• Basic Research •

Effects of brinzolamide on rabbit ocular blood flow *in vivo* and *ex vivo*

Ya-Ru Dong[1,2], Shi-Wei Huang[1], Ji-Zhe Cui[1], Takeshi Yoshitomi[2]

[1]Department of Ophthalmology, the Second Hospital of Jilin University, Changchun 130041, Jilin Province, China
[2]Department of Ophthalmology, Akita University School of Medicine, Akita 010-8543, Japan
Correspondence to: Ya-Ru Dong. Department of Ophthalmology, the Second Hospital of Jilin University, 218 Ziqiang Street, Changchun 130041, Jilin Province, China. dongyaru@sina.com; Takeshi Yoshitomi. Department of Ophthalmology, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan. yoshitom@med.akita-u.ac.jp
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Abstract

• AIM: To investigate if significant improvement of optic disc blood flow (ODBF) occurs after instillation of brinzolamide onto rabbit eyes.

• METHODS: Testing of bilateral intraocular pressure (IOP) and left ODBF in 10 male rabbits took place every 3h over a 24h period. Brinzolamide (1% ophthalmic solution, two drops at 9:00 and 21:00) was administered to the left eye. ODBF, assessed using laser speckle flowgraphy, was determined as the mean blur rate (MBR). Furthermore, the effect of brinzolamide on isolated rabbit ciliary arteries using isometric tension recording system was performed.

• RESULTS: After brinzolamide instillation, IOP was significantly decreased in the left eye. MBR-vessel was greater at 18:00 and 21:00 (P<0.05) than in the controls. MBR-tissue and MBR-average were greater at 18:00 (P<0.05) than in the controls. For isolated arteries pre-contracted with a high-K solution, brinzolamide induced concentration-dependent relaxation, reaching 46.1%±9% (n=21) at 1 mmol/L. In Ca^{2+}-free solutions, incubation with brinzolamide suppressed 1 μmol/L histamine-induced contractions (P<0.05).

• CONCLUSION: Brinzolamide decreases IOP and increases ocular blood flow. The direct vasodilatory effect of brizolamide is mediated by suppression of Ca^{2+} release from intracellular calcium stores.

• KEYWORDS: brinzolamide; ocular blood flow; vasodilation; intraocular pressure; ciliary artery
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INTRODUCTION

Brinzolamide, a compound structurally related to acetazolamide, was approved for the topical treatment of glaucoma in 1999[1]. Acetazolamide, dorzolamide, and brinzolamide are carbonic anhydrase inhibitors (CAIs), all of which belong to sulfonamide antiglaucomatous drugs. Brinzolamide, which is formulated as a 1% ophthalmic suspension and applied twice daily to lower intraocular pressure (IOP), has fewer systemic side effects than acetazolamide. Brinzolamide is a highly specific, noncompetitive, and reversible carbonic anhydrase II inhibitor[2]. Carbonic anhydrases are zinc-containing enzymes that catalyze the reversible hydration of carbon dioxide and bicarbonate. Inhibition of carbonic anhydrase II in the secretory cells of ciliary processes reduces the rate of aqueous humour formation, consequently lowering IOP[3-4].

Glaucoma, a multifactorial optic neuropathy, is the second leading cause of irreversible blindness worldwide[5]. Although elevated IOP is the principle risk factor, deterioration of ocular perfusion by the vascular system accelerates progression of glaucomatous optic nerve atrophy[6]. In glaucoma, reduced blood flow to the eye has been reported in many studies that used a variety of different techniques to assess ocular blood flow (OBF)[7-9]. Moreover, OBF of glaucoma patients is deficient in the retinal, choroidal, and retrobulbar circulations, and focal ischemia corresponds with areas of glaucomatous visual field loss[6]. Therapeutic agents that reverse this condition could be of great clinical benefit for glaucoma patients. The effect of IOP-lowering medications on OBF remains poorly understood.

CAIs may improve blood perfusion in the eyes in humans. It has been shown that acetazolamide leads to a dilatation of retinal vessel diameters and increases blood flow in the optic nerve head[10]. Similarly, topical application of dorzolamide leads to a significant increase of flow velocities of the retrobulbar vessels as measured by color Doppler imaging[11]. The mechanism by which IOP-lowering medications increase OBF remains unknown in glaucoma. It is difficult to determine if the CAI-induced increase in ocular perfusion is secondary

to IOP reduction or if it is a primary effect on the ocular vasculature.

There are very few studies that have reported the effect of brinzolamide on OBF. Moreover, the results of these studies have been contradictory due to the heterogeneous study designs and methodologies used to measure OBF. Barnes *et al*[12] found that brinzolamide significantly increased optic nerve head blood flow in Dutch rabbits. However, Martinez and Sanchez-Salorio[13] suggested that after 5y of treatment, brinzolamide did not augment retrobulbar blood flow when added to timolol in glaucoma patients. Based on these reports, we decided to focus on the relationship between brinzolamide and OBF.

In the current study, we sought to determine the effect of brinzolamide on optic disc blood flow (ODBF) by *in vivo* laser speckle flowgraphy and to assess the effect and mechanism of brinzolamide on isolated ciliary arteries by an isometric tension recording system.

MATERIALS AND METHODS

Animals This study was approved by the Animal Care Committee of Akita University, and was adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Ten male Dutch rabbits (weight 1.2-1.5 kg) were used to measure ODBF *in vivo*. Twenty-four male Japanese white rabbits (weight 2-3 kg) were killed, the ciliary arteries were removed and their isometric tension measured *ex vivo*. All rabbits were kept in a specialized animal center, and standard feed and water were supplied on demand.

In Vivo Studies

Experimental protocol Testing of bilateral IOP and ODBF in the left eye of the Dutch rabbits took place every 3h over a period of 24h at 9:00, 12:00, 15:00, 18:00, 21:00, 24:00, 3:00, 6:00, and 9:00. The pupils of the rabbits were not dilated pharmacologically during the experimental period. After IOPs were measured, the rabbits were given a 5min rest period, and then ocular circulation was assessed using LSFG-NAVI™ (Softcare Ltd., Fukuoka, Japan) in a dark room. For each rabbit, the 24h measurements of IOP and ODBF were performed twice. During the first 24h, control measurements were made without any drug treatment. After the rabbits rested for 4d, 1% brinzolamide ophthalmic suspension (Azopt®; Alcon Laboratories, Inc., Ft. Worth, TX, USA) was delivered topically as two drops instilled in the left eye at 9:00, and the measurements were immediately begun. The drug was applied again at 21:00, and the blood flow measurements were continued thereafter.

Measurement of intraocular pressure Bilateral IOP of rabbits was measured with a rebound tonometer (TonoVet®, Icare Finland Oy, Helsinki, Finland) without any topical anesthetic. In the first 24h measurements, circadian fluctuations of IOP were recorded. The effects of brinzolamide were assessed four days later when IOP was again recorded for 24h beginning immediately after brinzolamide was instilled into the left eyes.

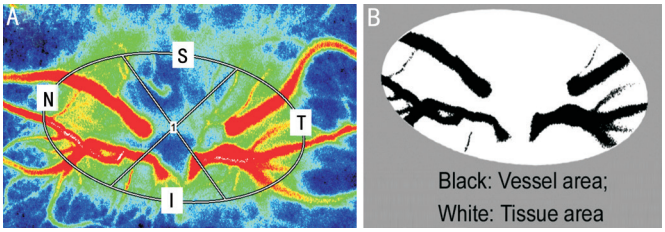

Figure 1 Map of the optic disc A: A representative map produced by LSFG-NAVI™, the investigator manually set an elliptical area at the outer edge of the optic disc, and LSFG-NAVI™ measured MBR of that areas (S: Superior quadrant; T: Temporal quadrant; I: Inferior quadrant; N: Nasal quadrant); B: In the elliptical area, the black part represented the blood vessel and the white part represented the tissue.

Laser speckle flowgraphy In order to assess ODBF, mean blur rate (MBR) of left eye of each rabbit was measured using LSFG-NAVI™. MBR is a parameter that yields a exact measurement of optic disc microcirculation, and shows good correlation with the blood flow parameters measured with other OBF assessment instruments[14-15]. MBR was measured in arbitrary units that make comparison in the same site in the same eye practical. Therefore, the investigator manually determined the measurement area of each rabbit's optic disk and saved it before measuring MBR (Figure 1A). MBRs of three areas of the optic disc were measured: the average MBR over the vessel area (MBR-V), the average MBR over the tissue area (MBR-T), and the average MBR over the entire optic disk (MBR-A; Figure 1B). All rabbits were examined by one experienced investigator. Detailed methods for measurement of MBR by LSFG-NAVI™ have been described by previous paper[16].

Ex Vivo Studies

Isolation of the ocular ciliary arteries Rabbits (weight 2-3 kg) were euthanized by intravenous injection of excess sodium pentobarbital (Abbott, North Chicago, IL, USA). Then, their eyes were immediately removed to ensure that the optic nerve attached to the eye is as long as possible. The eyes were placed in a Krebs solution bubbled with 95% O_2 and 5% CO_2. Our previous article has described in detail the composition of Krebs solution[17]. Under a dissecting microscope, the ciliary artery segments (length: 3-4 mm) and surrounding connective tissue were separated and cut off from the eyes.

Isometric tension recording Isolated vascular segments (2 mm in length) were mounted in the chamber of Myograph System® (JP Trading, Aarhus, Denmark). Our previous reports have described in detail the procedure for mounting the ciliary arteries[18-19]. After the equilibration period, contractions of the ciliary artery were evoked by a high-K solution and lasted for 20min. Then, 1 μmol/L carbachol was added to induce

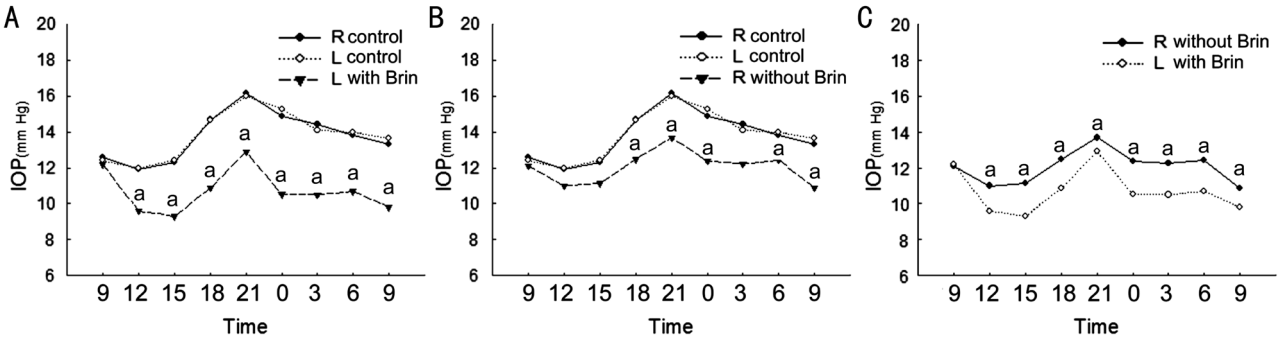

Figure 2 Effects of brinzolamide on rabbit IOP A: The left IOP decreased at all times except the first, which was taken immediately after instillation; B: The right IOP also decreased except at 9:00 (immediately after instillation), 12:00, 15:00 and 3:00; C: The right IOP was higher than the left at all times except immediately after instillation. Brinzolamide was instilled into the left eyes only. [a]P<0.05.

relaxation of ciliary artery. If high-K-induced contraction was less than 5 mmol/L or carbachol-induced relaxation was less than 1 mmol/L, such vascular segments were excluded from this study. After verifying the arterial responsiveness to high-K and carbachol, the medium in the chamber was replaced by Krebs solution.

Prior to testing the vasodilating effect of brinzolamide, dorzolamide, or acetazolamide, the isolated ciliary arteries were re-contracted with the high-K solution and maintained for 20min. Then, these drugs (10 μmol/L-1 mmol/L) were applied cumulatively every 10min. The amplitudes of high-K-induced contractions were defined as 100%.

To determine if nitric oxide (NO), prostacyclin, or large-conductance calcium-activated K^+ (BK_{Ca}) channels were involved in brinzolamide-mediated relaxation, these pathways were blocked with either 100 μmol/L L-NAME, a NO synthase inhibitor[20]; 10 μmol/L indomethacin, a cyclooxygenase inhibitor[21]; or 0.1 μmol/L iberiotoxin, a BK_{Ca} channel blocker[22]. These drugs were administrated prior to high-K-induced vasoconstriction. Furthermore, we evaluated the effect of brinzolamide on 1 μmol/L histamine-induced contractions in Ca^{2+}-free solutions. Based on the components of Krebs solution, Ca^{2+}-free solution was prepared by replacing $CaCl_2$ with isotonic equimolar $MgCl_2$ and adding 1 mmol/L ethylene glycol tetraacetic acid (EGTA).

Drugs The following drugs were used: brinzolamide and dorzolamide (Tokyo Chemical Industry, Tokyo, Japan), acetazolamide, indomethacin, iberiotoxin, histamine, L-NAME and carbachol (Sigma, St. Louis, MO, USA). The concentration of these drugs referred to the molarity in the myograph chambers.

Statistical Analysis Mean±standard deviations were used to express the measured results and 'n' represented the number of studied vessel segments unless specifically indicated. Unpaired two-tailed t-test was performed to analyze the statistical differences between the values. One-way analysis of variance was used to determine differences between these concentration-response curves. Statistical significant was set at probability values less than 0.05.

RESULTS

In Vivo Studies

Fluctuation of intraocular pressure In the first 24h measurements, analysis of 10 rabbits indicated that the peak IOP occurred at approximately 21:00. There was no significant difference between right and left eyes for any of the measurements (P>0.05). Instillation of brinzolamide reduced the left IOP at all periods except in the first one immediately after the drug was delivered (P<0.05; Figure 2A). Interestingly, with the exception of immediately after the instillation and at 12:00, 15:00, and 3:00, the IOPs in the right eyes of rabbits that received the brinzolamide in the left eye were also significantly decreased compared to the control IOPs taken four days earlier (Figure 2B). Nevertheless, the IOPs in the right eyes were significantly higher than in the left eyes except immediately after instillation (Figure 2C).

Increase of optic disc blood flow induced by brinzolamide In contrast to the circadian fluctuation of IOP, a nocturnal trough of MBR appeared, with a nadir at approximately 21:00 (Figure 3). Compared to control measurements, MBR-V (Figure 3A) was greater at 18:00 and 21:00 (P<0.05), and MBR-T (Figure 3B) and MBR-A (Figure 3C) were greater at 18:00 (P<0.05) after instillation of brinzolamide.

Ex Vivo Studies

Relaxation induced by brinzolamide, dorzolamide and acetazolamide For rabbit ciliary arteries pre-contracted with the high-K solution, with the exception of acetazolamide, brinzolamide and dorzolamide induced concentration-dependent relaxation (Figure 4A). For dorzolamide (Figure 4C), and brinzolamide (Figure 4D), the relaxations achieved at 1 mmol/L were 27.7%±5.2% (n=26) and 46.1%±9% (n=21), respectively.

Influence of nitric oxide, prostacyclin, and BK_{Ca} channels Isolated rabbit ciliary arteries were pretreated with 100 μmol/L L-NAME (n=7; Figure 5A), 10 μmol/L indomethacin (n=7; Figure 5B), or 0.1 μmol/L iberiotoxin (n=7; Figure 5C) 30min before application of the high-K solution. Brinzolamide-induced concentration-dependent relaxations were not altered by these drugs (P>0.05).

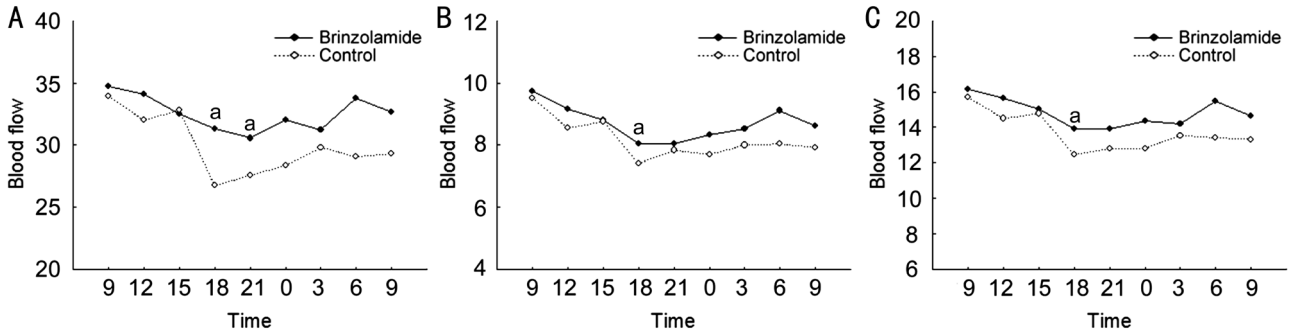

Figure 3 Effects of brinzolamide on ODBF Brinzolamide was instilled into the left eye of each rabbit. The MBR-V (A) was increased at 18:00 and 21:00, the MBR-T (B) and MBR-A (C) were increased at 18:00 compared with control. [a]P<0.05.

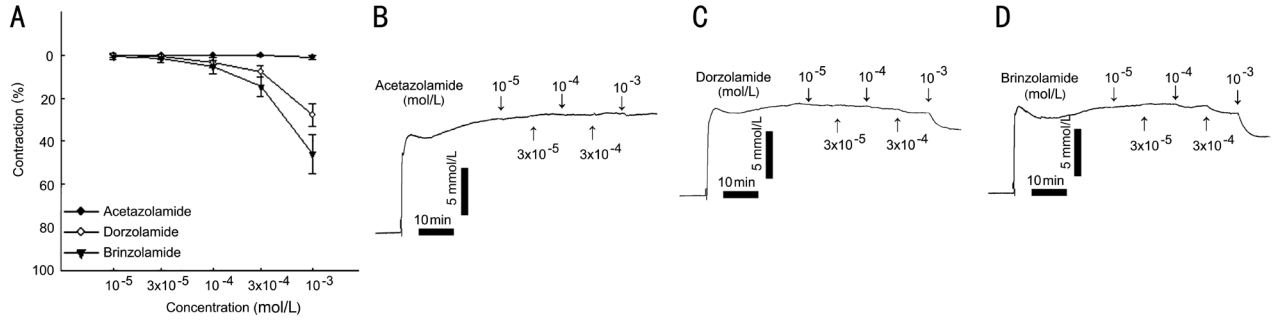

Figure 4 Brinzolamide-, dorzolamide- and acetazolamide-induced vasodilations Greater relaxation occurred with brinzolamide compared to dorzolamide and acetazolamide (A), representative relaxation curves of acetazolamide (B), dorzolamide (C), and brinzolamide (D).

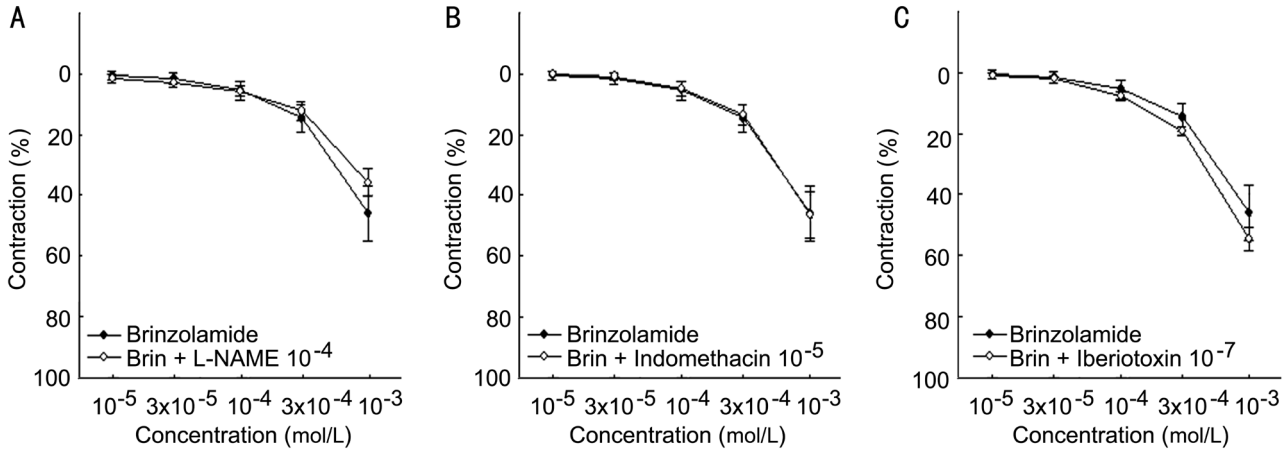

Figure 5 Effects of L-NAME, indomethacin, and iberiotoxin on brinzolamide-induced relaxation L-NAME (100 μmol/L; A), indomethacin (10 μmol/L; B), or iberiotoxin (0.1 μmol/L; C) were added to vessel segments prior to contraction induced by the high-K solution. There was no significant effect of L-NAME, indomethacin, or iberiotoxin on brinzolamide-induced vasodilatation.

Effect of brinzolamide on histamine-provoked vasoconstrictions in Ca^{2+}-free solutions In Ca^{2+}-free solutions, 1 μmol/L histamine induced smooth muscle contractions with or without 1 mmol/L brinzolamide (Figure 6A). We compared the areas under the histamine-induced contraction curves and found that incubation with brinzolamide suppressed histamine-provoked vasoconstrictions in the Ca^{2+}-free solution (n=4, P<0.05; Figure 6B).

DISCUSSION

In the present study, topical application of brinzolamide significantly decreased IOP and increased ODBF in rabbits. These observations raised the question of whether the brinzolamide-augmented ocular perfusion can be attributed to a primary effect on the ocular vasculature or if it is secondary to IOP reduction. Based on this question, rabbit ocular ciliary arteries were isolated, and brinzolamide was applied to the high-K pre-contracted preparations to measure the development of relaxation *ex vivo*. The vasodilating capacity of brinzolamide was superior to two other sulfonamide antiglaucoma drugs, dorzolamide and acetazolamide. Furthermore, incubation with brinzolamide suppressed histamine-induced contractions in the Ca^{2+}-free solution.

In vivo, IOP was decreased bilaterally after brinzolamide was instilled only in the left eye, though the right eye IOP remained

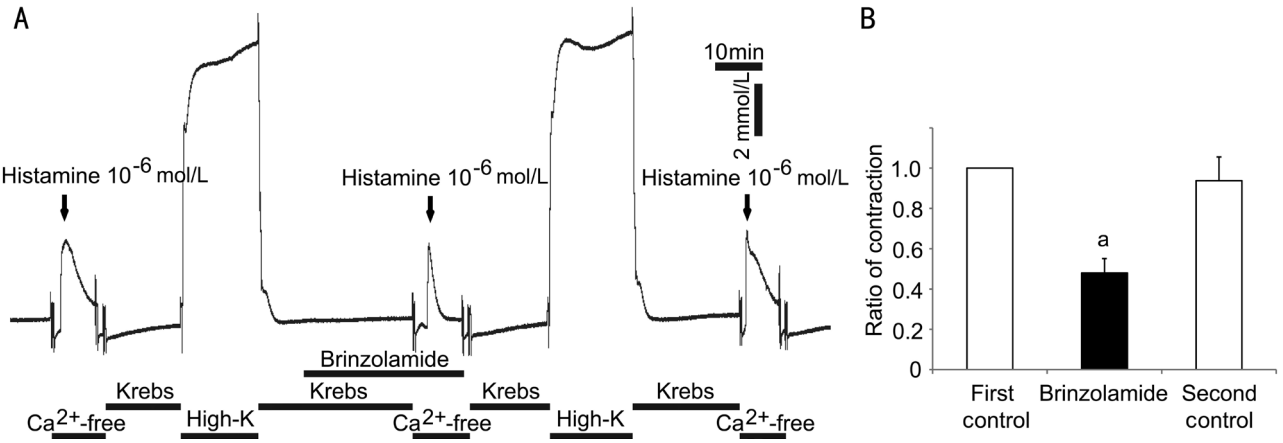

Figure 6 Effect of brinzolamide on histamine-provoked vasoconstrictions in Ca^{2+}-free solution A: A representative contractile curve in response to histamine in Ca^{2+}-free solution with or without brinzolamide (1 mmol/L); B: We compared the areas under the histamine-induced contraction curves, and found that the responses to histamine with brinzolamide were weaker than without brinzolamide. $^{a}P<0.05$.

higher than the left. It is unclear how instillation on the left eye can mediate a decrease in contralateral IOP. However, it is possible that after topical instillation in only the left eye, some of the drug was absorbed into the systemic circulation and reached the right eye through that route.

Brinzolamide not only decreased IOP, but it also augmented ODBF after topical application. This finding is consistent with another study indicating that brinzolamide significantly increased optic nerve head blood flow in Dutch rabbits[12]. Similarly, brinzolamide increased retinal blood flow as measured by confocal scanning laser Doppler flowmetry in glaucoma patients[23]. To achieve its effect on OBF, brinzolamide must reach the retina and optic nerve at therapeutically effective concentrations after topical application. However, Martinez and Sanchez-Salorio[13] suggested that after 5y of treatment, brinzolamide did not augment retrobulbar blood flow when added to timolol, a beta-blocker drug, in glaucoma patients. Whether the contradictory results between their study and our study is related to the different areas of measurements in the eye and/or to the different techniques used for assessment of OBF is unclear. From our results, it is possible that glaucoma patients, when treated with topically administered brinzolamide, may not only benefit from the IOP-lowering effect, but also an increase in OBF.

The brinzolamide-induced increase in OBF may be secondary to IOP reduction or it may represent a primary effect on the ocular vasculature. To differentiate between these two possibilities, we studied the effect of brinzolamide on isolated rabbit ciliary arteries. Brinzolamide at clinically relevant concentrations induced relaxation in arteries that were pre-contracted with high-K solution. These *ex vivo* results proved the hypothesis that brinzolamide possesses a direct vasodilating effect on the ocular vasculature. Supporting the importance of OBF, Gugleta[24] demonstrated that an improvement of OBF by dorzolamide resulted in preservation of visual fields in glaucoma patients. Thus, there may be an important correlation between the brinzolamide-mediated vasodilation of ocular blood vessels and the preservation of visual function in glaucoma.

We have previously demonstrated that dorzolamide directly induced relaxation of isolated rabbit ciliary arteries[25]. In the present study, we compared the effects of brinzolamide, dorzolamide and acetazolamide *ex vivo*. Our results showed that acetazolamide could not induce vasodilation, and dorzolamide-induced vascular relaxation was smaller than that induced by brinzolamide. Martinez and Sanchez-Salorio[13] reported that dorzolamide treatment resulted in a 3.8-fold higher peak systolic velocity and a 6.7-fold higher end diastolic velocity in the central retinal artery than did brinzolamide treatment in humans. However, they used the commercially available eye drops to compare the effects between two drugs. In those preparations, the dosing strength of the dorzolamide drops was 2%, which is twice that of the 1% brinzolamide eye drops. In our study, the vasodilating effects of the CAIs were compared at the same concentrations.

The mechanism underlying the direct vasodilating effect of CAIs is controversial. It has been suggested that dorzolamide-induced vasodilation of intraocular porcine ciliary arteries *ex vivo* depends on NO[26]. However, another study showed that the acetazolamide-induced vasodilation of pial arteriole is sensitive to indomethacin and not on NO release[27]. Moreover, Pickkers *et al*[28] reported that the vasodilator effect of acetazolamide is caused by opening of BK_{Ca} channels on vascular smooth muscle cells. In the present study, we found that the concentration-dependent vasodilating effect of brinzolamide is not mediated by NO, prostacyclin, or opening of BK_{Ca} channels.

We further investigated the relationship between brinzolamide-induced vasodilation and Ca^{2+} release from intracellular calcium stores. Histamine provokes smooth muscle contractions

using Ca^{2+} both entry from extracellular space and release from intracellular Ca^{2+} storages[29]. In Ca^{2+}-free solutions, histamine induces contractions that are mediated by the release of calcium from sarcoplasmic reticulum[30]. We incubated the arterial segments in Ca^{2+}-free solution and found that brinzolamide suppressed histamine-induced contractions. It demonstrated brinzolamide-induced vasodilation is mediated by suppression of Ca^{2+} release from intracellular calcium stores.

Taken together, our results show that brinzolamide decreased IOP and increased OBF. The direct vasodilatory effect of brizolamide is mediated by suppression of Ca^{2+} release from intracellular calcium stores. This effect of brinzolamide on OBF may enhance its beneficial action in preserving visual field in glaucoma patients.

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Conflicts of Interest: Dong YR, None; **Huang SW,** None; **Cui JZ,** None; **Yoshitomi T,** None.

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