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Netarsudil 0.02% Alters Episcleral Venous Flowrates: A Clinical Trial Using Erythrocyte-Mediated Angiography

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Objective: To characterize the effect of netarsudil 0.02% on episcleral blood flow in treatment-naive glaucoma suspect or ocular hypertension subjects.

Design: Prospective, unmasked, single-arm cohort study.

Participants: Ten treatment-naive patients with a diagnosis of glaucoma suspect or ocular hypertension.

Methods: Erythrocyte-mediated angiography (EMA) was used to measure episcleral erythrocyte velocity, vessel diameter, and blood flow at baseline before treatment, 1 hour after drop instillation (T1), 1 to 2 weeks after daily netarsudil 0.02% drop use (T2), and 1 hour after drop instillation at the 1-to-2-week time point (T3). Intraocular pressure (IOP) and blood pressure were measured at each visit.

Main Outcome Measures: Change in episcleral venous erythrocyte velocity, diameter, and blood flow between time points analyzed using generalized estimating equation models.

Results: Of the 18 eligible study eyes of 10 enrolled treatment-naive subjects, baseline IOP was 16.8 ± 3.6 mmHg (mean ± standard deviation), which significantly decreased to 13.9 ± 4.2 mmHg at T1, 12.6 ± 4.1 mmHg at T2, and 11.8 ± 4.7 mmHg at T3 ($P < 0.05$ at each time point compared with baseline). Episcleral vessels averaged 61.3 ± 5.3 μm in diameter at baseline which increased significantly at all posttreatment time points (78.0 ± 6.6, 74.0 ± 5.2, 76.9 ± 6.9 μm, respectively; mean ± standard deviation, $P < 0.05$ for each time point). Episcleral venous flowrates were 0.40 ± 0.22 uL/minute (mean ± standard deviation) at baseline, which increased significantly to 0.69 ± 0.45 uL/min at T1 ($P = 0.01$), did not significantly differ at T2 (0.38 ± 0.30 uL/minute), and increased significantly to 0.54 ± 0.32 uL/minute at T3 ($P < 0.05$ compared with baseline and T2).

Conclusions: Netarsudil causes episcleral venous dilation at all time points and resulting increases in episcleral venous flowrates 1 hour after drop instillation. Increased episcleral venous flow, associated with decreased episcleral venous pressure, may result in lowered IOP.

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Glaucoma is a leading cause of blindness worldwide, and reduction of intraocular pressure (IOP) is currently the only way to slow or stop progression of the disease. Medical therapy remains the mainstay of treatment with numerous formulations available from 6 major classes of topical medications.[1,2] These medications have multiple effects and target primarily aqueous production or outflow.

Netarsudil is a relatively new topical Rho kinase inhibitor and norepinephrine transporter inhibitor which has been shown to lower IOP[3] via increased trabecular outflow facility.[4,5] More recently, it has been shown to decrease episcleral venous pressures (EVPs), indicating that it may be an ideal candidate to assess changes in episcleral flow.

The majority of aqueous humor drains into the episcleral vasculature via the conventional pathway. The regulation of aqueous humor outflow may be impaired in glaucoma, with an array of molecular and structural abnormalities that span from the trabecular meshwork to episcleral veins which may be implicated in the disease.[6–9] As compared to other portions of the aqueous outflow system, including the uveoscleral pathway, episcleral vessels are visible without need for gonioscopy or manipulation of the eye and hence provide a uniquely accessible tool for assessing aqueous humor outflow. Precise quantification of flow in the episcleral veins may further allow for the development of pharmacologic agents that target increased episcleral outflow. Prior attempts at quantifying episcleral flowrates have shown promise but are limited by their reliance on more subjective metrics.[10] Vessel caliber and aqueous column caliber have been used to infer information about aqueous humor outflow, but in isolation this does not provide a metric for dynamic flowrates.[11,12]

Anterior segment erythrocyte-mediated angiography (EMA) is a technique developed to determine absolute episcleral blood flow in live humans.[13] Anterior segment EMA utilizes indocyanine green (ICG)—labeled

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erythrocytes in combination with conventional ICG angiography to simultaneously analyze erythrocyte velocity and vessel diameter, which could provide greater accuracy and precision to measurements of episcleral venous flow when compared with existing modalities. The aim of this study is to investigate short-term and long-term effects of netarsudil 0.02% on episcleral venous flowrates.

Methods

Study Design

This was a prospective, single center, single-arm cohort study determining the effect of netarsudil 0.02% on episcleral venous flow in a cohort of 10 treatment-naive ocular hypertension and glaucoma suspect subjects (http://www.clinicaltrials.gov, NCT04401982).

Participants

Human subjects were recruited from the Department of Ophthalmology and Visual Sciences at the University of Maryland, Baltimore from March 2021 to February 2022. Institutional review board approval was obtained. This study was carried out in accordance with the Declaration of Helsinki, and informed consent was obtained from all human subjects prior to enrollment. To be eligible for inclusion, subjects had to be $\geq$18 years of age, have open angles on gonioscopy, have clinical signs of ocular hypertension or glaucoma suspect (i.e., suspicious optic nerve head, suspicious retinal nerve fiber layer, or elevated IOP $>$21 mmHg associated with normal optic disc, retinal nerve fiber layer, and visual field) per the Preferred Practice Patterns of the American Academy of Ophthalmology,[14] and be treatment naive to topical IOP-lowering agents. Subjects were excluded from participation if they had secondary glaucoma, prior intraocular surgery other than uncomplicated cataract surgery, moderate or severe visual field deficits as per Hodapp-Anderson-Parish criteria, if they had a known allergy to ICG, iodine, or shellfish, were pregnant or nursing, had significant liver disease or uremia, or were participating in any other investigational drug study.

Human Erythrocyte Preparation

A description of erythrocyte preparation in EMA is detailed in prior work and is summarized here.[15] Approximately 34 mL of blood was drawn from each participant, and erythrocytes were isolated from whole blood for encapsulation with ICG dye. The osmotic shock method, as described by Flower et al, was used to load the human erythrocytes with ICG.[16] The resulting ICG-labeled erythrocytes were prepared for autologous intravenous injection in a sterile fashion.

Human In Vivo Imaging

Subjects underwent 4 imaging sessions in 2 scheduled study visits. Imaging took place at baseline (T0), 1 hour after drop instillation (T1), 1 to 2 weeks after once daily netarsudil usage (T2), and 1 hour after drop instillation at the 1-to-2-week time point (T3). Both visits followed the same imaging protocol and occurred at a similar time of day to account for potential diurnal rhythms associated with aqueous outflow or IOP.[17] We surveyed episcleral veins using EMA to determine erythrocyte velocity and conventional liquid ICG for vessel caliber. These veins were identified as thin-walled straight vessels visibly deep to the fine mobile conjunctival blood vessels with erythrocyte flow moving away from the limbus at baseline. The patient was asked to blink in order to differentiate episcleral vessels from conjunctival vessels, as the former do not move upon blinking. An eyelid speculum was not used for imaging. Baseline infrared autofluorescence images were taken before injecting boluses of ICG and fluorescent erythrocytes, confirming the lack of autofluorescence prior to imaging. Up to a 1.4 mL bolus of autologous ICG-loaded erythrocytes were injected intravenously followed by a 10 mL bolus of saline flush via intravenous access established by a trained nurse immediately prior to imaging (Fig 1). Cells remained visible for the duration of the imaging session. Zero point six mL of ICG was injected immediately before imaging to measure vessel diameter. Immediately after the erythrocytes were injected and $\geq$1 cardiac cycle had passed, angiograms were obtained with an Heidelberg retinal angiograph 2 (Heidelberg Engineering GmbH) using a 15-degree horizontal x 7.5-degree vertical field of view taken at 24.6 frames per second. Ten 60-second angiograms were taken in total for each eligible eye.

Subjects' blood pressure, heart rate, respiratory rate, temperature, and oxygen saturation were monitored using standard clinical instruments during each imaging session with a Carescape B450 (GE Healthcare). Demographic data and medical history including medications were also recorded. Race was self-reported by subjects.

To obtain scale values, pictures were taken with a Lindstrom roller with a known measured width. This was confirmed with an algorithm provided by Heidelberg Engineering accounting for the focus and distance from the camera.

Experimental Protocols

Repeatability of Multiple Graders

To assess the repeatability of multiple graders, 2 angiogram sequences were used to determine the intraclass correlation coefficient. Erythrocytes were tracked by 3 graders (V.C., J.P., and S.C.).

Intrasession Repeatability

To assess intrasession repeatability of EMA measures episcleral flowrates, the subjects had multiple angiograms (imaging sequences) acquired at each time point (T0−T3). Ten 60-second angiograms were taken in total for each eligible eye, each angiogram with 246 frames at 24.6 frames per second. Angiograms with the greatest number of trackable cells were chosen for analysis. Coefficient of variation (CV) was used to determine intravisit variability in overall erythrocyte velocity measurements for each session that had $\geq$2 tracked sequences.

Assessing IOP and Mean Ocular Perfusion Pressure Changes on Erythrocyte Velocity Measurements

Intraocular pressure was measured with Goldmann applanation tonometry by a trained ophthalmologist (O.J.S.). If the patient had tight eyelids, deep-set eyes, or squeezing affecting the reliability of the eye pressure, the iCare IC100 tonometer (Icare USA, Inc) was used. One measurement was taken at each time point. The same method was used at baseline and at each subsequent time point. Mean ocular perfusion pressure was calculated for each condition using the measured IOP, systolic blood pressure, and diastolic blood pressure.[18]

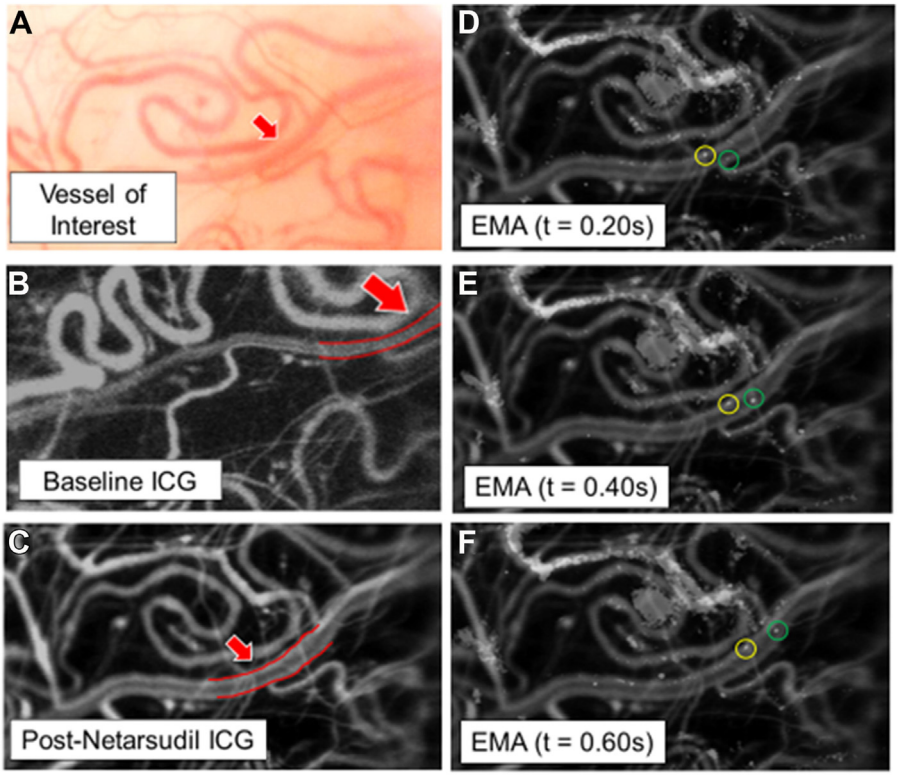

Figure 1. Representation of vessel of interest. Episcleral vessel shown in color photography (**A**), the red arrow indicates the segment of the vessel of interest at baseline (**B**), and after netarsudil (**C**). The yellow and green circles identify 2 tracked cells in successive frames (**D**–**F**). EMA = erythrocyte-mediated angiography; ICG = indocyanine green.

Image Analysis

Vessel inclusion criteria only allowed for episcleral vessels that did not move after blinks and were free of overlying conjunctival vessels. Vessels that failed to meet inclusion criteria were excluded from tracking and analysis. To correct for eye motion, images were registered by aligning the full angiogram sequence to a selected reference image, and a previously validated semiautomated MATLAB (MathWorks version R2022a) script was used to perform spatial domain image registration.[15] Episcleral veins were selected for analysis if the same vein was visible at all time points. The tracked segment of the vein was the same across all time points. Individual erythrocytes were tracked manually within episcleral veins by 3 graders (S.K., M.C., and G.C.). Erythrocyte velocity was defined as the vessel distance traveled by an erythrocyte between 2 adjacent frames divided by the time elapsed between frames. A MATLAB custom tracking software was used to determine tracked cell velocities. In the case of acquisition of multiple imaging sequences in 1 session, the angiograms with the greatest number of trackable cells were chosen after image registration and erythrocyte tracking.

Image Analysis for Vessel Diameters

Vessel diameter was measured using the Automated Retinal Image Analyzer program.[19] Vessels were clearly outlined after injection of ICG boluses. Diameter values were collected from 5 in-focus imaging sequences, 5 frames apart to measure changes in diameter over the cardiac cycle.[13] Vessel segments for diameter values matched the velocity path of the tracked erythrocytes. Scale values were obtained by averaging the vessel caliber from 5 images.

Flow Calculation

Episcleral venous flowrates were calculated assuming a circular cross-sectional area within a vessel of interest as described previously,[13] allowing for the calculation of flowrate from vessel diameter and erythrocyte velocity at each condition (Fig 2).

Statistical Analysis

To account for multiple observations per individual, generalized estimating equations were used to assess the effect of netarsudil on erythrocyte velocity, vessel diameter, and flowrates. All statistical analysis was completed on SPSS Statistics version 27.0.1.0 (IBM).

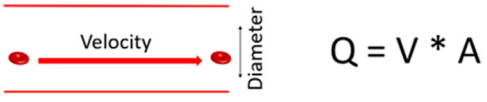

Figure 2. Illustration of velocity and diameter as it relates to flow calculations. Erythrocyte velocities multiplied by cross-sectional area of vessels result in flowrates. A = cross-sectional area; Q = flow; V = velocity.

Table 1. Subject Demographics and Baseline Characteristics

Characteristic	
Subjects, n	10
Eyes, n	18
Diagnosis by number of eyes	
Ocular hypertension, n (%)	1 (6)
Glaucoma suspect, n (%)	17 (94)
Age (yrs)	58.1 ± 13.4
Sex, n (%)	
Male	5 (50)
Female	5 (50)
Race/ethnicity, n (%)	
African American	3 (30)
Asian	2 (20)
White	5 (50)
Diabetes mellitus, n (%)	3 (33)
Hypertension, n (%)	6 (60)
Baseline IOP (mmHg)	16.8 ± 3.6
Baseline MAP (mmHg)	85.6 ± 10.8
Baseline MOPP (mmHg)	40.1 ± 7.2

IOP = intraocular pressure; MAP = mean arterial pressure; MOPP = mean ocular perfusion pressure.
Data are presented as mean ± standard deviation for continuous variables or as number (%) for categorical variables.

Results

A total of 18 eyes from 10 treatment-naive ocular hypertensive or glaucoma suspect subjects were enrolled for this study. One eye had a diagnosis of ocular hypertension while 17 eyes had a diagnosis of glaucoma suspect. Two subjects each had 1 eye ineligible for inclusion in the study due to past history of ocular trauma. No subjects withdrew from the study, and there were no adverse events. The demographic characteristics of the participants are summarized in Table 1.

Fifteen vessels from 13 eyes of 9 subjects were included in the vessel analysis. The first subject was excluded from this analysis due to the inability to track cells at baseline condition due to excessive eye motion, resulting in poor quality angiograms and the inability to identify episcleral veins at baseline. Five eyes of the 18 enrolled eyes did not have vessels imaged across all time points, and thus were not included in the analysis. Of the 13 remaining eyes, 1 or 2 vessels were analyzed for each eye. An average of 70.6 velocity measurements were taken for each episcleral vein (range from 17 to 388).

Mean velocity, diameter, and venous flowrates for all tracked vessels were quantified at baseline and at the 3 post-treatment time points (Table 2). The relationship between velocity, diameter, and flow is shown graphically in Figure 3. Average IOP (mean ± standard deviation) was 16.8 ± 3.6 mmHg at baseline, 13.9 ± 4.2 mmHg at time point 1 ($P = 0.01$ compared with baseline), 12.6 ± 4.1 mmHg at time point 2 ($P = 0.0004$ compared with baseline), and 11.8 ± 4.7 mmHg at time point 3 ($P < 0.001$ compared with baseline, $P = 0.18$ compared with T2).

The average episcleral venous velocity did not change 1 hour after netarsudil ($P = 0.70$) but was significantly lower after 1 week when compared with baseline velocity ($P < 0.01$). The average velocity then increased significantly after final drop instillation during the second visit (time point 3 as compared with time point 2; $P < 0.01$). Episcleral vessel diameter measurements remained significantly elevated at all time points when compared with baseline. Flowrates increased significantly from baseline 1 hour after initial drop instillation ($P < 0.01$), but returned to baseline rates at time point 2, prior to the final drop instillation. At time point 3, the episcleral flowrate increased compared with time point 2, 1 hour prior ($P = 0.03$).

Reproducibility of Episcleral EMA Blood Flow Measurements

Among different graders, the intraclass correlation coefficient for erythrocyte velocity measures was 0.986. The CV was calculated with respect to the variation between multiple measures of the same vessel. Table 3 lists the average CV across vessels that were tracked in ≥2 angiogram sequences for velocity measurements and ≥5 tracked diameter measurements for vessel diameter measurements. The CV for velocity measurements ranged from 0.002 to 0.195 across all time points and for vessel diameter, ranged from 0.02 to 0.05 across all time points.

Discussion

We present the first study to use EMA to assess both the baseline characteristics of episcleral venous flowrates as

Table 2. Mean Velocity, Diameter, and Flow at Baseline and after Netarsudil Treatment

	Velocity (mm/s) (n = 15)	Diameter (μm) (n = 15)	Flow (uL/min) (n = 15)
Baseline	2.23 ± 0.96	61.3 ± 5.3	0.40 ± 0.22
Time point 1	2.34 ± 1.19	78.0 ± 6.6*	0.69 ± 0.45*
Time point 2	1.40 ± 0.88*	74.0 ± 5.2*	0.38 ± 0.30
Time point 3	1.97 ± 1.04†	76.9 ± 6.9*	0.54 ± 0.32*,†

Baseline = initial measurement prior to treatment, time point 1 = 1 hr after drop instillation with 1 drop of netarsudil 0.02% (T1), time point 2 = 1 to 2 wks after daily netarsudil 0.02% drop use (T2), time point 3 = 1 hr after drop instillation at the 1 to 2 wk time point (T3). Generalized estimating equation was used for statistical analysis.
*Denotes statistical significance at $P < 0.05$ when compared with the baseline time point.
†Denotes statistical significance at $P < 0.05$ for comparisons of time point 3 to time point 2.

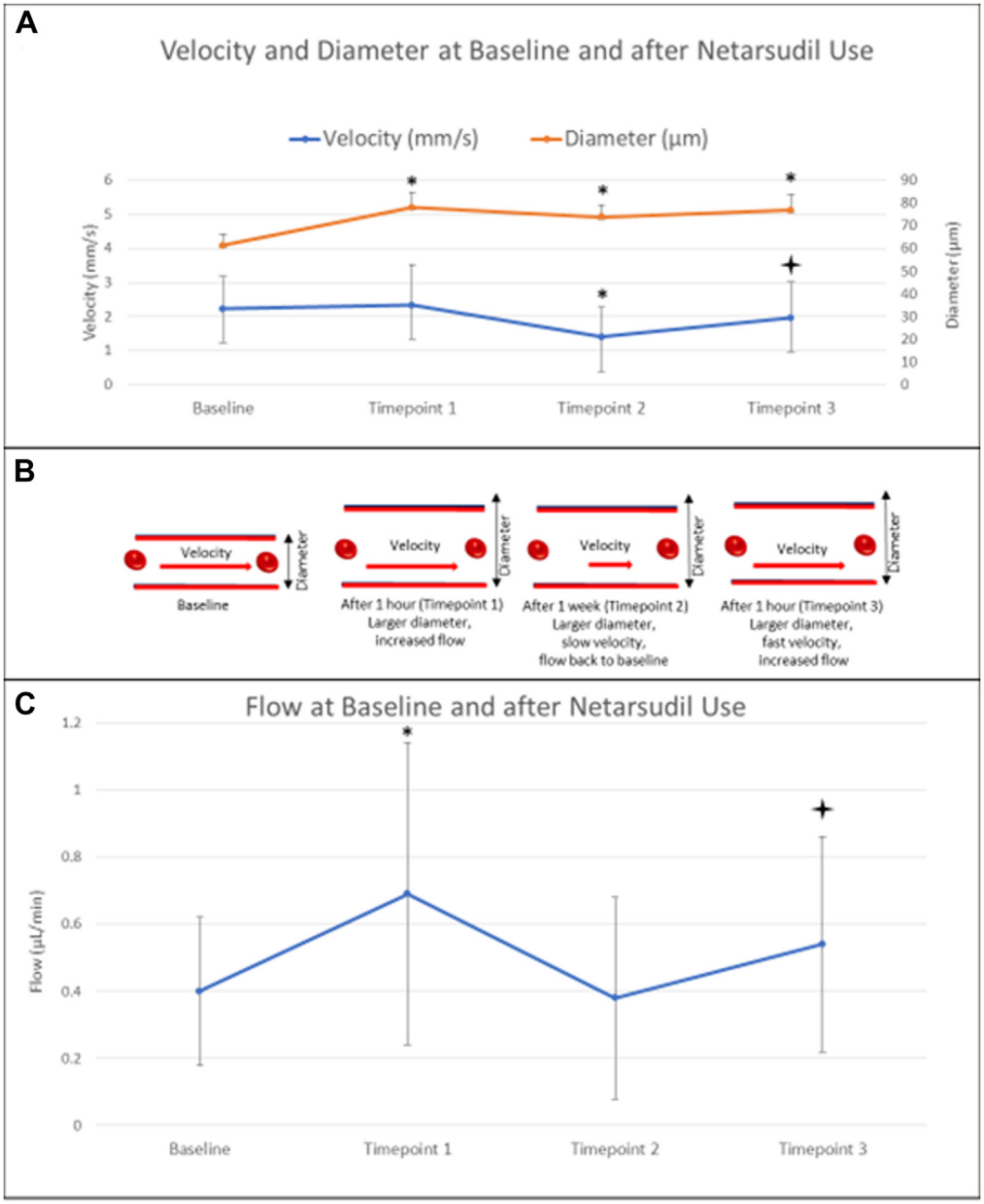

Figure 3. Velocity, diameter, and flow at baseline and after netarsudil use. Velocity and diameter at baseline and after netarsudil use with error bars showing standard deviation (**A**), illustrations of changing velocity, diameter, and flow at 4 time points (**B**), flowrates at baseline and after netarsudil use with error bars showing standard deviation (**C**). Baseline = before treatment, time point 1 = 1 hour after drop instillation (T1), time point 2 = 1 to 2 weeks after daily netarsudil 0.02% drop use (T2), time point 3 = 1 hour after drop instillation at the 1 to 2 week time point (T3). * denotes statistically significant difference from baseline at $P < 0.05$. † denotes a statistically significant difference from time point 2 at $P < 0.05$.

well as the role of netarsudil on episcleral venous flowrates. This study demonstrated that netarsudil has significant short-term effects on episcleral vein diameter as early as 1 hour after instillation as well as increased velocity of ICG-labeled erythrocytes, suggestive of increased episcleral vein flowrates. After 1 week of instillation, the vasodilation of episcleral veins persisted, but velocity significantly decreased; thus, the calculated flowrates were not significantly different from baseline. These results reflect an increase in episcleral outflow resulting in lower IOP after 1 hour, a dynamic change. After 1 week of persistent use, the eye reaches a new steady state at lower IOP with larger episcleral vein diameter, significantly slower erythrocyte velocity, and ultimately episcleral flow that is unchanged from baseline. Furthermore, we showed a low intrasession variability of episcleral flowrates as measured by EMA, potentially paving the way for further studies using this technique.

The persistence of vasodilation that we observed at 1 week is consistent with prior studies of Rho kinase

Table 3. Reproducibility

Measurement	Average CV (Mean ± SD)
Velocity (n = 5 eyes of 4 subjects)	0.12 ± 0.023
Baseline	0.12 ± 0.08
Time point 1	0.20 ± 0.22
Time point 2	0.002 ± 0.18
Time point 3	0.18 ± 0.37
Diameter (n = 7 eyes of 4 subjects)	0.04 ± 0.02
Baseline	0.05 ± 0.02
Time point 1	0.05 ± 0.03
Time point 2	0.02 ± 0.02
Time point 3	0.02 ± 0.01

CV = coefficient of variation; SD = standard deviation.
Average coefficient of variation in all time points for velocity and diameter. Baseline = before treatment, time point 1 = 1 hr after drop instillation (T1), time point 2 = 1 to 2 wks after daily netarsudil 0.02% drop use (T2), time point 3 = 1 hr after drop instillation at the 1 to 2 wk time point (T3).

inhibitors.[20,21] The corresponding change in erythrocyte velocity may ultimately be, as we noted, a new steady state after the initial increase in flow corresponding to the acute lowering of IOP. This is also consistent with data showing a decrease in EVP with netarsudil use.[5] With increased episcleral vein diameter and related decline in EVP, the pressure gradient first results in increased episcleral flowrate, but after reduction of IOP, the flowrates decline to baseline as the pressure gradient has equilibrated. According to Goldmann's modified equation, IOP = [(Fin-Fu)/Ctot] + EVP, where IOP = intraocular pressure, Fin = total aqueous humor inflow rate, Fu = uveoscleral outflow rate, Ctot = total outflow facility (=1/total resistance to aqueous humor outflow), and EVP = episcleral venous pressure; episcleral vein dilation may result in reduction of EVP, thus resulting in a reduction of IOP. Interestingly, at both the first and second visit, flowrates increased 1 hour after drop instillation. This may suggest that even with the return to steady state, Rho kinase promote an acute increase in outflow facility, correlating with the peak of IOP-lowering effect within 24 hours of drop instillation.[22] This may be similar to an increase in aqueous pulse waves that is also seen with IOP-reducing medications from other classes, which had acute flow-increasing effects for ≥1 hour for brimonidine and prostaglandins.[23]

Erythrocyte-mediated angiography offers potential advantages and complementary information to other techniques developed to measure aqueous outflow in live subjects. Aqueous angiography is a technique that involves the use of fluorescein and ICG intracamerally to assess the proximal outflow system.[24] By contrast, EMA, as used in this investigation, assesses the distal outflow system. While the number of episcleral vessels assessed per quadrant was small, the distal outflow pathway is downstream to thousands of collector channels allowing for a more comprehensive assessment of aqueous humor outflow. Hemoglobin video imaging, a noninvasive technique that enhances the contrast of red blood cells for imaging, has been primarily used as a means of assessing changes in cross-sectional area of episcleral veins.[12] Hemoglobin video imaging, while promising, is best at assessing cross-sectional area and does not easily lend itself to analysis of dynamic patterns such as pulsatile flow.[10,25,26] In future work, preoperative assessment of the aqueous outflow of a patient undergoing a minimally invasive glaucoma surgery could allow for improved surgical planning and more targeted placement of devices.[27]

We note the following limitations to this initial study. While the sample size was small, as each individual served as their own control, we did find significant changes in episcleral metrics at each time point. The use of systemic antihypertensive agents by subjects could confound the data through effects on IOP and episcleral venous tone, such as the IOP-lowering effects of systemic beta blocker therapy or potential vasodilation by calcium channel blockers.[28,29] Cell processing and semi-manual analysis are highly reproducible but require training. As part of this investigation, we assessed 1 or 2 major episcleral vessels in each eye repeatedly. This is consistent with the methodology for assessing episcleral pressure, but further work needs to be done to assess the variability of response to netarsudil between vessels, as studies demonstrate variations in aqueous outflow into the episcleral venous system.[30,31] Another factor to account for is that the distal outflow system drains from multiple aqueous veins draining from a larger area. Additionally, episcleral veins were assumed to be cylindrical for flow calculations. A more accurate assessment of episcleral vein cross-sectional area may be achieved using tools such as anterior segment OCT in the future.

In conclusion, this study demonstrates that netarsudil effectively increases episcleral flowrates in the short-term, at 1 hour after drop instillation, and that this leads to a new steady state that persists at 1 week. Anterior segment EMA may be a useful tool to evaluate therapeutic interventions which target aqueous humor outflow, including topical medications and minimally invasive glaucoma surgery procedures, ultimately leading to more effective lowering of IOP for patients with glaucoma.

Footnotes and Disclosures

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HUMAN SUBJECTS: Human subjects were included in this study. Institutional Review Board (University of Maryland, Baltimore) approval was obtained. This study was carried out in accordance with the Declaration of Helsinki, and informed consent was obtained from all human subjects prior to enrollment.

No animal subjects were included in this study.

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Author Contributions:

Conception and design: Saeedi, Magder

Analysis and interpretation: Saeedi, Magder, Park, Cruz, Damani, Mayo, Kim, Chen, Chen, Pottenburgh

Data collection: Chen, Pottenburgh, Chen, Kim, Mayo, Damani, Cruz, Park, Im

Obtained funding: Saeedi

Overall responsibility: Saeedi

Abbreviations and Acronyms:
CV = coefficient of variation; **EMA** = erythrocyte-mediated angiography; **EVP** = episcleral venous pressure; **ICG** = indocyanine green; **IOP** = intraocular pressure.

Keywords:
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