rats [48]; similar effects were also observed with other ROCK inhibitors [18, 49, 50]. Therefore, neuroprotective effect by ripasudil is expected to show beneficial effect on visual field in humans.

Furthermore, ROCK inhibitors have direct anti-inflammatory effects [51, 52] compared with other antiglaucoma agents. Increased production of proinflammatory cytokines has been reported to result in POAG and secondary, including exfoliation and uveitic glaucomas [53, 54]. Glucocorticoid-induced ocular hypertension is a form of secondary open-angle glaucoma induced by steroid administration. Its underlying mechanisms are associated with increasing outflow resistance through the conventional outflow route caused by accumulation of extracellular matrix (ECM) [55]. Fujimoto et al. reported that ROCK inhibitor improved dexamethasone-induced reduction of the outflow facility and inhibited the increase in ECM, such as collagen type IV α1 and fibronectin mRNA expression in porcine eyes [56]. Therefore, anti-inflammatory effect of ripasudil and its suppressive effect of ECM via ROCK inhibition may provide a strategy to treat and prevent secondary glaucoma, with additive IOP-lowering effects when combined regimens are used with other antiglaucoma agents.

5. Conclusions

In this study, we demonstrated that ripasudil showed additional maximum IOP-lowering effect or prolongation

of IOP-lowering effect in combined regimens with β-blocker, $\alpha\beta$-blocker, $\alpha 2$-agonist, CAI, PG analog, and fixed combination of these agents. The mechanisms of action are due to increment of conventional outflow by ripasudil treatment. Ripasudil is expected to have substantial utility when used in combined regimens with existing agents and provide a greater choice in pharmacological treatment options for glaucoma.

Conflicts of Interest

Yoshio Kaneko, Masayuki Ohta, Tomoyuki Isobe, Yuto Nakamura, and Ken Mizuno are employees of Kowa. The authors declare that there is no conflict of interest regarding the publication of this paper.

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