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Original Article

Effects of fasudil on the cytoskeleton of trabecular meshwork cells and outflow facility in bovine eyes

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Abstract: This study aimed to investigate the effect of fasudil on outflow facility and its underlying mechanism on conventional outflow passway. Primary cultured trabecular meshwork (TM) cells derived from bovine eyes were treated with fasudil at various concentrations and observed for the changes of cell morphology, filamentous actin (F-actin) arrangement as well as amount of vinculin. A perfusion system for evaluating the outflow facility of fasudil was established in bovine eyes. Afterwards the eyes were perfused with red fluorescent microspheres to label the outflow pattern and the fixed samples were viewed under a confocal microscopy to calculate the percent effective filtration length (PEFL). The cultured TM cells after fasudil treatment at higher concentration changed morphologically into a longer and thinner appearance. Fasudil also induced the depolymerization of F-actin and reduction of vinculin in cultured TM cells at higher concentration. All these effects of fasudil were in a time- and dose-dependent manner. In addition, the outflow facility in the fasudil-perfused eyes increased significantly compared with control eyes (18.69 ± 3.44% *vs* 48.00 ± 9.33%, $P < 0.001$). There was an improvement of outflow pattern in the fasudil-perfused eyes since tracer distribution was more uniform and extensive compared to the controls. The PEFL was significantly larger in the fasudil-perfused eyes than in the controls (56.56 ± 4.55% *vs* 13.36 ± 3.58%, $P < 0.001$). Fasudil can increase aqueous humor outflow facility through inducing the cytoskeletal changes in the bovine eyes and may be a potential drug for the treatment of glaucoma.

Keywords: Glaucoma, fasudil, trabecular meshwork, aqueous humor

Introduction

Glaucoma, a progressive optic neuropathy, is the leading cause of irreversible blindness, affecting over 67 million people in the global [1]. Primary open angle glaucoma (POAG) which is the most prevalent form of glaucoma accounts for over 80% of glaucoma cases [2]. Although the pathogenesis of POAG is not fully understood, the development of POAG is thought to be tightly associated with the increased intraocular pressure (IOP) [3]. As previously described, IOP is caused by a defect in drainage of aqueous humor and an increased resistance to aqueous humor outflow through the trabecular meshwork (TM) and Schlemm's canal (SC) [4, 5]. Thus, understanding the potential regulatory mechanisms involved in aqueous humor outflow is of great importance for revealing the cause of POAG and developing better therapy.

The normal aqueous humor outflow facility is maintained with cellular contraction and relaxation as well as cell-substratum and cell-cell adhesive forces of human TM and SC cells [3, 6-9]. Factors that can alter cell morphology and cytoskeletal architecture of TM cells within the outflow pathways is able to decrease the aqueous humor outflow resistance and induce a reduction of intraocular pressure, as demonstrated by several perfusion studies [6, 7, 10, 11]. Rho GTPase-mediated signaling pathway is reported to play a vital role in the regulation of TM function and the maintenance of normal aqueous humor outflow [12]. Rho-associated protein kinase (ROCK) is a critical kinase located downstream of this signaling pathway and its inactivation has been shown to induce changes in cell morphology, cell-cell and cell-extracellular matrix (ECM) interactions [13]. Thus, the inhibitors of ROCK have been considered to be an attractive agent for treating POAG.

Among these ROCK inhibitors, fasudil is the only one that has been introduced to the market and applied in the clinic for treating several cerebral disease, such as cerebral vasospasm and ischemic cerebral vascular disease [14]. In addition, it has been reported to significantly lower IOP and increase aqueous humor outflow in rabbit eyes [8].

Therefore, in the present study, we attempted mechanistically to explore the underlying molecular and cellular mechanisms of the IOP-lowering effects of fasudil. First, we detected the changes in cell morphology and cytoskeletal architecture of TM cells after fasudil treatment. Then we explored the effects of fasudil on aqueous humor outflow in fasudil-perfused bovine eyes. We also observed the changes in the hydrodynamic patterns of outflow after fasudil treatment under confocal microscopy. Our results would provide a reference for revealing the molecular mechanism of the IOP-lowering effects of fasudil.

Material and methods

Isolation and primary culture of bovine TM cells

A total of five bovine eyes were extracted from three one- or two-year-old animals from a local slaughter-house and immediately sterilized in 7% benzalkonium bromide and 75% ethanol for about 5 min, respectively. Then the TM tissue was dissected into strips of 2×1 mm size and incubated in Dulbecco's modified Eagle medium (DMEM) (Hyclone, Logan, Utah, USA) containing 10% fetal bovine serum (FBS) at 37°C under 5% CO_2 as previously described [16]. Cells that passaged three to five times were used in this study. All animal experiments performed in our study were approved by the Animal Ethics Committees of Shanghai Jiao Tong University.

Identification of TM cells by immunofluorescence

After three times of passage, cultured primary TM cells were identified by immunofluorescence. Cells were first seeded in 24-well plates that were coated with poly-L-lysine, then fixed in 4% paraformaldehyde and treated with 0.5% Triton-100. After washing with phosphate buffer solution (PBS) for three times, wells were blocked with normal non-immune goat serum and then incubated with rabbit anti-bovine fibronectin (1:100), laminin (1:100), and neuron-specific enolase (1:50) antibody (Boster, Wuhan, Hubei, China) at 4°C overnight, respectively. The next day, allophycocyanin (APC)-conjugated goat anti-rabbit antibody (Boster, Wuhan, Hubei, China) was added into all wells at a dilution of 1:100 and the mixtures were incubated at 37°C for 1 h. Then cell nuclei were dyed by 4', 6'-diamidino-2-phenylindole (DAPI) diluted in 1:2000 for 2 min. The stained TM cells were viewed under fluorescence microscope.

Detection of cell morphology changes after fasudil treatment

The cultured TM cells were inoculated in 24-well plates and treated with fasudil at different concentrations (1 μM, 10 μM, 100 μM and 1 mM) at 37°C and then viewed for the changes of cell morphology at 0 min, 30 min, 60 min, 2 h, withdrawal of fasudil (withdrawal) 2 h, and withdrawal 16 h under light microscope, respectively.

Detection of cytoskeletal architecture changes after fasudil treatment

The cultured TM cells were inoculated in 24-well plates and treated with fasudil at different concentrations (1 μM, 10 μM, 100 μM and 1 mM), respectively, TM cells incubated with PBS were set as controls. Then cultured TM cells were divided into two parts. One part was stained with Phalloidin-FITC (0.05 mg/ml; Sigma-Aldrich, St Louis, MO, USA) at 37°C for 1 h. The other part of TM cells was stained with mouse anti-vinculin antibody (1:100; Sigma-Aldrich, St Louis, MO, USA) at 4°C overnight, followed by Cy3-labeled goat anti-mouse secondary antibody (1:100; Jackson, US) at 37°C for 1 h in dark. After washing with PBS, the cell nuclei of all cultured TM cells were counterstained with DAPI and observed under a confocal laser scanning microscopy (Leica, Wetzlar, Germany) for the cytoskeletal changes at 0 min, 30 min, 60 min, 2 h, withdrawal 2 h and withdrawal 16 h, respectively.

Bovine eye perfusion

A total of 12 bovine eyes extracted from six bovines were used in this study and the test for

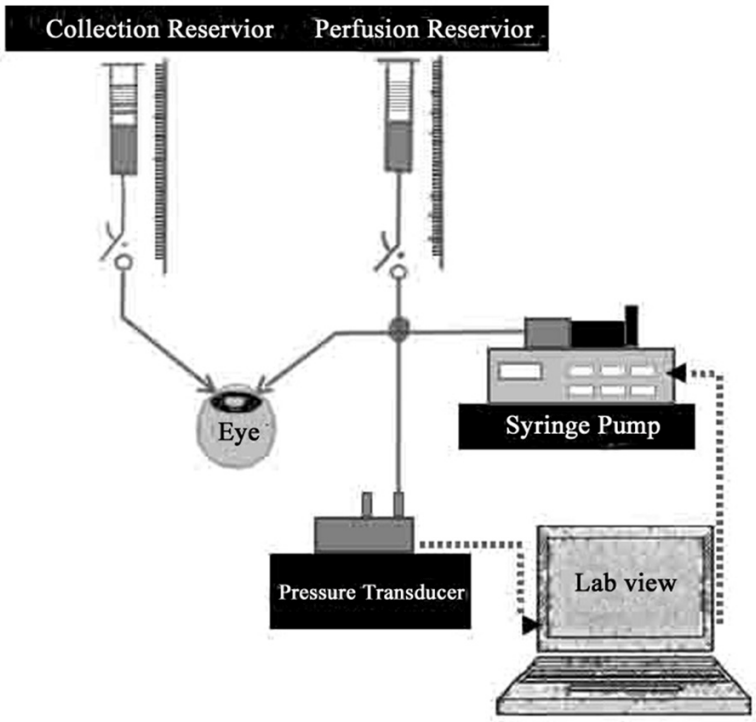

Figure 1. Flow diagram of the perfusion system.

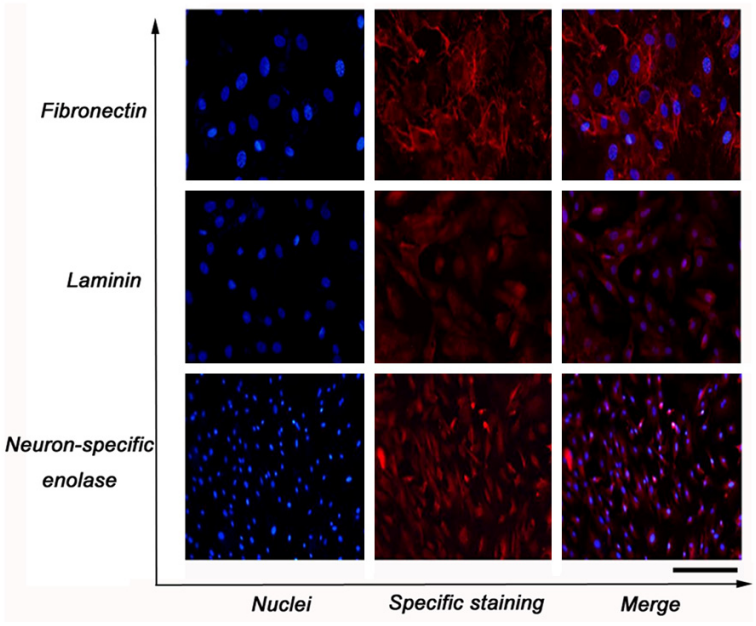

Figure 2. Characterization of TM cells identified by immunofluorescence for the expression of fibronectin, laminin, and neuron-specific enolase. Scale bar: 100 μm.

aqueous humor outflow was performed following the previous description with several modifications [17]. Bovine eyes were cleared of extraocular tissues and submerged to PBS at 34°C. A 23-gauge infusion needle (Hilmton, US) was carefully inserted intraocularly through the transparent cornea and the needle tip was positioned within the posterior chamber to prevent deepening of the anterior chamber that would otherwise lead to an artificial increase in outflow facility. Another 23-gauge needle was also inserted through the transparent cornea and positioned within the anterior chamber upon the surface of the iris that was connected to the collecting reservoir of the perfusion system. During perfusion, the catheter of collecting reservoir was clamped until the liquid exchange was conducted. The baseline facility of the eye was determined after perfusion with perfusion medium containing Dulbecco's PBS (DPBS) at 15 mmHg for 30 min to get a steady state flow value. Then anterior chamber solution was replaced and refilled with DPBS in six eyes (control eyes) or refilled with fasudil solution (500 μM fasudil in DPBS) in another six eyes. The perfusion was continued for 30 min and measurements of outflow facility were recorded. The outflow facility (C) was calculated with this formula: perfusion rate (μl/min)/IOP (mmHg). A computer system (Labview, US) was used to measure the perfusion rate and calculate the mean outflow facility during the whole process (**Figure 1**). The anterior chamber content in all eyes was then perfused with DPBS containing red fluorescent microspheres (0.5 Mm; 0.002% v/v; Molecular Probes, Ins. Eugene, Oregon, USA) to label the hydrodynamic outflow patterns. Finally, the anterior chamber

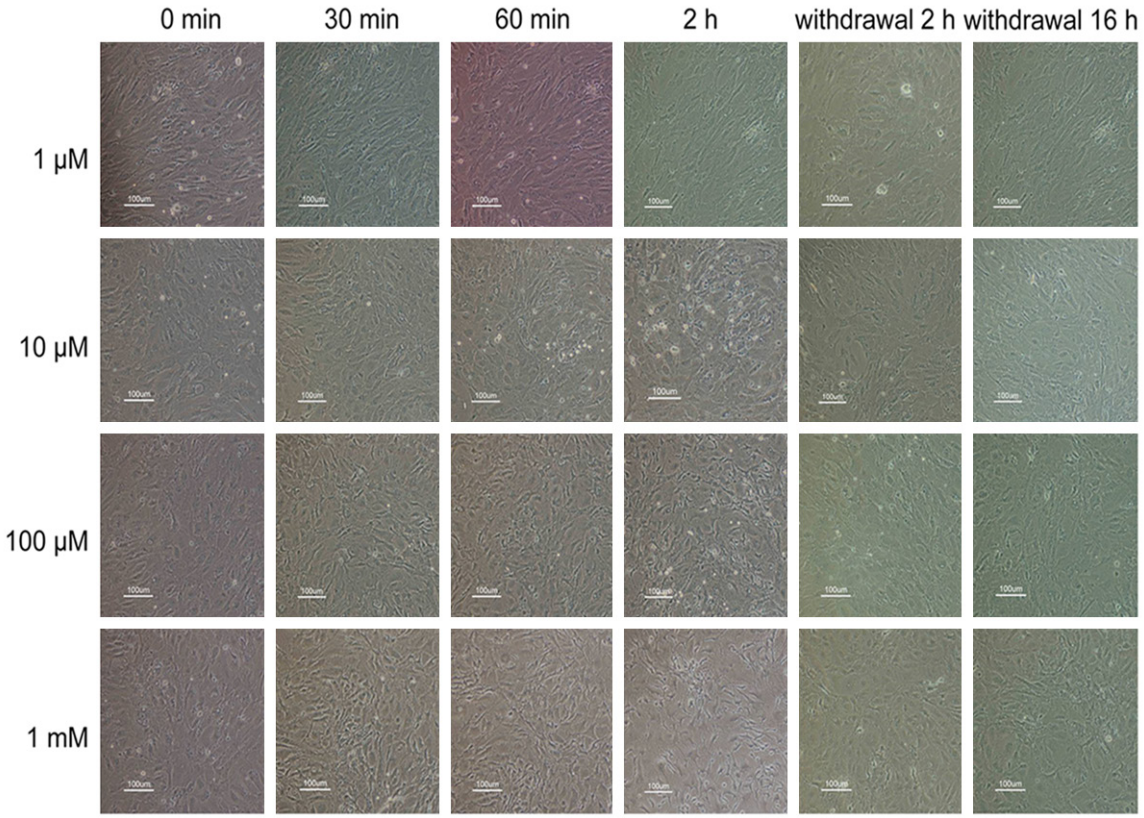

Figure 3. Effects of fasudil on TM cells morphology. The morphological changes of TM cells were observed after fasudil treatment at various concentrations (1 μM, 10 μM, 100 μM and 1 mM) and time point (0 min, 30 min, 60 min, 2 h, withdrawal 2 h and withdrawal 16 h) under light microscope. Scale bar: 100 μm.

content was perfused with formalin solution and perfusion-fixed in formalin.

Measurement of percent of the effective filtration length (PEFL)

The fixed eyes were cut in half at the equator. After carefully removing the vitreous body and lens of each eye, the anterior segments were divided into four quadrants (nasal, temporal, superior, and inferior). Then tissue sections were cut into 1.0-1.5 mm (2-3 μm thick) strips referred to frontal sections and counterstained with DAPI and Hoechst to visualize cell nuclei. The sections were viewed under a confocal microscope (Carl Zeiss 510, Axiovert 100M Laser Scanning Microscope, Heidelberg, Germany) and LSM 510 software version 3.2 PS2 (Carl Zeiss, Heidelberg, Germany) was used to scan and analyze the image. In order to properly analyze the tracer distribution along the inner wall and the TM, images were taken along the aqueous plexus (AP) with or without accompanying collector channel ostia. The PEFL was calculated as the formula: PEFL=FL/TL [18], PEFL: percent of the effective filtration length, FL: tracer-decorated length of AP, TL: total length of AP. A minimum of sixteen images four images per quadrant were taken in each eye for the measurement of PEFL.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Comparison was made with one-way ANOVA or student's *t*-test when appropriate. $P < 0.05$ was considered statistically significant.

Results

TM cell characterization

Six days after adherent culture, a few spindle-shaped cells were viewed to creep from the cultured TM tissue block. After three to five times of passage, the cultured wells were crowded

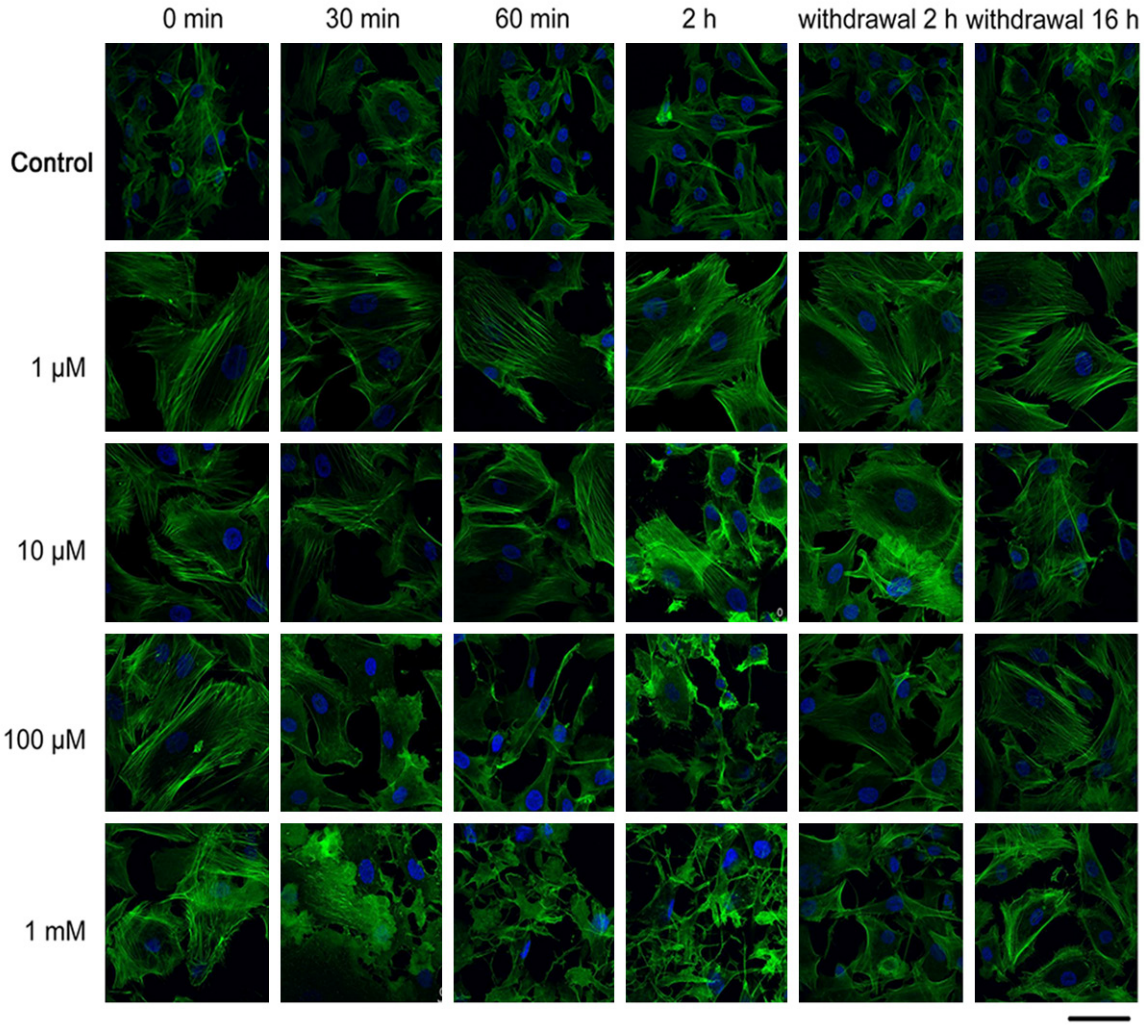

Figure 4. Effects of fasudil on F-actin arrangement in TM cells. TM cells were stained with Phalloidin-FITC and changes in TM cells were viewed after fasudil treatment at various concentrations (1 μM, 10 μM, 100 μM and 1 mM) and time point (0 min, 30 min, 60 min, 2 h, withdrawal 2 h and withdrawal 16 h) under a confocal confocal microscope. Scale bar: 10 μm.

with spindle or triangle cells which were the typical morphology of TM cells. Meanwhile, according to the results from immunofluorescence analysis, the positive signals for fibronectin, laminin, and neuron-specific enolase were viewed under fluorescence microscope (**Figure 2**). All these results demonstrated the TM cells were successfully prepared.

Effects of fasudil on TM cell morphology

The effects of fasudil on TM cell morphology were shown in **Figure 3**. Treatment with fasudil at different concentration (1, 10, 100 and 1000 μM) for 30 min, 60 min and 2 h resulted in retraction and thinning of the cells. In addition, the changes of cell morphology caused by fasudil were in a dose- and time-dependent manner. The morphology of TM cells with fasudil at higher concentrations (100 μM and 1 mM) changed a lot. After treatment with higher concentration (100 μM and 1 mM) of fasudil, the spindle or triangle cells changed into a longer and thinner morphology. In addition, the longer time for fasudil treatment could accelerate these morphological changes. However, recovery of normal morphologic status with a cluster of spindle or triangle cells was observed 2 and 16 hours after withdrawal of the fasudil treatment. Based on these results, the effects of fasudil on the TM cell morphology within

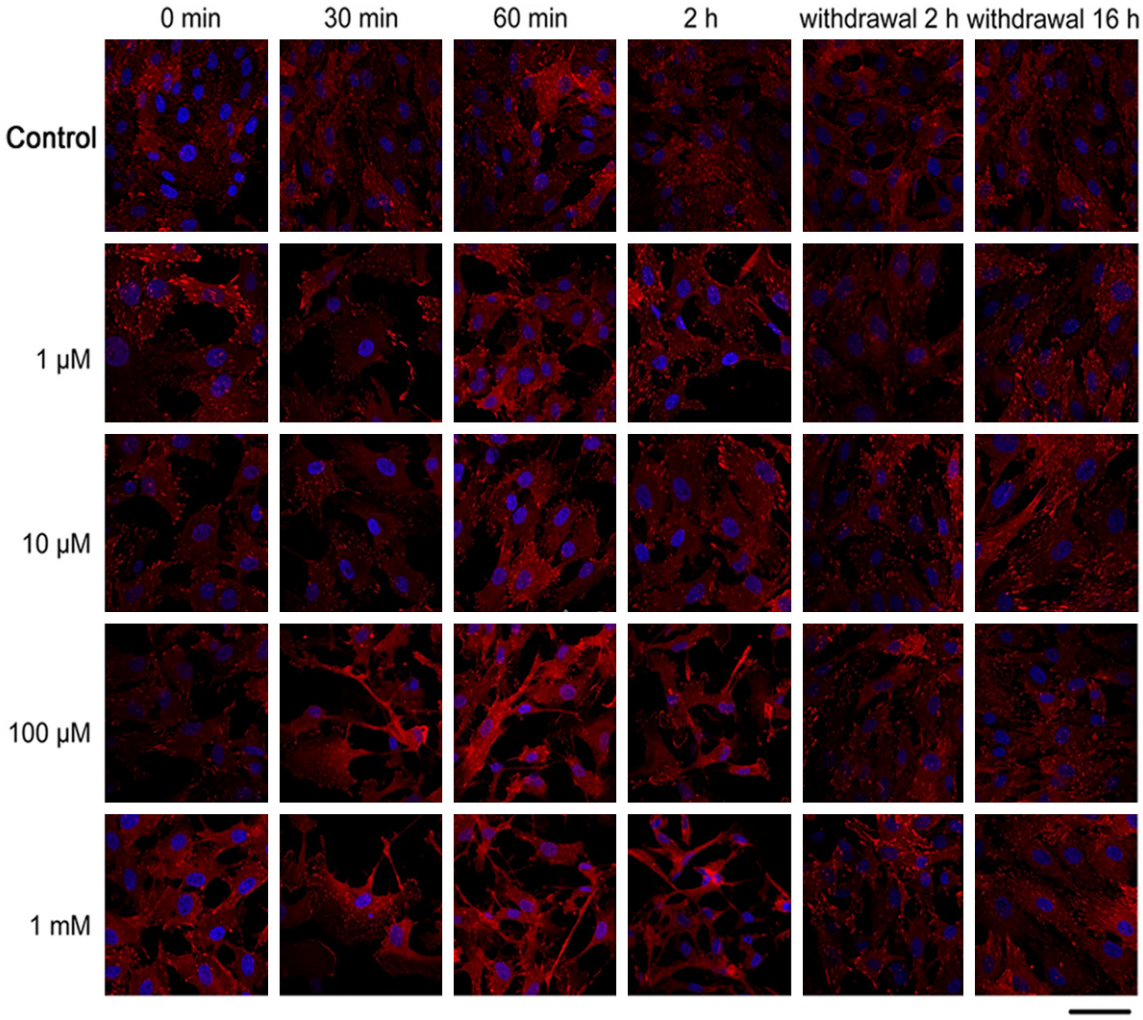

Figure 5. Effects of fasudil on vinculin amount in TM cells. TM cells were stained with mouse anti-vinculin antibody, followed by Cy3-labeled goat anti-mouse secondary antibody. Changes in TM cells were viewed after treatment at various concentrations (1 μM, 10 μM, 100 μM and 1 mM) and time point (0 min, 30 min, 60 min, 2 h, withdrawal 2 h and withdrawal 16 h) under a confocal confocal microscope. Scale bar: 10 μm.

Table 1. Effects of fasudil on the outflow facility of bovine eyes

	DPBS-perfused eyes (n=6)	Fasudil-perfused eyes (n=6)	*P*-value
C_0	1.86 ± 0.31	1.91 ± 0.32	
C_1	2.20 ± 0.29	2.77 ± 0.38	0.0153
ΔC	0.34 ± 0.03	0.87 ± 0.13	< 0.001
ΔC (%)	18.69 ± 3.44%	48.00 ± 9.33%	< 0.001

Data presented as Mean ± SD. C_0 (μl/min/mmHg): outflow facility at baseline; C_1 (μl/min/mmHg): outflow facility after perfusion; ΔC (μl/min/mmHg): increase of outflow facility from baseline; ΔC (%): increase rate of outflow facility from baseline.

indicated duration and concentration were reversible.

Effects of fasudil on cytoskeletal architecture in TM cells

The effects of fasudil on cytoskeletal architecture in TM cells were shown **Figures 4** and **5**. There were no obvious changes in cytoskeletal architecture in TM cells after PBS treatment during the whole experiment. The changes in cytoskeletal architecture caused by fasudil were in a dose- and time-dependent manner. Fasudil treatment at lower concentration (1 μM and 10 μM) induced no significant changes in F-actin arrangement and vinculin amount in TM cells. However, treatment with higher dose of fasudil (100 μM and 1 mM) induced changes in depolymerization of F-actin and loss of vinculin. In addition, treatment of fasudil with longer

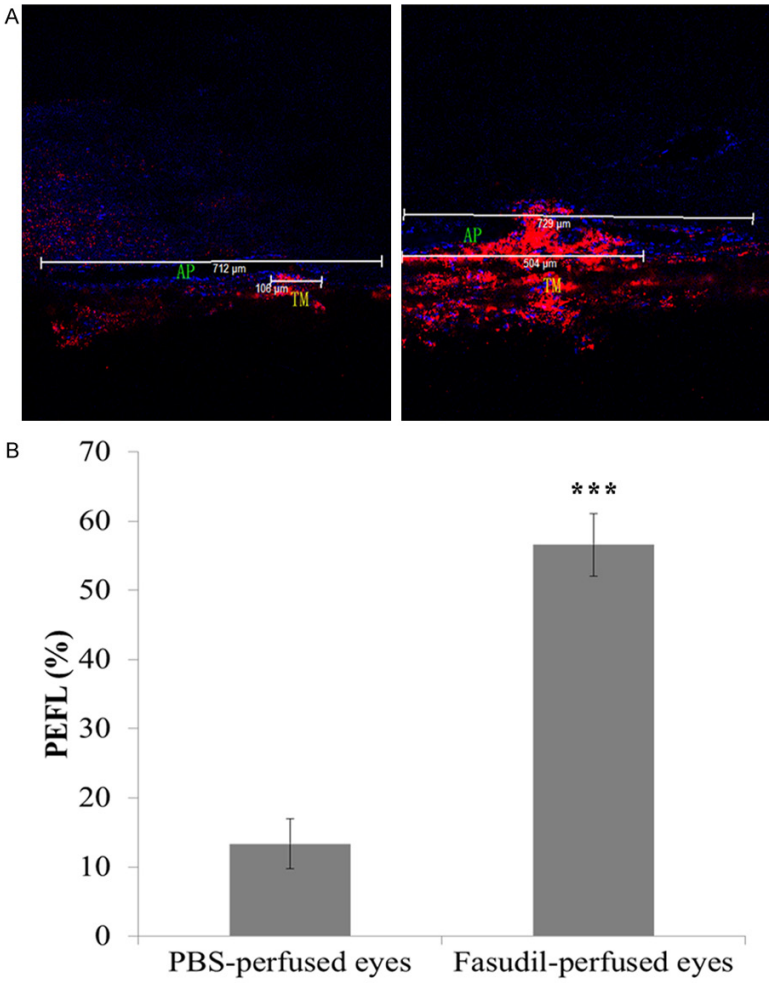

Figure 6. Effects of fasudil on outflow pattern and Percent effective filtration length (PEFL) in the bovine eyes. A: The red fluorescent microspheres were perfused in the bovine eyes to label the hadeodynamic outflow patterns and visualized by a confocal microscopy under 20× magnifications. Left: Tracer distribution in the DPBS-perfused eyes; Right: Tracer distribution in the control eyes. AP: aqueous plexus; TM: trabecular meshwork. B: The comparison of average PEFL between the DPBS-perfused and control eyes after statistical analysis. Data were presented as mean ± S.D. $P < 0.05$ was considered as significant difference. ***, $P < 0.001$.

time would aggravate these effects. However, two hours after withdrawal of the fasudil treatment, the depolymerized F-actin began to aggregate and the amount of vinculin also started to increase. Then sixteen hours after withdrawal of the fasudil treatment, depolymerized structure reorganized to F-actin bundles and the amount of vinculin-positive spots recovered to the level similar to the controls. Following these results, these effects induced fasudil on TM cytoskeletal architecture were reversible.

Changes in aqueous outflow facility after fasudil perfusion

The changes of outflow facility in both fasudil-perfused and control groups were shown in **Table 1**. Based on these results, the average outflow facility in control group increased 18.69 ± 3.44% from baseline (from 1.86 ± 0.31 to 2.20 ± 0.29 μl/min/mmHg) while in fasudil-perfused group the outflow facility increased 48.00 ± 9.33% from baseline (from 1.91 ± 0.32 to 2.77 ± 0.38 μl/min/mmHg). The outflow facility in fasudil-perfused group increased significantly than that in the control group ($P < 0.001$) (**Table 1**).

Effects of fasudil on outflow pattern and PEFL

The partial distribution of tracer in the TM cells viewed by confocal microscopy in both control and fasudil-perfused eyes was shown in **Figure 6A**. The punctate labeling along AP clustered near collector channel ostia in the control ayes, while tracer distribution was more uniform and extensive throughout the TM in the fasudil-perfused group. In addition, the average PEFL in the fasudil-perfused eyes was significantly larger than that in the control eyes (56.56 ± 4.55% *vs* 13.36 ± 3.58%, $P < 0.001$) following the statistic results in **Figure 6B**.

Discussion

Fluid flow in the anterior chamber maintains IOP and globe shape. Moreover, it can supply oxygen and nutrients to TM [3]. However, elevated IOP can bring abnormally high resistance to aqueous humor drainage in the TM outflow pathways, thus leading to POAG [5]. So agents which can increase the aqueous humor outflow

facility are considered to be potential therapies for the POAG. In the present study, we demonstrated that a specific ROCK inhibitor, fasudil [6], induce a significant decrease in IOP in bovine eyes. To explore the underlying mechanisms of IOP-lowering effects and hydrodynamic patterns changes induced by this inhibitor, we have conducted a series of experiments.

In our study, the primary TM cells were identified by detecting the expression of fibronectin, laminin, and neuron-specific enolase according to previous studies [18-20]. Fibronectin and laminin are two extracellular matrix molecules, and neuron-specific enolase is a cytoskeletal molecule. All of them are specific markers for TM cells and immunopositive for TM cells. Our cytochemical studies demonstrated that fasudil disrupted F-actin bundles and impaired local adhesion formation. Previous studies indicated agents that can decrease IOP are always shown to disrupt cytoskeletal architecture and contractility of TM cells [21, 22]. Y-27632, another ROCK inhibitor, has been identified to significantly reduce outflow resistance in the live monkey eye. However, it is identified to be not applicable for clinic for its poor pharmacokinetics with very short half-life [23]. Therefore we performed the present study to explore the effects of another ROCK inhibitor fasudil on outflow resistance, which is a mature clinical preparation. Following the results in our study, fasudil at higher concentrations changed the cell morphology and disrupted the cytoskeleton through inducing the depolymerization of F-actin and reduction of cell adhesion in TM tissue that were similar to the previous description of Y-27632. While different from Y-27632, fasudil has been applied in the clinic for several cerebral diseases without severe side effects having been reported. Consequently, fasudil may be a new potential drug against POAG.

Fasudil is known to specifically inhibit the activity of ROCK that is a downstream effector of Rho GTPase [8]. As previously described, ROCK modulates phosphorylation states of myosin light chain (MLC) by inhibiting the activity of myosin light chain phosphatase (MLCP) as well as directly phosphorylating MLC [24-26]. MLC phosphorylation can promote the crosslink between actin and myosin, induce actin stress fiber assembly and focal adhesions formation as well as regulate cellular contraction and cytoskeleton organization in TM tissue [27]. So it is tightly associated with aqueous humor outflow facility. The inactivation of ROCK would directly lead to the reduction of MLC phosphorylation and then result in the destruction of actomyosin system, thus induce the cytoskeletal disruption as well as alteration in cell shape, cell contraction and cell adhesion [13, 27]. Consequently, we speculated that fasudil induced the depolymerization of stress fiber bundle, degradation of focal adhesion as well as the morphological changes through inducing the inactivation of ROCK and then reducing MLC phosphorylation. In addition, these effects of fasduil were reversible within indicated duration and concentration.

We also explored the hydrodynamic patterns changes induced by fasudil through establishing a perfusion system (**Figure 1**) in the bovine eyes and also calculated the outflow facility as well as the PEFL. Outflow facility is widely used to measure the aqueous humor flow and evaluate the outflow resistance [28-30]. In our study, fasudil could decrease the outflow resistance and increase the outflow facility compared to the control eyes. Several studies showed that the primary aqueous outflow resistance was derived from a region including the juxtacanalicular (JCT) and inner wall endothelium of SC in the eyes of primates [31]. PEFL is considered to be the most appropriate anatomical parameter when describing the correlation of several physiological factors to the outflow system [11, 13, 32]. So we detected the influence of fasudil on the outflow pattern and the PEFL (redefined) in the bovine eyes. There was a significant improvement of the outflow facility after perfusion with fasudil in bovine eyes and the outflow facility positively correlated with PEFL in our study. Lu *et al.* [5] proposed that outflow facility, outflow hydrodynamics and cell morphology were likely coupled in monkey eyes. Following our results, we suggested that fasudil could increase the outflow facility through changing TM cell morphology as well as cell contractility. However, the anatomical structures are different between bovines and humans. The bovine eyes lack the Schlemm's canal but possess AP with similar functions meanwhile TM tissues in bovine eyes are less developed. Therefore, the specific effects of fasudil on the outflow pattern and outflow facility in human eyes need further investigation.

In conclusion, the effects of fasudil on inducing the reduction of outflow resistance were associated with its effects on cytoskeleton in bovine eyes. This revelation would provide a reference for the further exploration of fasudil on the treatment of POAG.

Disclosure of conflict of interest

None.

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References

[1] Quigley HA. Glaucoma. Lancet 2011; 377: 1367-1377.
[2] Weinreb RN, Aung T and Medeiros FA. The pathophysiology and treatment of glaucoma: a review. JAMA 2014; 311: 1901-1911.
[3] Tian B, Geiger B, Epstein DL and Kaufman PL. Cytoskeletal involvement in the regulation of aqueous humor outflow. Invest Ophthalmol Vis Sci 2000; 41: 619-623.
[4] Lütjen-Drecoll E. Functional morphology of the trabecular meshwork in primate eyes. Prog Retin Eye Res 1999; 18: 91-119.
[5] Johnson M and Erickson K. Mechanisms and routes of aqueous humor drainage. Principles and Practice of Ophthalmology 2000; 4: 2577-2595.
[6] Epstein DL, Rowlette LL and Roberts BC. Actomyosin drug effects and aqueous outflow function. Invest Ophthalmol Vis Sci 1999; 40: 74-81.
[7] Kaufman PL, Gabelt B, Tian B and Liu X. Advances in glaucoma diagnosis and therapy for the next millennium: new drugs for trabecular and uveoscleral outflow. Semin Ophthalmol 1999; 14: 130-143.
[8] Honjo M, Inatani M, Kido N, Sawamura T, Yue BY, Honda Y and Tanihara H. Effects of protein kinase inhibitor, HA1077, on intraocular pressure and outflow facility in rabbit eyes. Arch Ophthalmol 2001; 119: 1171-1178.
[9] Rao PV, Deng P, Maddala R, Epstein DL, Li CY and Shimokawa H. Expression of dominant negative Rho-binding domain of Rho-kinase in organ cultured human eye anterior segments increases aqueous humor outflow. Mol Vis 2005; 11: 288-297.
[10] Epstein DL, Roberts BC and Skinner LL. Nonsulfhydryl-reactive phenoxyacetic acids increase aqueous humor outflow facility. Invest Ophthalmol Vis Sci 1997; 38: 1526-1534.
[11] Tian B, Kaufman PL, Volberg T, Gabelt BT and Geiger B. H-7 disrupts the actin cytoskeleton and increases outflow facility. Arch Ophthalmol 1998; 116: 633-643.
[12] Rao PV, Deng PF, Kumar J and Epstein DL. Modulation of aqueous humor outflow facility by the Rho kinase-specific inhibitor Y-27632. Invest Ophthalmol Vis Sci 2001; 42: 1029-1037.
[13] Somlyo AP and Somlyo AV. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J Physiol 2000; 522: 177-185.
[14] Asano T, Suzuki T, Tsuchiya M, Satoh S, Ikegaki I, Shibuya M, Suzuki Y and Hidaka H. Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. Br J Pharmacol 1989; 98: 1091-1100.
[15] Yue BY, Higginbotham EJ and Chang IL. Ascorbic acid modulates the production of fibronectin and laminin by cells from an eye tissue-trabecular meshwork. Exp Cell Res 1990; 187: 65-68.
[16] Rao PV, Deng P, Sasaki Y and Epstein DL. Regulation of myosin light chain phosphorylation in the trabecular meshwork: role in aqueous humour outflow facility. Exp Eye Res 2005; 80: 197-206.
[17] Zhu JY, Ye W and Gong HY. Development of a novel two color tracer perfusion technique for the hydrodynamic study of aqueous outflow in bovine eyes. Chin Med J (Engl) 2010; 123: 599-605.
[18] Lin S, Lee OT, Minasi P and Wong J. Isolation, culture, and characterization of human fetal trabecular meshwork cells. Curr Eye Res 2007; 32: 43-50.
[19] Ueda J and Yue BY. Distribution of myocilin and extracellular matrix components in the corneoscleral meshwork of human eyes. Invest Ophthalmol Vis Sci 2003; 44: 4772-4779.
[20] He Y, Ge J and Tombran-Tink J. Mitochondrial defects and dysfunction in calcium regulation in glaucomatous trabecular meshwork cells. Invest Ophthalmol Vis Sci 2008; 49: 4912-4922.
[21] Johnson DH. The effect of cytochalasin D on outflow facility and the trabecular meshwork of the human eye in perfusion organ culture. Invest Ophthalmol Vis Sci 1997; 38: 2790-2799.
[22] Tian B and Kaufman PL. Effects of the Rho kinase inhibitor Y-27632 and the phosphatase inhibitor calyculin A on outflow facility in monkeys. Exp Eye Res 2005; 80: 215-225.
[23] Ishizaki T, Uehata M, Tamechika I, Keel J, Nonomura K, Maekawa M and Narumiya S. Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. Mol Pharmacol 2000; 57: 976-983.

[24] Etienne-Manneville S and Hall A. Rho GTPases in cell biology. Nature 2002; 420: 629-635.
[25] Burridge K and Wennerberg K. Rho and Rac take center stage. Cell 2004; 116: 167-179.
[26] Ai S, Kuzuya M, Koike T, Asai T, Kanda S, Maeda K, Shibata T and Iguchi A. Rho-Rho kinase is involved in smooth muscle cell migration through myosin light chain phosphorylation-dependent and independent pathways. Atherosclerosis 2001; 155: 321-327.
[27] Kaibuchi K, Kuroda S and Amano M. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem 1999; 68: 459-486.
[28] Li A, Banerjee J, Peterson-Yantorno K, Stamer WD, Leung CT and Civan MM. Effects of cardiotonic steroids on trabecular meshwork cells: search for mediator of ouabain-enhanced outflow facility. Exp Eye Res 2012; 96: 4-12.
[29] Hays CL, Gulati V, Fan S, Samuelson TW, Ahmed II and Toris CB. Improvement in outflow facility by two novel microinvasive glaucoma surgery implants. Invest Ophthalmol Vis Sci 2014; 55: 1893-1900.
[30] Dastiridou AI, Tsironi EE, Tsilimbaris MK, Ginis H, Karyotakis N, Cholevas P, Androudi S and Pallikaris IG. Ocular rigidity, outflow facility, ocular pulse amplitude, and pulsatile ocular blood flow in open-angle glaucoma: a manometric study. Invest Ophthalmol Vis Sci 2013; 54: 4571-4577.
[31] Mäepea O and Bill A. Pressures in the juxtacanalicular tissue and Schlemm's canal in monkeys. Exp Eye Res 1992; 54: 879-883.
[32] Rao PV, Deng P, Sasaki Y and Epstein DL. Regulation of myosin light chain phosphorylation in the trabecular meshwork: role in aqueous humour outflow facility. Exp Eye Res 2005; 80: 197-206.