

Full length article

Visualization of conventional outflow tissue responses to netarsudil in living mouse eyes

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ABSTRACT

Visual impairment due to glaucoma currently impacts 70 million people worldwide. While disease progression can be slowed or stopped with effective lowering of intraocular pressure, current medical treatments are often inadequate. Fortunately, three new classes of therapeutics that target the diseased conventional outflow tissue responsible for ocular hypertension are in the final stages of human testing. The rho kinase inhibitors have proven particularly efficacious and additive to current therapies. Unfortunately, non-contact technology that monitors the health of outflow tissue and its response to conventional outflow therapy is not available clinically. Using optical coherence tomographic (OCT) imaging and novel segmentation software, we present the first demonstration of drug effects on conventional outflow tissues in living eyes. Topical netarsudil (formerly AR-13324), a rho kinase/ norepinephrine transporter inhibitor, affected both proximal (trabecular meshwork and Schlemm's Canal) and distal portions (intrasceral vessels) of the mouse conventional outflow tract. Hence, increased perfusion of outflow tissues was reliably resolved by OCT as widening of the trabecular meshwork and significant increases in cross-sectional area of Schlemm's canal following netarsudil treatment. These changes occurred in conjunction with increased outflow facility, increased speckle variance intensity of outflow vessels, increased tracer deposition in conventional outflow tissues and decreased intraocular pressure. This is the first report using live imaging to show real-time drug effects on conventional outflow tissues and specifically the mechanism of action of netarsudil in mouse eyes. Advancements here pave the way for development of a clinic-friendly OCT platform for monitoring glaucoma therapy.

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1. Introduction

Optical coherence tomography (OCT) is a non-contact imaging technology that provides cross-sectional information, quantitative analysis of ocular tissues and is widely used for imaging both anterior and posterior segments. Twenty years ago the first OCT images of the cornea and anterior segment were visualized (Izatt et al., 1994). Today, anterior segment OCT imaging is a crucial tool in the clinical practice

of ophthalmology, including glaucoma, the leading cause of irreversible blindness worldwide. In glaucoma practices, OCT is primarily used to monitor retinal ganglion cell axon loss, however it is also used anteriorly for angle assessment (Narayanawamy et al., 2010) and evaluating the efficacy of laser procedures (Lee et al., 2011; See et al., 2007). Moreover, OCT is routinely used to appraise tissue responses following minimally invasive glaucoma surgeries (Jung et al., 2015; Qian et al., 2015), as well as to evaluate bleb architecture following trabeculectomy (Mastropasqua et al., 2014).

Major advancements in OCT technology for the anterior eye have resulted in increased reproducibility and accuracy in the assessment of progressive tissue damage (Kagemann et al., 2015; Li et al., 2007) and enhanced penetration of the OCT signal into ocular tissues, including conventional outflow structures (Francis et al., 2012; Kagemann et al., 2014a, 2010). Interest in imaging the

Abbreviations: OCT, optical coherence tomography; SC, Schlemm's canal; TM, trabecular meshwork; C57, C57BL/6; IOP, intraocular pressure; DBG, Dulbecco's phosphate-buffered saline containing 5.5 mM D-glucose; PBS, phosphate buffered saline; NET, norepinephrine transporter

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trabecular meshwork/Schlemm's canal (TM/SC) has increased in recent years since reduced aqueous humor drainage through these tissues is the root cause of ocular hypertension in glaucoma. For example, OCT was used to monitor mechanical responses of the TM to acute intraocular pressure (IOP) elevation in living human eyes. Elevated IOP reduced SC cross-sectional area (Kagemann et al., 2015, 2014b), which was enhanced in the presence tropicamide, a drug that disables the ciliary muscle (Kagemann et al., 2015).

Resolution limitations due to optical penetration constraints of OCT into human and non-human primate eyes, has prompted study of mouse eyes as a surrogate to learn more about conventional outflow behavior. Conventional outflow in mouse eyes is very similar to human eyes in terms of anatomy, physiology and response to a number of conventional outflow drug treatments (Aihara et al., 2003; Bous-sommier-Calleja et al., 2012; Li et al., 2014a; Millar et al., 2011; Overby et al., 2014a, 2014b; Smith et al., 2001). Importantly, unlike many other models, mouse eyes have a true SC, through which ~70% of aqueous humor exits (Bous-sommier-Calleja et al., 2012). In terms of imaging, there are great advantages in using OCT to monitor mouse outflow tissues compared to primate eyes: mouse eyes are small, have a large SC lumen, thin sclera, and can be immobilized under anesthesia during imaging sessions. For example, behavior of TM/SC can be monitored at different IOP levels (Li et al., 2014a, 2014b) and at different disease stages (Li et al., 2014b). Similar to healthy human volunteers (Kagemann et al., 2015), we observed that mouse SC dimensions are reduced in a pressure-dependent manner that is quickly reversible (Li et al., 2014a, 2014b). The mouse eyes also respond to topical cholinergic treatment similar to that of human eyes (Flocks and Zweng, 1957; Li et al., 2014a; Lutjen-Drecoll, 1973), with the ciliary muscle contracting to maintain a patent conventional outflow tract (Li et al., 2014a).

Using our custom-built OCT imaging system for mouse eyes, we set out to monitor the effects of a new class of glaucoma drug, netarsudil (Sturdivant et al., 2016), currently in late-stage human clinical trials. Netarsudil primarily targets cells in the conventional outflow tract, efficiently decreasing IOP in both human (Bacharach et al., 2015; Levy et al., 2015; Lewis et al., 2015) and non-human primate eyes (Wang et al., 2015). In addition, netarsudil has been shown to increase outflow facility in non-human primate eyes (Wang et al., 2015) and to decrease episcleral venous pressure in rabbit eyes (Kiel and Kopczynski, 2015). In the present study, we found that netarsudil lowered IOP, increased outflow facility and increased tracer deposition in the trabecular meshwork of mouse eyes. More importantly, using our newly developed OCT imaging technologies, we discovered a novel action of the drug on the living mouse outflow tissues. We observed that netarsudil induced widening of the opening to the trabecular meshwork and increases the cross-sectional area of SC without pupillary constriction.

2. Materials and methods

2.1. Animals

Mice were handled in accordance with animal care and use guidelines of Duke University and in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 (C57) and CD1 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA), bred/housed in clear cages and kept in housing rooms at 21 °C with a 12 h: 12 h light-dark cycle. Mice were examined at ages between 2 and 5 months old.

2.2. Intraocular pressure measurement

The right eyes from age and gender-matched C57 and CD1 sedated mice (5 mice per group) were given either 10 µl 0.04% topical netarsudil mesylate (from here on referred to as netarsudil) or 10 µl placebo (CF324-01) eye drop once/day for five consecutive days. IOP of both eyes was measured prior to eye drop administration using rebound tonometry (TonoLab) between 1 and 4 pm daily (Li et al., 2014a). Briefly, mice were anesthetized with ketamine (60 mg/kg) and xylazine (6 mg/kg). IOP was immediately measured just as the mice stopped moving (light sleep). Each IOP recorded was the average of six measurements, giving a total of 36 rebounds from the same eye.

2.3. Recovery from provocative IOP elevation

The effects of netarsudil on recovery from IOP spikes in living mice were conducted by analyzing pressure decay curves in paired eyes. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and maintained with ketamine (50 mg/kg at 20–30 min intervals). One microglass needle was back filled with the active metabolite of netarsudil (netarsudil-M1) (100 nM) and the other with vehicle (0.001% DMSO). The two needles were connected to two fluid reservoirs and two pressure transducers (model 142PC01G; Honeywell, Fort Washington, PA). Before the two needles were placed into mouse eyes, the two needle tips were guided and immersed into tear film using micromanipulators. After pressure transducers were zeroed, anterior chambers of both eyes were cannulated simultaneously and the spontaneous IOPs were recorded. Both eyes were perfused with drug or vehicle at 15 mmHg for 30 min, allowing drug or vehicle to reach outflow tissues. The pressure in both eyes was then elevated to 40 mmHg and held for 5 min. Once the IOPs in both eyes were stable, the stopcocks were turned to close off the reservoir and maintain the opening between pressure transducers and eye interiors. Pressure was continuously recorded until returning back to spontaneous IOP. To characterize the decline in pressure over time, the data were fitted with Eq. (1).

$$p = p_{ss} + (p_0 - p_{ss})\exp(-\alpha t) \quad (1)$$

where p_0 is the IOP at the start of the experiment, which was 40 mmHg in our case, and p_{ss} is the IOP when the pressure decay reached a steady state. α is a constant that characterized the rate of pressure decay. The regression analysis yields the values of α and p_{ss} in Eq. (1).

2.4. Outflow facility measurement using iPerfusion system

A system especially designed to simultaneously measure low outflow facilities in paired mouse eyes, the iPerfusion system (Sherwood et al., 2016), was used in the present study. Each freshly enucleated mouse eye was mounted on a stabilization platform in a perfusion chamber using a small amount of cyanoacrylate glue (Loctite, Westlake Ohio, USA). The perfusion chamber was filled with pre-warmed D-glucose in phosphate-buffered saline (DBG, 5.5 mM) and temperature regulated at 35 °C. A glass microneedle, back filled with either 100 nM netarsudil-M1 or vehicle (0.001% DMSO), was connected to the system and the microneedle was inserted into anterior chamber using a micromanipulator.

Both eyes were perfused at 9 mmHg for 45–60 min to allow acclimatization and deliver the drug to the outflow pathway, followed by 9 sequential pressure steps of 4.5, 6, 7.5, 9, 10.5, 12, 15, 18 and 21 mmHg. Data analysis was carried out as described previously (Sherwood et al., 2016). Briefly, a non-linear flow-pressure model was used to account for the pressure dependence of

outflow facility in mice, and the reference facility was analyzed at a reference pressure of 8 mmHg (approximates the physiological pressure drop across the conventional outflow pathway in living mice). A two-tailed paired weighted *t*-test carried out on the log transformed outflow facility data was used to determine whether any observed difference in reference facility between contralateral eyes was statistically significant.

2.5. Microbeads infusion

Two pulled glass microneedles secured onto two micro-manipulators were filled with green fluorescent beads (100 nm, carboxylate-modified FluoSpheres[®], Molecular Probes, 1:750 dilutions) mixed with 100 nM netarsudil-M1 or vehicle (0.001% DMSO) in 1 × DBG (Dulbecco's phosphate-buffered saline, GIBCO, pH 7.3 containing 5.5 mM D-glucose). A microneedle containing netarsudil-M1 was inserted into the anterior chamber of one eye and a microneedle containing vehicle into the contralateral eye. The two needles were connected to two 1 ml syringes via tubing that were locked into a single syringe pump. Both eyes were perfused simultaneously at a rate of 0.167 μ l/min for 1 h resulting in a total of 10 μ l of liquid infused into each anterior chamber. After infusion, needles were withdrawn and mice were maintained for ~1–2 h before euthanizing. Both eyes were collected and bisected at the equator. Lens, iris and ciliary body were removed and anterior segments were flat mounted with corneal epithelium side facing up. The fluorescence images were captured using a 2.5 × lens on a fluorescence microscope (AxioPlan2, Carl Zeiss MicroImaging, Thornwood, NY) using identical settings for both eyes. The automatic computation of the width and intensity was done by a number of image processing operations. The original image (changed to grayscale) was converted to an edge map by applying Canny edge detector (Canny, 1986). The edges from same region get disconnected due to noise. Hence, the edge map was enhanced and disjoint edges were connected by applying a morphological dilation (Serra, 1982) operation using a circular structuring element of size 5. Afterwards, a morphological region filling operation recovered the bounded regions. At this stage, the applied image processing recovered the TM/SC regions along with noisy isolated regions and the central region, since the edge detection and subsequent dilation also enhance noisy pixels as well as the central part of the image (containing high intensity). Hence, two attributes were computed for each segmented region: area (number of pixels) and elongation (major axis length of the best fitted ellipse to the region). Since the desired regions (TM/SC) have large areas and elongated structures, a high threshold on area values and elongation values each keeps only the desired regions while removing unwanted noisy segmentation as well as the central region. After final segmentation, the center of this circular region was located, and the width was computed at 1/10th of each degree to be more precise. Average of the intensity of all pixels with identical angles to the center, represents the mean intensity at the corresponding width.

2.6. Optical coherence tomographic imaging

In vivo imaging was performed utilizing an Envisu R2200 high-resolution spectral domain (SD)-OCT system (Biophtigen Inc., Research Triangle Park, NC). This 840 nm SD-OCT system utilized a customized 180 nm Superlum Broadlighter source providing two micron axial resolution and a 12 mm telecentric lens bore for anterior segment OCT imaging. For experiments, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and continued with ketamine (60 mg/kg) to maintain anesthesia. The OCT probe was aimed at the inferior lateral limbus and the image was centered and focused at SC. A sequence of repeated OCT B-scans (each with 1000 A-scans spanning 0.5 mm in lateral

length) from spatially close positions was captured, registered, and averaged to create a high signal-to-noise-ratio image from the iridocorneal angle region of each animal. The images from the same SC location captured before and an average of 45 min after 10 μ l 0.04% netarsudil or placebo control (CF324-01) were compared.

For experiments in which IOP was controlled, iridectomy was conducted on mice as previously described (Li et al., 2014a, 2014b) 2–3 weeks before OCT imaging. Iridectomies were performed to avoid artificial enhancement of outflow due to anterior chamber deepening observed with single needle perfusions (Boussommier-Calleja et al., 2015). A pulled glass micro-needle was filled with phosphate buffered saline (PBS) and inserted into anterior chamber of mice. The micro-needles were connected to both a manometric column to adjust IOP and a pressure transducer to continuously monitor IOP levels (using PowerLab software). The OCT probe was aimed at the inferior lateral limbus and the image was centered and focused at SC. While collecting images, mouse eyes were subjected to a series of IOP steps (10, 15, 30 mmHg) by adjusting the height of the fluid reservoir. At each IOP step, a sequence of repeated OCT B-scans (each with 1000 A-scans spanning 0.5 mm in lateral length) from spatially close positions was captured, registered, and averaged to create a high signal-to-noise-ratio image from the iridocorneal angle region of each animal. After IOP was returned to 10 mmHg for 5 min, the eye was treated with a 10 μ l 0.04% netarsudil or placebo control (CF324-01) eye drop. After 30–60 min, the same area of SC was imaged and again subjected to the sequence of pressure steps. As an example, supplemental Fig. 2 contains averaged OCT images and corresponding live videos. Here real-time speckling can be visualized in both SC and scleral vessels in the presence or absence of netarsudil.

2.7. Assessment of OCT images

OCT B-scans of iridocorneal angle tissues were automatically registered using our newly developed software for SC segmentation, called Schlemm I. This version provides the automatic registration and segmentation based on three variables: (i) computation of the visual and statistical cues of temporal mean, (ii) successive frame differences and the speckle variance from the data, followed by (iii) successive gray-level morphological operations to extract the vessel regions reliably from the rest (Huang et al., 2009). Hence, the outcome of Schlemm I contains segmented SC along with several other vessels. Note that this stage of segmentation is important for analysis of vessels in general. With the options provided in the second version of the software, called Schlemm II, SC is manually separated from the other vessels. Manual separation is required since the visual and statistical cues cannot properly differentiate SC from other vessels. SC is easily differentiated from other vessels due to its size and location. In terms of manual control, Schlemm II has options for freehand drawing, erasing and choosing appropriate regions to incorporate in the final segmentation. The important feature for Schlemm II is that the registered B-scans are periodically displayed as live images alongside the averaged image during segmentation, which makes comparison and segmentation much easier. The segmentation results in this study are based on the outputs from this stage. The inter-observer repeatability is also tested based on the outputs of this stage, since the automatic segmentation provides robust output to the point of separating the important vessel structures from the background, but not a complete segmentation of the SC. Hence, the robustness of the final segmentation is assured via a repeatability test.

After the second stage of the segmentation, the background regions are segmented automatically from the foreground since the foreground is already defined in Schlemm II, containing the required vessel structure. Also, the outcome of Schlemm II containing all ves-

sels is still available. Based on these segmented regions, speckle variance regions generated by blood cells or other reflectors in vessels (Hendargo et al., 2013; Huang et al., 2009; Li et al., 2014a; Mariampillai et al., 2008; Poole et al., 2014) were automatically segmented by the third version of the software, Schlemm III, that includes several statistical analysis tests to measure the shape, size, location and variation of speckling in different vessels as well as the SC.

2.8. Reproducibility

To validate application of our new software, we performed two studies to assess interobserver and intraobserver reproducibility. The segmentation of SC was independently performed by two individuals; one reviewed the images without knowing the treatments to assess the interobserver reproducibility. The other observer repeated the measurement one to two months after the first examination to determine intraobserver reproducibility.

2.9. Histology

C57 and CD1 mice were each given one 10 μ l drop of 0.04% netarsudil in one eye and a 10 μ l drop of placebo in the contralateral eye. Fifty minutes after treatment, both eyes were collected, immersed into 4% paraformaldehyde, and kept at 4 °C overnight. The eyes were then bisected and the posterior segments and lenses were removed. The anterior segments were cut into four quadrants and each quadrant was embedded into Epon (Electron Microscopy Sciences, ON, Canada). The blocks were cut into 0.5 μ m semi-thin sections and stained with 1% methylene Blue. The images were captured digitally using light microscopy.

3. Results

3.1. Topical netarsudil decreases intraocular pressure in mice

To test the effects of netarsudil on IOP in young mice (2–4 months old), 10 μ l of 0.04% netarsudil and placebo eye drops were given topically once daily to a single eye of two different cohorts of mice. IOP was measured before eye drops were given and for five consecutive days of treatment. As shown in Fig. 1A, netarsudil significantly

lowered IOP at every time point examined in pigmented C57 mice (range 3.7–6.8 mmHg), with an average IOP-lowering of 5.2 mmHg over the course of the experiment compared to the contralateral untreated eye. Interestingly, netarsudil was not as effective in albino, CD1 mice, significantly lowering IOP an average of 2.2 mmHg (ranging 1.3–2.8 mmHg) (Fig. 1B). IOP in eyes receiving placebo eye drops increased on average by 1.4 mmHg compared to their contralateral untreated eyes in both strains of mice (1.37 ± 0.21 mmHg in C57 and 1.38 ± 0.23 mmHg in CD1).

3.2. Netarsudil improves IOP recovery and outflow facility in mouse eyes

We used two methods, a pressure-decay technique in living eyes and an automated perfusion assay of enucleated mouse eyes, to examine whether reduction of IOP by netarsudil was a result of increased outflow facility. Recovery from provocative IOP elevation over time was evaluated simultaneously in paired eyes of living mice, with one eye perfused with the active metabolite of netarsudil and the other with vehicle. As shown in Fig. 2A, pressure dropped faster in the netarsudil-M1 perfused eyes compared to vehicle control. After fitting the curves to Eq. (1) described in Methods, we found that overall netarsudil-M1 treatment resulted in a significant 22% decrease in p_{ss} and 25.3% increase in α . The decrease in p_{ss} can be caused by a decrease in episcleral venous pressure, aqueous humor secretion, an increase in outflow facility, or the combination of all three factors. While netarsudil has known effects on all three parameters, its primary mechanism of action involves effects on outflow facility, which is likely a dominant factor in determining α .

We next tested netarsudil effects on outflow facility more specifically in a controlled experimental environment, where conventional outflow function was isolated from other physiological parameters that affect IOP such as aqueous humor inflow, uveoscleral outflow and episcleral venous pressure. Using an iPerfusion system (Sherwood et al., 2016), we subjected enucleated mouse eyes to 9 sequential pressure steps (4.5–21 mmHg) and measured flow. A sample flow-pressure plot for a C57 mouse is given in Fig. 3A, showing a higher rate of outflow for a given pressure in the netarsudil-M1 treated eye. Whilst accounting for measurement uncertainty, we observed that treatment with netarsudil-M1 yielded a proportional increase of

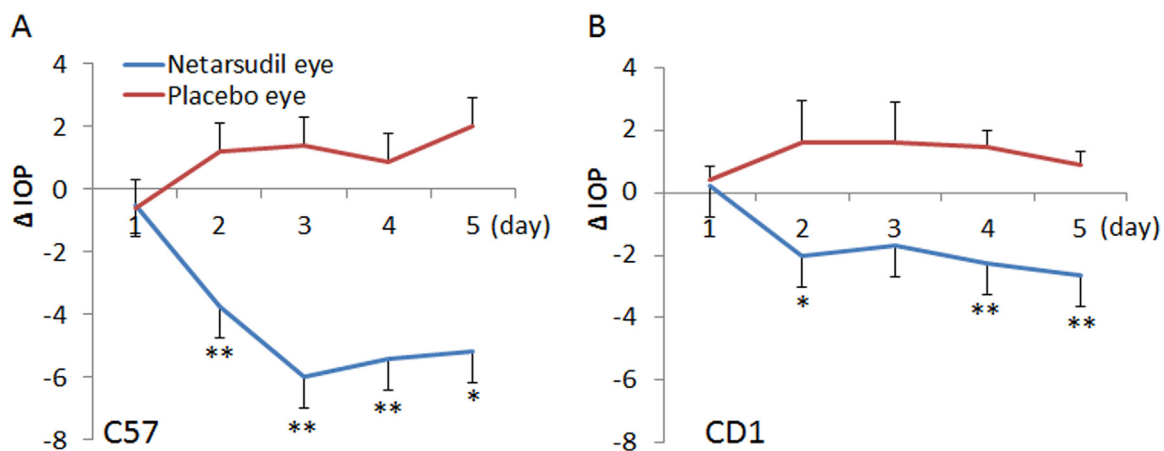


Fig. 1. Netarsudil lowered intraocular pressure (IOP) in both pigmented and nonpigmented mice. Ten week-old C57 and 6–14 week-old CD1 mice were divided into 2 age and gender-matched groups (5 mice/group, 4 groups total). For each strain of mice, one group was given 10 μ l of 0.04% netarsudil topically, and another group received 10 μ l of a placebo (CF324-01) eye drop. All eye drops were applied to right eyes. IOP was measured in both eyes before eye drops were given. The data represent mean $\pm$ S.E.M. of Δ IOP compared between netarsudil and placebo-treated group analyzed by Mann Whitney U test. *, $P < 0.05$; **, $P < 0.01$.

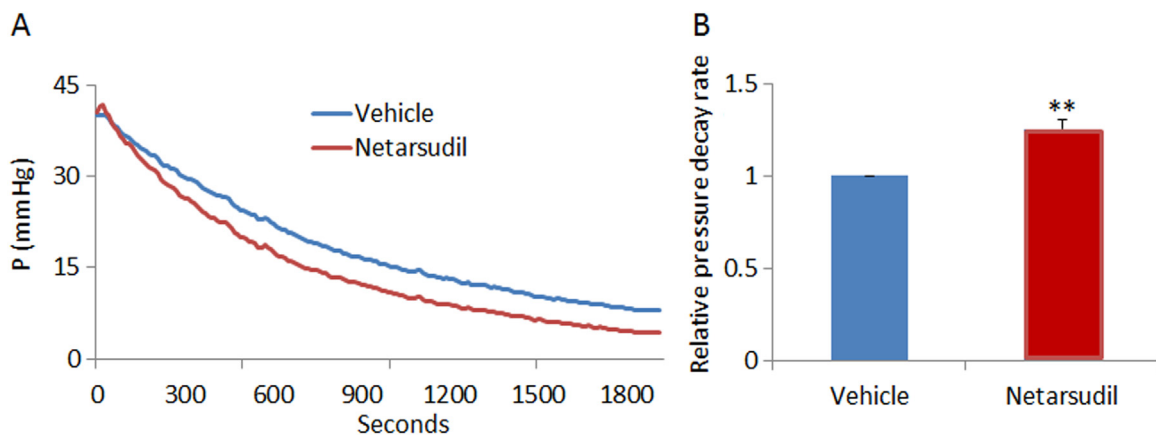


Fig. 2. Netarsudil enhanced IOP recovery in living mouse eyes. Vehicle (0.001% DMSO) or 100 nM netarsudil-M1 was preloaded into one of two perfusion needles and inserted intracamerally into contralateral eyes of living mice. Both eyes were simultaneously exposed to intraocular pressure (IOP) of 15 mmHg, allowing drug/vehicle to enter eyes for 30 min IOP was artificially raised to 40 mmHg in both eyes and held for 5 min using a fluid reservoir. Connection to the fluid reservoir was closed, but remained open to pressure transducers, which monitored IOP in both eyes over time. Panel A. Representative IOP decay curves in paired eyes exposed to vehicle (DMSO) or netarsudil-M1. Panel B. Relative changes in α (a constant characterizing the rate of pressure decay). The data represent mean $\pm$ S.E.M., $N=8$, **, $P < 0.01$ by student t -test.

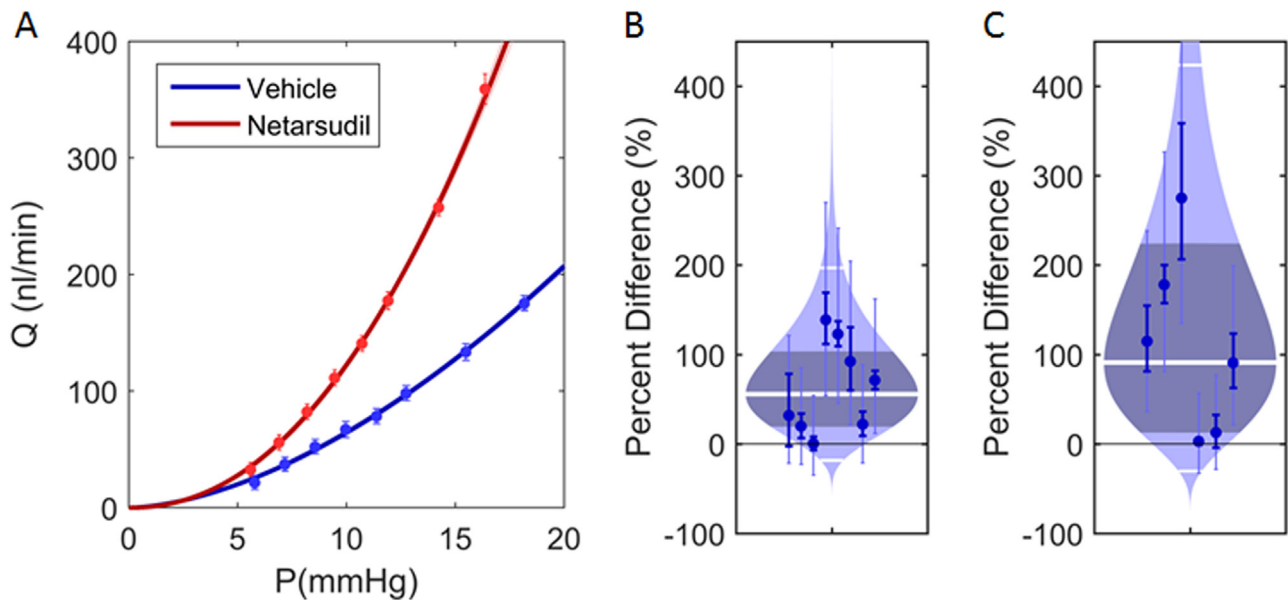


Fig. 3. Netarsudil increased outflow facility in perfused mouse eyes *ex vivo*. Outflow facility measurement was conducted in paired enucleated eyes using an iPerfusion system. The drug (netarsudil) and vehicle (0.001% DMSO) were preloaded in microneedles and perfused for 45–60 min before exposing eyes to 9 sequential pressure steps. Panel A. Representative experiment showing flow rate (Q) vs pressure (P) that was used to calculate outflow facility in a single pair of eyes. Panel B. Cello plot (Sherwood et al., 2016) shows percentage change in facility for each pair of eyes in response to treatment with netarsudil. Dark blue error bars indicate confidence interval from the fitting of the model to the flow-pressure data and lighter error bars include additional uncertainty arising from differences between contralateral eyes of C57BL/6 mice (Sherwood et al., 2016). Shaded region shows estimated lognormal distribution of percentage change in facility. Outer white lines show 2 standard deviations and central lines shows the average increase. Dark central band shows the 95% confidence interval on the mean increase. $N=8$, $P=0.006$ by a paired weighted t -test. Panel C. Cello plot shows percentage change in facility for each pair of eyes in response to treatment with netarsudil for CD1 mice. Lighter error bars assume that the additional uncertainty arising from differences between contralateral eyes in CD1 mice is similar to that of C57 mice. $N=6$, $P=0.025$.

outflow facility over paired controls by 56% (95% confidence interval [19, 104%], $N=8$, $P=0.006$) for C57 mice and 91% (95% confidence interval [12, 225%], $N=6$, $P=0.025$) for CD1 mice (Fig. 3C). The range of treatment effect at two standard deviations was [–1,146%] for C57 mice and [35, 223%] for CD1 mice.

3.3. Netarsudil increases fluorescent tracer deposition in outflow tissues

To test whether netarsudil effects on outflow in living mouse eyes includes modifications in TM/SC flow patterns, fluorescent tracer was perfused simultaneously under constant flow conditions into paired eyes in the presence or absence of netarsudil-M1. As observed

previously (Swaminathan et al., 2013; Zhang et al., 2009), green fluorescent beads in control eyes deposited in outflow tissues in a segmental pattern in both strains of mice, with high and low flow regions marked by high and low fluorescence intensity, respectively (Fig. 4A). In netarsudil-M1 perfused eyes, the fluorescence intensities were clearly increased in both high flow and low flow regions. Quantitative measurements of the fluorescence intensities, showed a significant increase in fluorescence by 2.2-fold in C57 and 1.5-fold in CD1 mouse eyes perfused with netarsudil-M1 compared to vehicle control eyes (Fig. 4B). In order to examine whether increase of fluorescence intensities correspond to the increase of tracer deposition of outflow tissues, the width and areas of the fluorescence in TM/SC were also quantified as shown in Fig. 4B. Significant increase of both

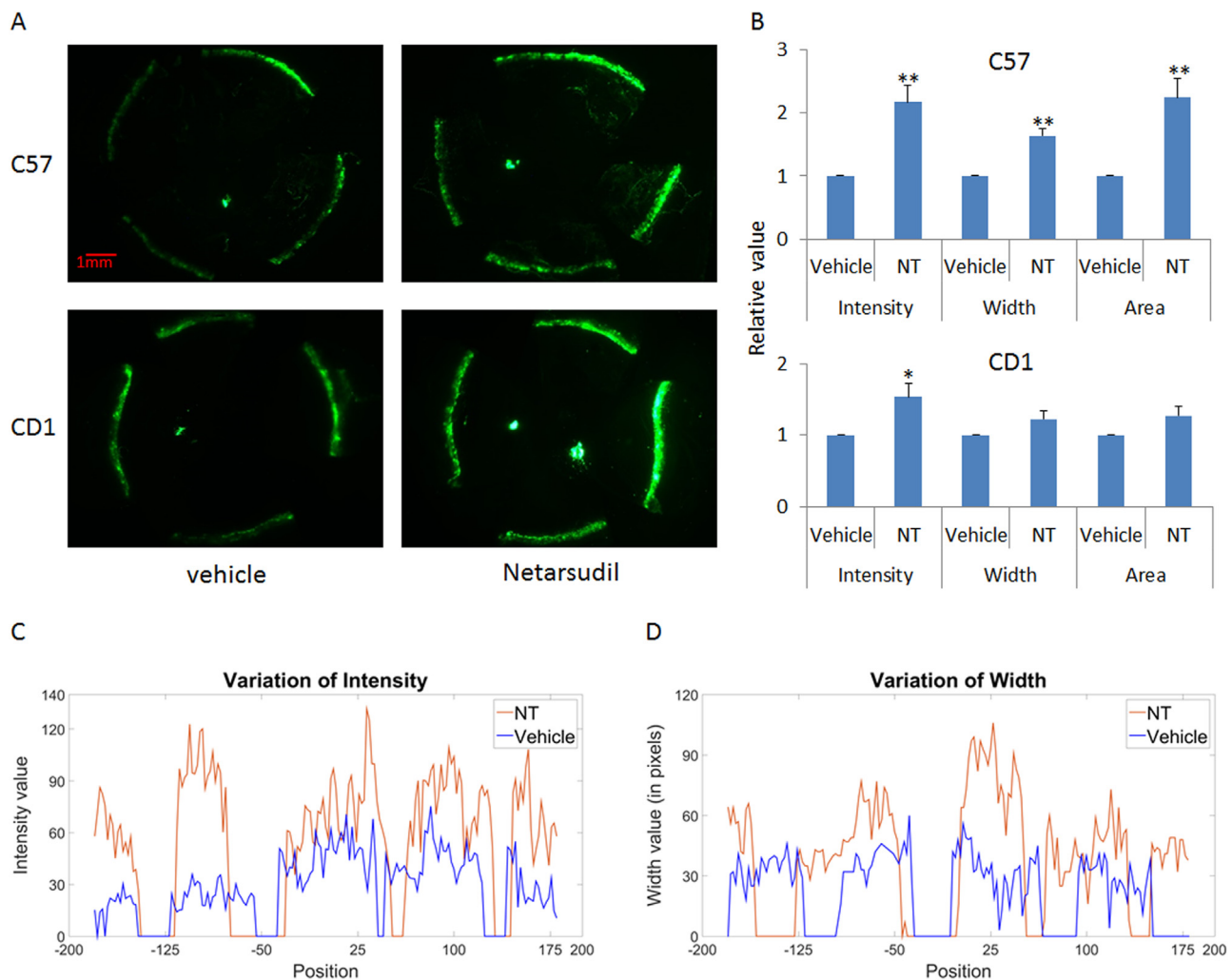


Fig. 4. Enhanced tracer deposition in outflow tissues of living mice subjected to netarsudil treatment. Fluorescent microbeads were loaded into microneedles in the presence or absence of netarsudil-M1 anterior chambers were cannulated and paired eyes simultaneously perfused at a constant flow rate of 0.167 $\mu\text{l}/\text{min}$. Perfusion was stopped after one hour and the mice were maintained for another hour before being euthanized. Shown are flat mounted anterior segments of C57 and CD1 mouse eyes that were visualized using epifluorescence microscopy (panel A). Summary of comparisons between netarsudil-M1-treated (NT) and vehicle (0.001% DMSO)-treated groups are shown in panel B. Fluorescence intensity, the width and area of fluorescence in conventional outflow region were automatically quantified. Data represent mean $\pm$ S.E.M, $N=5$ for both strains of mice. *, $P < 0.05$, **, $P < 0.01$ compared to their contralateral vehicle treated eyes by student t -test. Panels C and D show representative fluorescence intensity and width maps of conventional outflow regions from four quadrants of paired eyes. The variation of mean intensity (of all pixels with identical angles to the center) (panels C) and the variation of width (panels D) are shown with the variation of angle from the center of the circular TM/SC regions (from -180 to 180 degrees). The 0 (180) degree is represented by the positive X-axis.

fluorescence width (1.63-fold) and area (2.24-fold) were found in C57 mouse TM/SC treated with netarsudil-M1. In CD1 mice, both width and area were also slightly increased (1.22- and 1.27-fold), but did not reach statistical significance. Representative fluorescence intensity and width maps for four quadrants of paired eyes are shown in Fig. 4C and D, respectively displaying quantitative spatial differences between placebo and netarsudil-M1 treatments.

3.4. Dynamic changes in conventional outflow tissues of mouse eyes treated with netarsudil visualized by OCT

In order to visualize behavior of conventional outflow tissues treated with netarsudil, we used our custom-build OCT imaging system for live mice. Iridocorneal angle tissues were imaged immediately before, and 30–60 min after topical netarsudil application. A representative series of OCT images of treated and untreated mouse eyes are shown in Fig. 5A, displaying averaged images of 200 B-scan frames with segmentation of SC lumen using Schlemm II software. Alongside the segmented images are speckle variance OCT images showing location of moving reflectors in iridocorneal vessels. Data

illustrate that the SC cross sectional area was significantly increased by 48.2% after topical netarsudil treatment ($P=0.00005$) compared to 11.5% decrease of the SC area after placebo treatment ($P=0.33$, panel B). Coincident with changes in SC lumen area, we also found that netarsudil expanded the opening to the TM as shown in Fig. 5A (red arrow). This OCT finding was confirmed in a subsequent histological study (supplementary Fig. 1), where both C57 and CD1 mice were treated with topical netarsudil or placebo and eyes were processed and analyzed. Consistent with the observation in living animals, TM area was clearly expanded by netarsudil treatment in both strains of mice. While area of scleral vessels did not change with treatment, we did observe that their speckle variance intensity increased by 61.6% ($p=0.03$, Fig. 5 panel C). These changes were comparable to that of SC, whose speckle variance increased by 34.2% after netarsudil treatment. We found there was no effect of netarsudil on pupil diameter in OCT images (data not shown).

We next examined the effects of netarsudil on outflow structures in the presence of increasing IOPs. IOP was controlled by a single needle inserted into the mouse anterior chamber in iridotomized eyes. Similar to naive eyes, when IOP was held at near physiological IOP

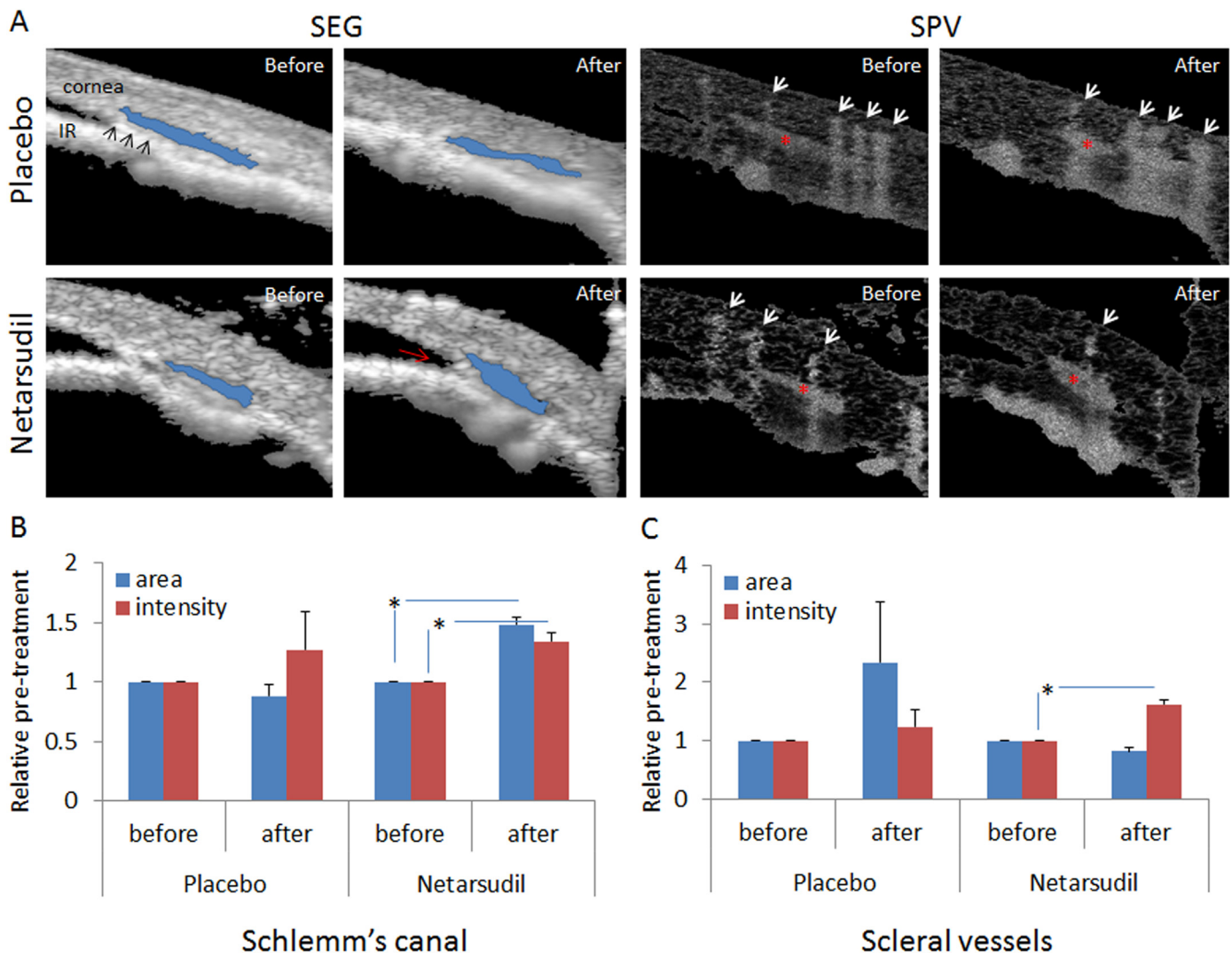


Fig. 5. Netarsudil-induced changes in conventional outflow tissue morphology of living mice visualized by optical coherence tomography (OCT). Panels A shows representative averaged OCT images from 200 B-scans of iridocorneal angles (identical positions) before and 45 min after topical netarsudil or placebo treatment of living C57 mice (IR: iris; black arrows point to TM tissue). Overlaid atop averaged images are segmentations (SEG, blue) of SC using Schlemm II software. Arrow in red, shows expanded TM after netarsudil treatment. The speckle variance images (SPV) of same eyes are shown alongside averaged images. Arrows in white point out scleral vessels conducting aqueous humor outflow and asterisks indicate SC. Panels B and C show quantitative results summarizing effect of treatments on area and speckle variance intensity of SC and scleral vessels conducting aqueous humor outflow using Schlemm III software. The data represent mean $\pm$ S. E. M., N=5 for each treatment. * $P < 0.05$ (student *t*-test).

(10 mmHg) netarsudil significantly increased SC cross sectional area by 32% and 29% in C57 and CD1 mice, respectively (Fig. 6). We observed that SC lumen dimensions were decreased at elevated pressures, which is consistent with our previous observations using manual segmentation (Li et al., 2014a). However, after topical netarsudil treatment SC was significantly less compressed at all IOPs tested. Thus, netarsudil functions to prevent SC collapse as IOP increases. In contrast, placebo drops had no effect on the SC area, whether IOP was elevated or not (Fig. 6B and C).

3.5. Effects of netarsudil on scleral vessel cross-sectional area, flow patterns and speckle variance intensity

We next visualized and analyzed effects of netarsudil on scleral vessels that conduct aqueous humor when IOP was held constant at 10 mmHg. We found that netarsudil changed the flow patterns and speckling intensity in scleral vessels of living C57 and CD1 mice as shown in Fig. 7A. Quantification of total speckle variance in

cross-sections (indicating vessels carrying aqueous humor) and intensities are shown in Fig. 7B and C. The total cross-sectional area of scleral vessels conducting aqueous humor outflow increased from $433.4 \pm 170.1 \mu\text{m}^2$ before netarsudil treatment to $734.9 \pm 243 \mu\text{m}^2$ after treatment in C57 mice ($P=0.037$). Similarly, vessel area increased from $531.3 \pm 147.2 \mu\text{m}^2$ to $965.9 \pm 233.2 \mu\text{m}^2$ in presence of netarsudil in CD1 mice ($P=0.0009$). In vehicle-treated groups, the areas changed from $377.4 \pm 122.4 \mu\text{m}^2$ before placebo to $446.8 \pm 119.3 \mu\text{m}^2$ after placebo in C57 mice ($P=0.46$) and from $559.1 \pm 184.9 \mu\text{m}^2$ to $474.4 \pm 186.5 \mu\text{m}^2$ after placebo in CD1 mice ($P=0.79$). The relative speckle variance intensity within single vessels also increased after netarsudil treatment, suggesting an increase in fluid flow. In C57 and CD1 mice, speckle variance intensity increased by 72.9% ($P=0.023$) and 27% ($P=0.43$), respectively. There was no difference after placebo treatment in either C57 mice or CD1 mice (Fig. 7B and C). These data are similar to experiments conducted in naive eyes (not cannulated) whereby speckle intensity increased, but interestingly cross-sectional area remained the same (Fig. 5C).

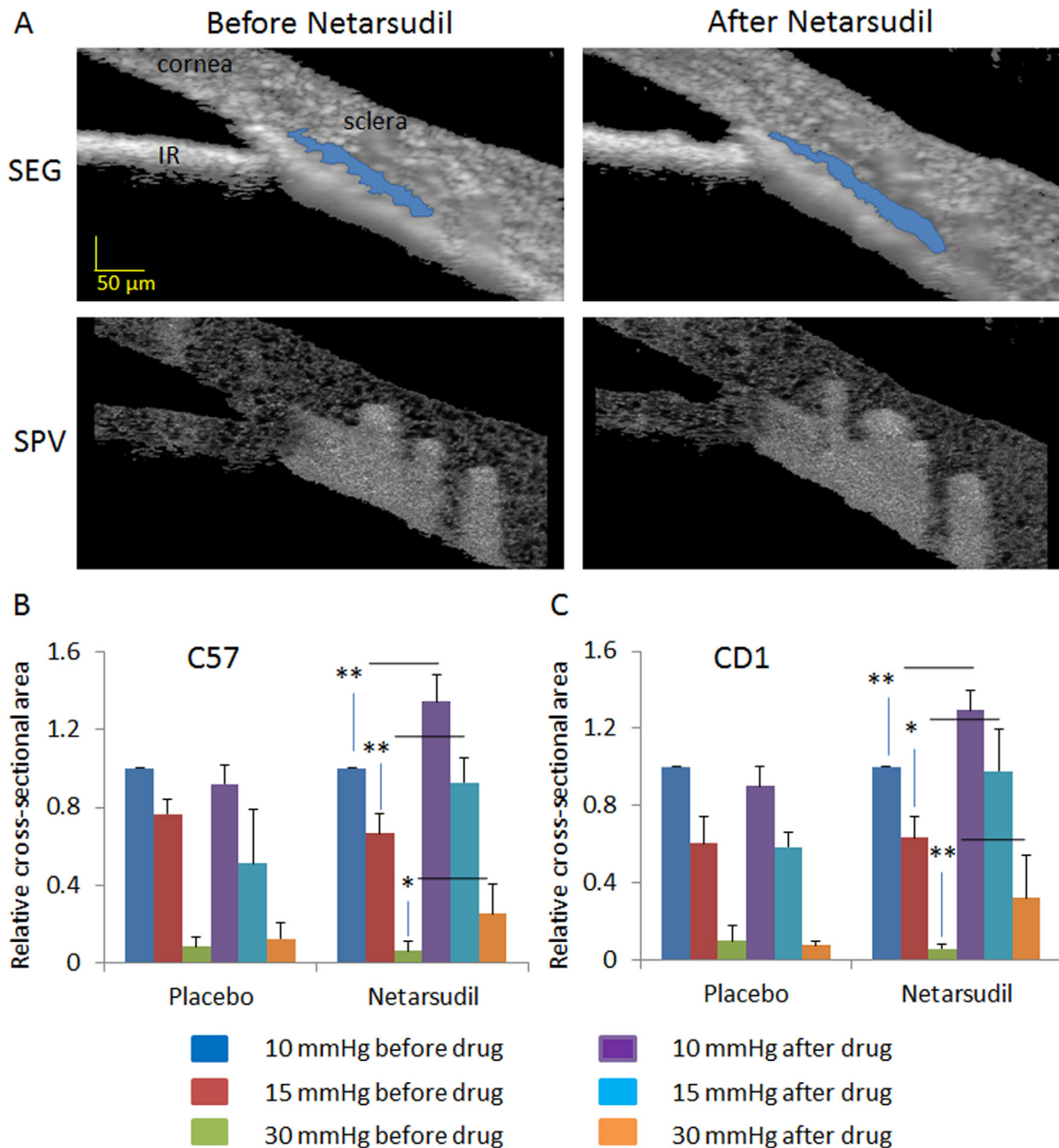


Fig. 6. Netarsudil increased cross-sectional area of Schlemm's canal (SC) lumen in living mice with elevated intraocular pressure (IOP) visualized by optical coherence tomography (OCT). Panel A shows representative averaged images from 200 B-scans of iridocorneal angles (identical positions) of a live C57 mouse before and 40 min after netarsudil treatment, with IOP held at 10 mmHg. Semi-automatic segmentation (SEG) of SC is shown overlaid in blue. Bottom panels show OCT speckle variance (SPV) images. (IR = iris). Panels B and C show results of quantitative SC segmentation from OCT images analyzed using Schlemm II software. C57 and CD1 mouse eyes were imaged at the same location by OCT with a glass needle inserted into anterior chamber to control intraocular pressure sequentially at 10, 15, and 30 mmHg before and 30–60 min after treatment (topical netarsudil or placebo eye drops). The data show SC area ($\pm$ S. D.) relative to the area of SC measured just prior to treatment at the starting IOP of 10 mmHg. Eleven mice were examined in this set of experiments. * $P < 0.05$; ** $P < 0.01$ (Mann Whitney U test).

4. Discussion

The results of this study provide further evidence that mice are a useful model to study drugs that target conventional outflow function. Netarsudil has previously been shown to lower IOP in humans (Bacharach et al., 2015; Lewis et al., 2015; Wang et al., 2015) and to increase outflow facility in non-human primates (Wang et al., 2015). We demonstrated that netarsudil also effectively lowers IOP and increases outflow facility in both albino and pigmented mice. To better understand its mechanism of action, we probed for real time drug effects on conventional outflow dynamics using OCT of living mice. We observed that netarsudil treatment expanded the opening to conventional outflow tissues,

increased the cross-sectional area of SC, and prevented its collapse at elevated IOPs. Netarsudil also increased speckle variance intensity in both SC and scleral vessels that conduct aqueous humor outflow. These changes were reproducibly quantified using our newly developed segmentation software. Taken together, these results demonstrate that netarsudil robustly lowers IOP by affecting both proximal and distal portions of the conventional outflow tract.

Contractile tone of conventional outflow tissue determines outflow facility (Dismuke et al., 2014; Zhou et al., 2012). Studies have shown that TM cells possess smooth muscle-like properties, and that drugs relaxing TM cells increase outflow while drugs contracting TM cells decrease outflow (McKee et al., 2011; Rao et al., 2001; Rosenthal et al.,

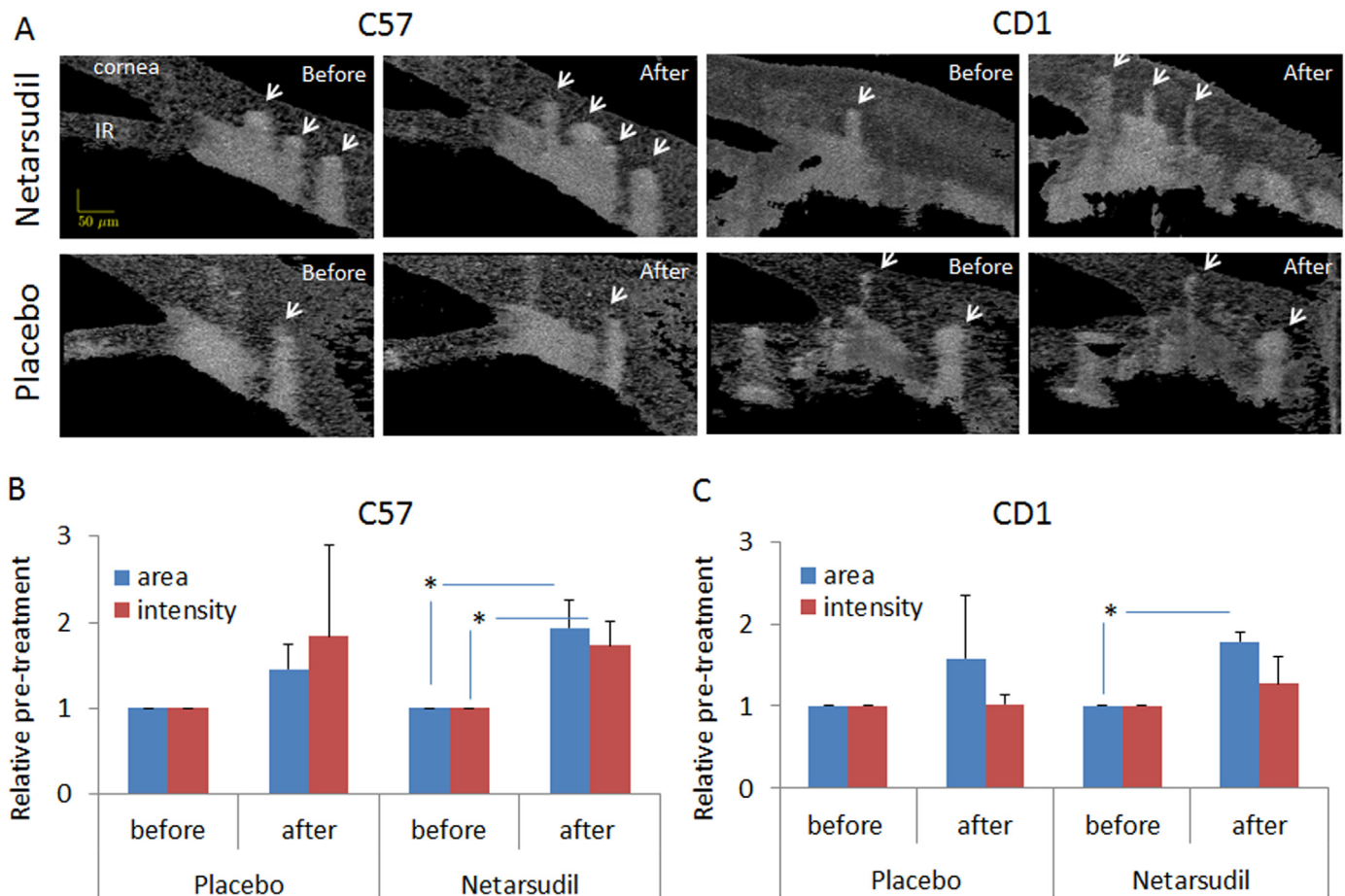


Fig. 7. Netarsudil-induced changes in flow area and intensity in scleral vessels visualized on OCT speckle variance images. Panel A shows representative speckle variance images of C57 and CD1 mouse outflow tissues before and after netarsudil or placebo eye drops. Arrows show scleral vessels before and 30–60 min after netarsudil treatment. Panels B and C show a summary of quantitative segmentation data expressed as relative changes in scleral vessel cross-sectional area and intensity after netarsudil or placebo treatment in C57 pigmented versus CD1 non-pigmented mice. Speckle variance images were visualized by Schlemm II software, and the areas of the intrascleral plexi near SC were selected for analysis. Speckle variance intensities in those areas were analyzed using Schlemm III software. The data represent mean $\pm$ S.E.M., $N=4-5$ from each group and each strain of mice, * $P < 0.05$ (Student's t -test).

2005). At the cellular level, inhibition of rho-associated kinases is known to decrease actomyosin-based cellular contraction by reducing the number of actin stress fibers and focal adhesions in conventional outflow cells (Nakajima et al., 2005; Rao et al., 2001). These changes in the actomyosin cytoskeleton and cell adhesive properties result in separation of the outermost cribriform plate from the inner wall of SC (Gong and Yang, 2014; Yang et al., 2013). This separation increases access of aqueous humor to the inner wall and theoretically eliminates resistance-generation due to “funneling” (Overby et al., 2009). Consistent with these previous studies (Yang et al., 2013), we visualized expansion of the TM using two different methods. Importantly, we demonstrate for the first time that rho-kinase-induced changes in TM shape can be resolved in living mice using OCT. Coincident with these changes, we observed that the cross-sectional area of SC increased 30–60 min (time period the experiments were conducted) after netarsudil treatment in both naïve and IOP-controlled eyes. Theoretically, relaxation of TM and inner wall separation should reduce SC lumen area, not increase it. However, since netarsudil is also known to lower episcleral venous pressure (28), reduction of downstream pressure in the context of reduction of resistance at SC/JCT prevents SC compression and promotes flow into and filling of SC lumen.

Fluorescent microbead tracer was used in the current study to determine netarsudil's effects on the patterns of aqueous humor outflow in the conventional tract. Netarsudil increased tracer deposition width and area, plus increased fluorescent intensity, indicating that more fluid passed through those regions to deposit

beads in the TM. It has been reported that reduced deposition of fluorescent tracer in outflow tissues correlates with elevated IOP and increased outflow resistance in both mouse and monkey eyes (Swaminathan et al., 2013; Zhang et al., 2009). Y27632, a relatively weak, non-selective rho kinase inhibitor increases tracer deposition in outflow tissues of enucleated bovine, monkey and human eyes ex vivo (Gong and Yang, 2014; Lu et al., 2008; Lu et al., 2011). We observed a similar pattern of increased bead decoration in mouse outflow tissues after netarsudil treatment in both low and high flow regions, which corresponded to increased outflow facility and lower IOP.

Impairment in outflow facility is responsible for ocular hypertension and large diurnal IOP fluctuations in glaucomatous eyes (Brubaker, 2003). Treatments, such as rho kinase inhibitors, that act by enhancing outflow facility are beneficial because they correct the physiological deficit in aqueous humor dynamics. Additionally, they may flatten diurnal IOP curves plus help alleviate the pressure spikes that occur with everyday events, such as choroidal expansion upon awakening from sleep (Brown et al., 1988; Zeimer et al., 1990). In the present study we modeled in living mice the IOP spike that occurs every morning upon awakening. As theorized (Brubaker, 2001), we show experimentally for the first time that a conventional outflow drug speeds IOP recovery by 25%. While enhancement of outflow facility by netarsudil is likely the primary effect on IOP decay, other parameters such as ocular compliance, inflow, episcleral venous pressure and

choroid volume need to be considered. Regardless, this demonstration shows that drugs that primarily target outflow facility reduce the integrated IOP insult that are imparted to susceptible retinal ganglion cell axons at the optic nerve head.

Besides its primary mechanism as a rho kinase inhibitor, netarsudil also functions as an inhibitor of norepinephrine transporter (NET) (Kiel and Kopczynski, 2015; Wang et al., 2015). NET inhibition in the ciliary body is thought to increase norepinephrine levels and thereby decrease aqueous humor secretion via activation of α_2 adrenergic receptors. As a third mechanism of action, netarsudil has been shown to decrease episcleral venous pressure in rabbits (Kiel and Kopczynski, 2015), which may be related to the rho kinase inhibitors' ability to promote vasodilation (Somlyo, 1997). Consistent with this possibility, we detected by OCT netarsudil-mediated increase in cross-sectional area of scleral vessels and SC. Studies have shown that elevated rho kinase pathway activity is associated with a variety of vascular diseases (Noma et al., 2006) and may play a pivotal role in cardiovascular diseases such as vasospastic angina, ischemic stroke, and heart failure. It is unclear whether elevated rho kinase activity may underlie ocular hypertension in glaucoma.

The development of software that can quantitatively measure the cross sectional area of SC and scleral vessels from OCT images, plus detect the flow pattern of perfused scleral vessels and changes of speckle variance intensities is another major achievement in this study. In two previous reports we quantified SC areas from OCT images using manual segmentation methods (Li et al., 2014a, 2014b). Not surprisingly, this is a laborious and subjective task, requiring extensive experience working with OCT images of mouse outflow tracts, multiple readers and masking of samples. In order to provide a more objective and rapid method to evaluate ocular vessels, we have designed novel segmentation software for use in the anterior segment of the eye. This new software sequentially processes images, reproducibly and efficiently segmenting SC based upon three factors: stable pixels of the outer wall of SC, speckle variance in SC delimiting its inner wall, plus successive B-scan differences to enhance contrast of the subtle flow patterns. Importantly, we found that the interobserver-reproducibility of the software was $91.7\% \pm 7.43\%$ and intraobserver-reproducibility was $99.2\% \pm 0.48\%$. This represents a significant improvement in reproducibility compared to the ImageJ software we used in our previous study (intraobserver-reproducibility = $86.2\% \pm 11.5\%$) (Li et al., 2014a).

In the current study, we also found that netarsudil increased speckle variance areas and intensities inside of scleral vessels that likely conduct aqueous humor. Speckle variance is a phenomenon caused by moving OCT reflectors, such as lipid and blood cells. Changes in speckle on OCT images in regions of flowing blood have been described earlier (Barton and Stromski, 2005; Barton et al., 2001). Whether the increase of speckle variance areas and intensities by netarsudil correlates with increased flow velocity in scleral vessels is unknown and needs to be further investigated. Also important is determining the exact identity of the reflectors in the conventional outflow tract.

A limitation of the present study is that we only monitored the acute effects of netarsudil by OCT in young mice. Our next goal is to chronically treat mice (young and aged) with netarsudil and examine the effect of long-term treatment on outflow facility, outflow tissue morphology and behavior via OCT. A second limitation is that we only imaged one OCT volume of each mouse eye. We are developing methods to image larger regions (i.e. imaging the entire interval between two adjacent collector channels), with the ultimate goal of imaging outflow structures for 360 degrees following different treatments or manipulations. These advances will provide much needed information about conventional outflow dynamics, and may ultimately assist physicians in medical decision-making regarding glaucoma care.

5. Conclusions

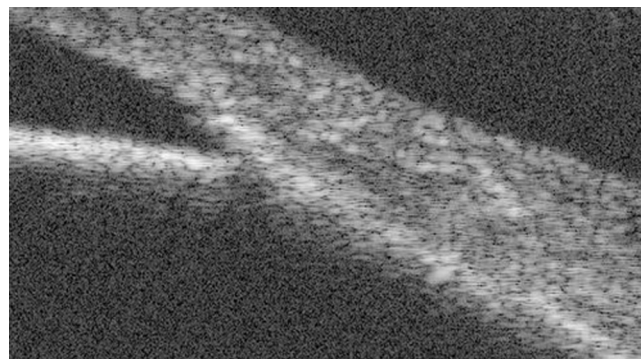
The present study documents the dynamic effects of a novel glaucoma drug, netarsudil, currently in phase III clinical trials. We examined IOP, outflow function and conventional outflow morphology in living mice. Results show that netarsudil impacted both proximal and distal portions of the conventional outflow tract to reduce outflow resistance and lower IOP. This is the first report showing real-time, non-invasive drug effects on conventional outflow tissues in living eyes, paving the way for development of a clinic-friendly OCT platform for glaucoma.

Acknowledgments

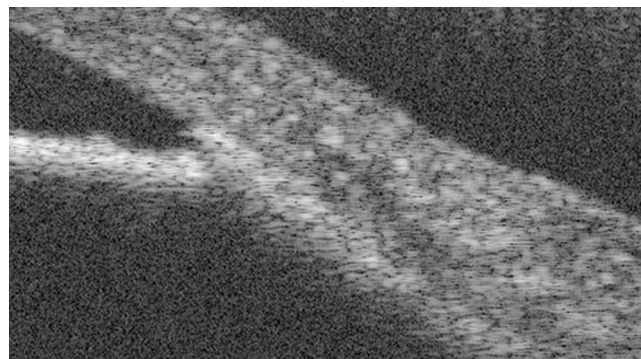
This work is supported in part by the BrightFocus Foundation (G2015100) and that National Eye Institute (EY005722 and EY019696). The authors thank Ying Hao from Duke Eye Center core facility for help with histology studies.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ejphar.2016.04.002>.



Video 2A. Comparison of outflow tissue behavior of living mouse eyes before and after Netarsudil treatment using OCT OCT videos show representative behavior of iridocorneal angle tissues from linear 200 B-scans (identical positions) of a live C57 mouse before (Video S2a) with intraocular pressure held at 10 mmHg. Video S2a shows speckling in SC, collector channel and scleral vessels. A video clip is available online. Supplementary material related to this article can be found online at <http://dx.doi.org/10.1016/j.ejphar.2016.04.002>.



Video 2B. A Comparison of outflow tissue behavior of living mouse eyes before and after Netarsudil treatment using OCT OCT videos show representative behavior of iridocorneal angle tissues from linear 200 B-scans (identical positions) of a live C57 mouse 40 min after topical 0.04% netarsudil treatment (Video S2b) with intraocular pressure held at 10 mmHg. In Video S2b, the speckling area of SC lumen enlarges, and collector channels widen after netarsudil treatment. Supplementary material related to this article can be found online at <http://dx.doi.org/10.1016/j.ejphar.2016.04.002>.

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