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Technical brief: Direct, real-time electrochemical measurement of nitric oxide in ex vivo cultured human corneoscleral segments

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Chronic elevation of intraocular pressure (IOP) is a major risk factor associated with primary open angle glaucoma (POAG), a common form of progressive optic neuropathy that can lead to debilitating loss of vision. Recent studies have identified the role of nitric oxide (NO) in the regulation of IOP, and as a result, several therapeutic ventures are currently targeting enhancement of NO signaling in the eye. Although a low level of NO is important for ocular physiology, excess exogenous NO can be detrimental. Therefore, the ability to directly measure NO in real time is essential for determining the role of NO signaling in glaucomatous pathophysiology. Historically, NO activity in human tissues has been determined by indirect methods that measure levels of NO metabolites (nitrate/nitrite) or downstream components of the NO signaling pathway (cGMP). In this proof-of-concept work, we assess the feasibility of direct, real-time measurement of NO in ex vivo cultured human corneoscleral segments using electrochemistry. A NO-selective electrode (ISO-NOPF200) paired to a free radical analyzer (TBR1025) was placed on the trabecular meshwork (TM) rim for real-time measurement of NO released from cells. Exogenous NO produced within cells was measured after treatment of corneoscleral segments with esterase-dependent NO-donor O2-acetoxymethylated diazeniumdiolate (DETA-NONOate/AM; 20 µM) and latanoprostene bunod (5–20 µM). A fluorescent NO-binding dye DAF-FM (4-Amino-5-methylamino- 2',7'-difluorofluorescein diacetate) was used for validation. A linear relationship was observed between the electric currents measured by the NO-sensing electrode and the NO standard concentrations, establishing a robust calibration curve. Treatment of ex vivo cultured human donor corneoscleral segments with DETA-NONOate/AM and latanoprostene bunod led to a significant increase in NO production compared with vehicle-treated controls, as detected electrochemically. Furthermore, the DAF-FM fluorescence intensity was higher in outflow pathway tissues of corneoscleral segments treated with DETA-NONOate/AM and latanoprostene bunod compared with vehicle-treated controls. In conclusion, these results demonstrate that NO-sensing electrodes can be used to directly measure NO levels in real time from the tissues of the outflow pathway.

Glaucoma is a complex multifactorial neurodegenerative disease characterized by progressive optic neuropathy. It is the leading cause of irreversible loss of vision, with more than 70 million people affected worldwide [1], and this number is expected to increase to 111.6 million by 2040 [2]. Primary open angle glaucoma (POAG) is the most common form of glaucoma, accounting for approximately 70% of all cases [1]. Elevated intraocular pressure (IOP) is a major risk factor associated with the development and progression of the disease [3,4], and it is the only modifiable risk factor in POAG. Therefore, most medical and surgical interventions are targeted toward reduction of IOP [5].

IOP is tightly regulated by the balance in secretion of the aqueous humor (AH) from the ciliary epithelium and its rate of elimination from the eye by the conventional and unconventional outflow pathways. The conventional outflow pathway, consisting of a sieve-like tissue called the trabecular meshwork (TM), is responsible for most of the resistance to AH outflow [5-7]. An increase in resistance at the TM leads to chronic IOP elevation and glaucomatous pathology [8-10]. Therefore, current drug therapies are targeted at the TM outflow pathway for lowering IOP and reducing resistance to AH outflow.

Nitric oxide (NO) has been implicated in numerous physiological processes of the eye, including IOP homeostasis and ocular blood flow [11-13]. A recent body of evidence strongly implicates NO in the regulation of IOP by modulating the TM's contractile tone and outflow resistance [14-20]. It has been shown that exogenous delivery of NO via donor compounds reduces IOP in mouse [14], rabbit [21], nonhuman primate [19], and human ex vivo tissue models [20]. Latanoprostene bunod, a chimeric prostaglandin analog with a NO-donating moiety, has recently been approved by the United Sates Food and Drug Administration (FDA) as

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an IOP-reducing therapeutic drug after successful clinical trials in humans [22-27]. Although the benefits of exogenous NO delivery in glaucoma are apparent, it is important to acknowledge the paradoxical role of NO that may also contribute to disease pathology. The effect of NO is largely concentration dependent. At lower concentrations, NO can be beneficial to cell survival and act as an important homeostatic mediator, whereas at high concentrations, excess NO can lead to nitrosative stress and physiologic dysregulation [28]. Exogenous NO-donor drugs are activated in cells via nonspecific esterase-dependent NO release mechanisms, which can lead to unwanted release of NO in nontargeted tissues. In the eye, the nitric oxide synthase (NOS) family of enzymes is primarily responsible for producing endogenous NO. This endogenous NO produced in extremely low amounts is responsible for regulation of enzymatic processes and physiological pathways [20,29,30].

Given the benefits on NO, the intrinsic regulatory pathways controlling its endogenous production are yet to be elucidated. The spatial and temporal concentrations of NO are of extreme importance for determining the role of NO signaling in the pathophysiology of glaucoma. The high reactivity, rapid diffusion, and short half-life of NO make it challenging to accurately measure its level in biological tissues. Although several techniques have been used for indirect measurement of NO, electrochemical (amperometric) detection of NO is the only available technique sensitive enough to determine physiologically relevant concentrations of NO in real time [31,32]. To assess the role of NO in glaucomatous pathophysiology and determine the release profile of NO-donor drugs, it is crucial to directly and accurately measure NO levels in real time. A previous study used a derivative of the Clark-type NO-sensing electrode setup for measuring NO produced in enucleated bovine eyes [33]. Due to advancements in electrode technology, these Clark-type electrodes can now be made at the microscale size and be highly flexible for use in ex vivo or in vivo settings. Here, we used a commercially available, microscale (200 µm diameter) version of the Clark-type electrode for detection of NO in human ex vivo cultured corneoscleral tissue.

Although animal and human models share remarkable physiological and anatomical similarities, the results obtained in animal models rarely translate to disease-modifying outcomes. We have previously reported the use of ex vivo cultured human corneoscleral segments in modeling glaucomatous pathology (manuscript in review). These human corneoscleral segments with intact TM and outflow pathway tissues are gifted postmortem for corneal transplant. Corneoscleral segments that are ineligible for transplant are made available for biomedical research at a fraction of the processing cost compared with whole globes. This ex vivo cultured human corneoscleral model allows for the study of physiological processes and disease pathology in a system that closely mimics the in vivo state.

In this proof-of-concept study, we utilize the ex vivo cultured human corneoscleral segment model to electrochemically measure the NO generated from donor compounds in real time at the TM outflow pathway. We further validate these results using a secondary, fluorescence-based assay involving the NO-binding dye DAF-FM (4-Amino-5-methylamino- 2',7'-difluorofluorescein diacetate) for NO detection.

METHODS

Ex vivo culture of human corneoscleral segments: Transplant ineligible, deidentified human corneoscleral segments were acquired from Lions Eye Institute (Tampa, FL) in conformity with the guidelines outlined in the Declaration of Helsinki (Appendix 1). Upon receipt, the corneoscleral segments were washed three times with PBS (7.4 pH; Sigma-Aldrich; catalogue # 806552; St. Louis, MO) and then cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich; catalogue# D6046-500ML) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), L-glutamine (Sigma-Aldrich), and 1% penicillin–streptomycin (PS; Sigma-Aldrich). The culture medium was exchanged daily with fresh medium.

Electrode calibration and NO-sensing electrode setup: Direct measurements of NO were performed using a TBR1025 free radical analyzer and NO-sensing electrode (ISO-NOPF200) obtained from World Precision Instruments (WPI; Sarasota, FL). Current readings were recorded using the LabScribe3 software (iWorx; Dover, NH). Electrodes were calibrated before experiments according to the manufacturer's instructions by the decomposition of a NO-donor S-nitroso-N-acetyl-penicillamine (SNAP; WPI) using $CuCl_2$. SNAP was prepared by dissolving 5 mg of EDTA (Sigma-Aldrich) and 5.0 mg of SNAP in 250 ml of high-performance liquid chromatography (HPLC)-grade water. The electrode was immersed in 20 ml of 0.1 M $CuCl_2$ (Sigma-Aldrich) in distilled water until the electrode stabilized (1–2 h). A calibration curve was created by sequentially adding bolus volumes of SNAP after each signal reached a plateau. The sampling rate used was 10 samples/s. The change in recorded current was converted to corresponding molarities of NO produced by SNAP addition. The efficiency of the conversion of SNAP to NO was 0.6. All measurements were performed under a chemical hood in constant airflow and at room temperature to avoid any temperature fluctuations and electrical

interference. Prior to each measurement, the electrode was polarized for 2 h in PBS. Human corneoscleral segments with intact TM rims were washed three times with PBS and placed in a 24-well plate submerged in 1 ml of PBS. Measurements were performed in PBS instead of complete media to avoid interference from other nitrogen-containing compounds. We anticipated that the length/area of TM could influence the amount of NO generated. To normalize this variable, we used the entire corneoscleral segment with an intact TM rim for real-time electrochemical measurement of NO. Furthermore, to reduce the variability between different donors, we used pairs of corneoscleral segments from the same donor for vehicle and drug treatments. The electrode was lowered into the well and clamped in such a way that the tip of the electrode remained in contact with the TM rim throughout the experiment (Figure 1A). A stable baseline was achieved, and following this, the corneoscleral segments were treated with NO-donor drugs or vehicle. Troubleshooting of common problems encountered during application of the technique are listed in Table 1. DETA-NONOate/AM (O_2-acetoxymethylated diazeniumdiolate; MilliporeSigma; Burlington, MA) [34] and 0.024% latanoprostene bunod (Bausch & Lomb; Bridgewater, NJ) are the two NO-donor compounds tested in this study. Latanoprost was used as the vehicle against the NO-donor latanoprostene bunod. Both ophthalmic solutions (latanoprostene bunod and latanoprost) were obtained from a local pharmacy (Daniel Drug, Inc; Fort Worth, TX).

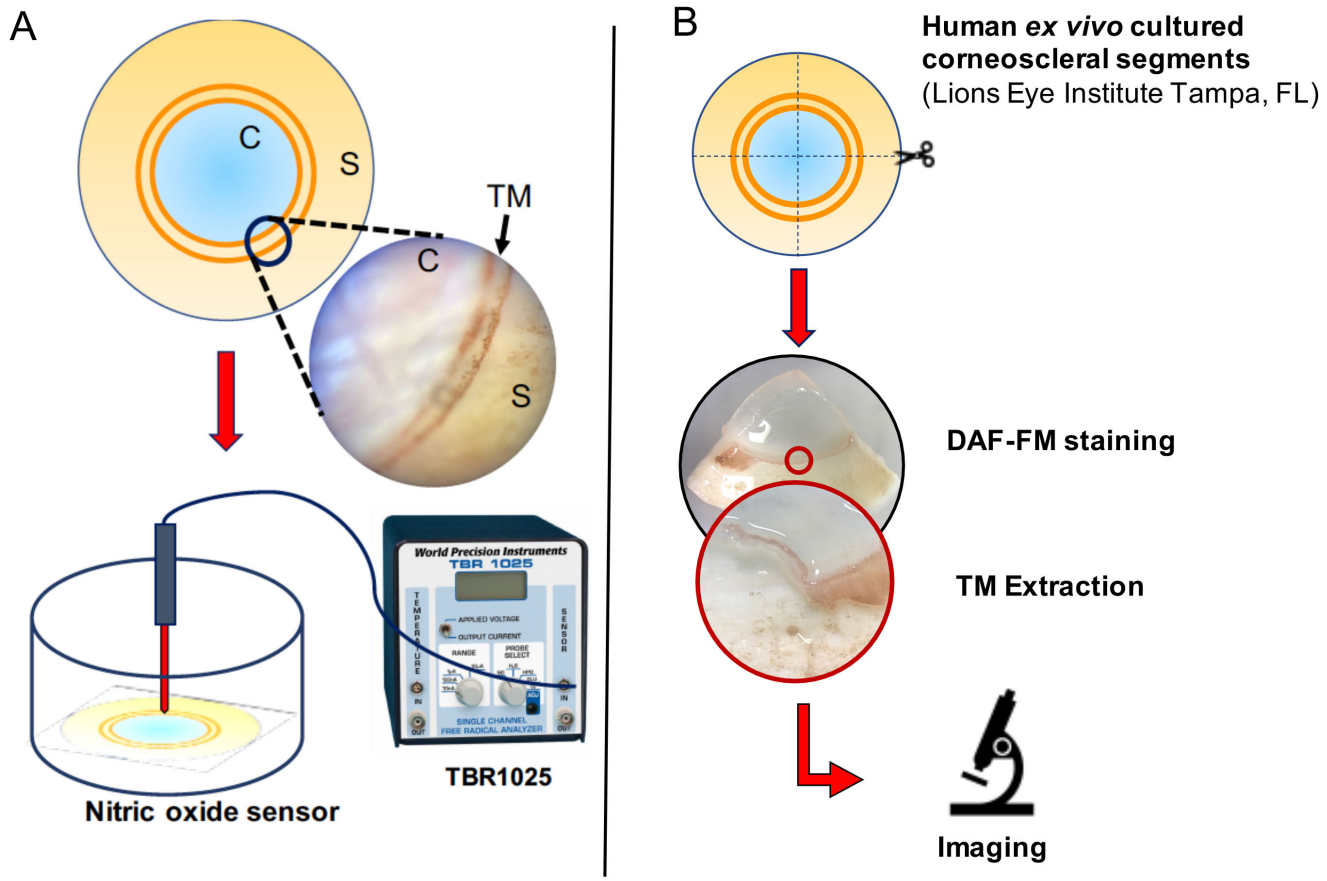

Figure 1. Experimental setup for the study. **A:** Workflow for electrochemical nitric oxide (NO) measurement using NO-sensing electrode. Cultured human donor corneoscleral segments (C = cornea; S = sclera; TM = trabecular meshwork) were washed 3X with PBS and placed in a 24-well plate with 1 ml of PBS. Drug treatments were performed after a stable baseline was achieved. The output current corresponds to the level of NO generated (~10 pA/nM), detected using a free radical analyzer (TBR1025). The signal is amplified by a four-channel Lab-Trax amplifier and analyzed using LabScribe3 software. **B:** Workflow for fluorochemical measurement of NO using DAF-FM assay (intracellular NO-binding dye). Quadrants of the human donor corneoscleral segment were cultured at 37 °C in DMEM media supplemented with 2% fetal bovine serum (FBS) and 1% penicillin–streptomycin (PS) and treated with 10 µM NO-binding fluorescent DAF-FM dye for 30 min. Cells and tissues were washed 1X with PBS and treated with different drugs or vehicle and incubated at 37 °C for an additional 30 min. Tissues were then washed 3X with PBS, and the trabecular meshwork (TM) rim was removed and imaged using fluorescence microscopy.

Fluorochemical DAF-FM assay for NO labeling: Human donor corneoscleral segments were divided into quadrants and cultured in phenol-free DMEM media (Sigma-Aldrich) supplemented with 0.2% FBS and 1% PS (Sigma-Aldrich). The vehicle and experimental treatments were performed on the same eye to reduce variability associated with the contralateral eye. Quadrants from each eye were first treated with 10 µM DAF-FM (MilliporeSigma) dye and incubated at 37 °C for 30 min. Quadrants were then washed three times with PBS and incubated for an additional 30 min at 37 °C to allow the proper incorporation and activation of dye within cells. The quadrants were then treated either with different NO-donor compounds or appropriate vehicle controls and further incubated for 30 min at 37 °C. After the incubation period, the quadrants were washed three times with PBS and prepared for imaging (Figure 1B). The TM rim (including the inner wall of Schlemm's canal) was carefully dissected from the unfixed corneoscleral quadrants and placed between coverslips for imaging under the microscope (Keyence; Itasca, IL). DAF-FM fluorescence images were analyzed by quantifying fluorescence intensity per unit area (IntDen/µm^2) using ImageJ (National Institutes of Health; Bethesda, MD) as described previously [35,36].

Statistical analysis: Statistical analysis was performed using GraphPad Prism 8 (San Diego, CA). Data are expressed in means ± standard error of the mean (SEM). Two-group comparisons were analyzed by an unpaired Student *t* test. Multiple comparisons were analyzed by two-way analysis of variance (ANOVA) with the Bonferroni post hoc test. Significance was designated at *p<0.05, **p<0.01, and ***p<0.001.

RESULTS

Electrochemical detection of NO involves the oxidation of NO on the electrode surface and subsequent measurement of the redox current generated. When the NO microelectrode (consisting of a working and reference electrode pair) is immersed in a solution containing NO and a positive potential is applied, NO is oxidized at the working electrode surface, producing a redox current. Thus, the amount of NO oxidized is proportional to the current flow at the electrode, which is measured by the free radical analyzer. As recommended by the manufacturer, NO generated by decomposition of SNAP was used to calibrate the NO-sensing electrode (ISO-NOPF200). The calibration curve demonstrates a linear relationship between the amount of NO generated from SNAP and the current detected at the electrode (Figure 2).

A previous study demonstrated the electrochemical measurement of NO in enucleated bovine eyes [33]. Here, we examined whether ex vivo cultured human corneoscleral

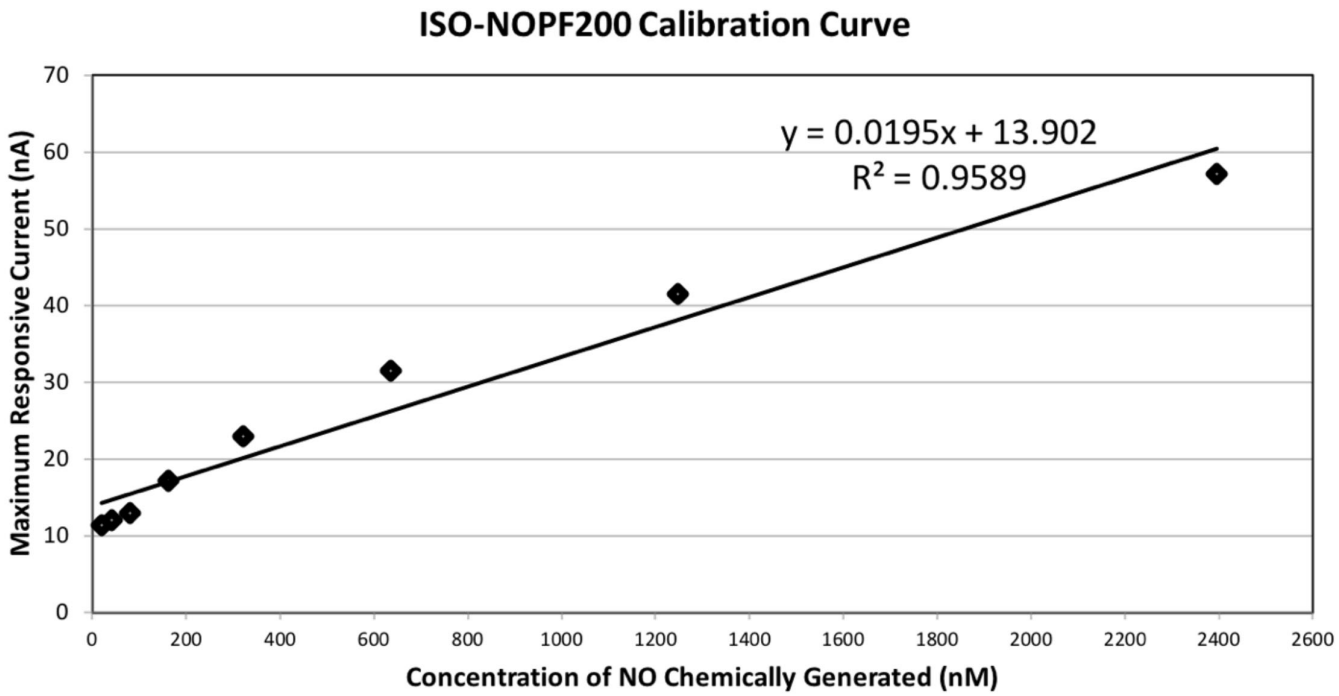

Figure 2. Linear regression analysis of the relationship between the amount of nitric oxide (NO) added and the electric current obtained from the NO electrode (ISO-NOPF200).

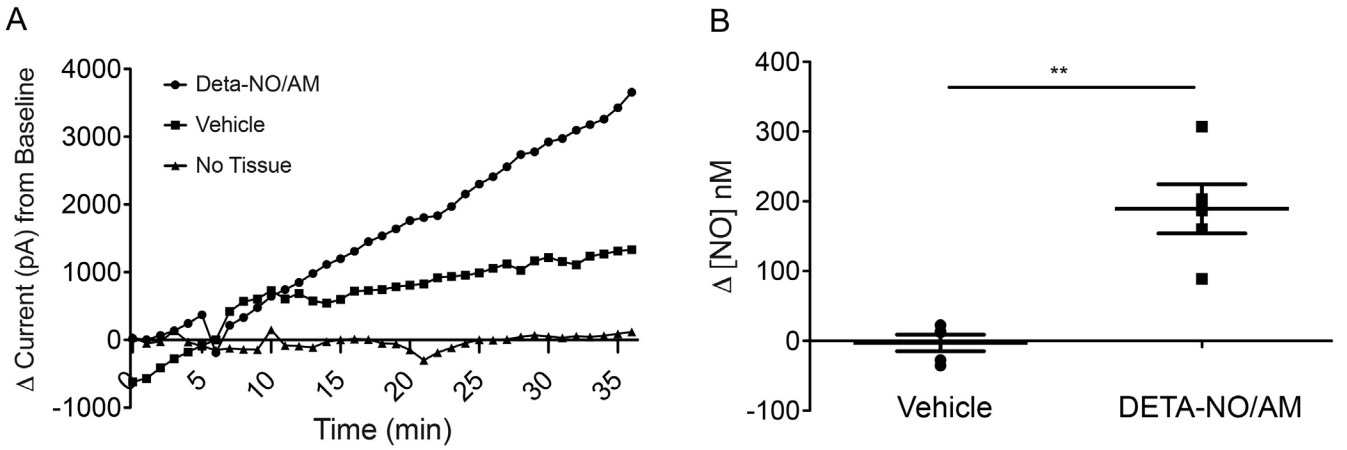

Figure 3. Detection of nitric oxide (NO) in human corneoscleral segments after treatment with DETA-NONOate/AM (exogenous NO donor). **A:** Representative response plot showing amperometric current readings obtained after treatment of ex vivo cultured human corneoscleral tissues with an exogenous NO-donor DETA-NONOate/AM or equivalent vehicle. A visible spike in recorded current was observed as a result of DETA-NONOate/AM treatment on human corneoscleral segments. A "no tissue" control was employed to ensure that the detection of NO signal was tissue dependent and not a result of NO release in aqueous PBS. **B:** Change in NO concentration from baseline after 30 min of treatment with DETA-NONOate/AM (20 µM) or equivalent vehicle (0.1% dimethyl sulfoxide [DMSO]) at room temperature. Data are expressed as means ± standard error of the mean (SEM); n = 5 for each group; ** p<0.001; two-tailed unpaired Student *t* test.

segments can be used to detect chemically generated NO in the outflow pathway tissues. Our improved understanding of the NO signaling pathway and its involvement in several disease pathologies has aided in the development of several esterase-dependent NO-donor compounds for therapy. We asked whether this NO-sensing electrode setup can be used for measuring NO in human tissues. We tested two NO-donor compounds, DETA-NONOate/AM and the clinically tested and FDA-approved latanoprostene bunod. DETA-NONOate/AM is a commercially available intracellular NO-donor compound with an esterase-dependent NO release mechanism [34]. Each pair of human corneoscleral segments was treated with 20 µM DETA-NONOate/AM or an equivalent vehicle. The representative response graph shows an increase in detected current from the segments treated with DETA-NONOate/AM compared with vehicle-treated control (Figure 3A). A "no tissue" control was used to determine whether the extracellular degradation of NO in aqueous solution was contributing to the detected current. We did not observe an increase in the NO current after the addition of DETA-NONOate/AM in aqueous PBS (Figure 3A). Furthermore, treatment of corneoscleral segments with 20 µM DETA-NONOate/AM for 30 min resulted in a significant increase in NO production compared with vehicle-treated controls (Figure 3B). We further used the DAF-FM assay to fluorescently label NO in the outflow pathway tissues to validate the results obtained via electrochemistry. Quadrants of human corneoscleral segments were pretreated with DAF-FM and subsequently treated with 20 µM DETA-NONOate/AM or vehicle control. A significant increase in DAF-FM fluorescence intensity was observed in quadrants of corneoscleral segments treated with DETA-NONOate/AM compared with vehicle-treated control (Figure 4A-B).

Latanoprostene bunod is an aqueous-soluble chimeric compound constituting latanoprost (an IOP-lowering prostaglandin analog) linked to butanediol mononitrate (NO-donor moiety). The NO released from latanoprostene bunod is implicated in regulating AH outflow resistance at the conventional outflow pathway, and it has been recently approved for IOP-lowering therapy in human glaucoma patients [22-25,37]. Each pair of ex vivo cultured human corneoscleral segments was treated with increasing concentrations of 5–20 µM latanoprostene bunod or equivalent vehicle latanoprost. The NO-sensing electrode setup was used to measure the subsequent NO production. A dose-dependent increase in NO production was observed in the segments treated with latanoprostene bunod compared with vehicle latanoprost (Figure 5). Furthermore, the DAF-FM staining intensity was significantly higher in the segments treated with 20 µM latanoprostene bunod compared with the latanoprost vehicle (Figure 6A-B).

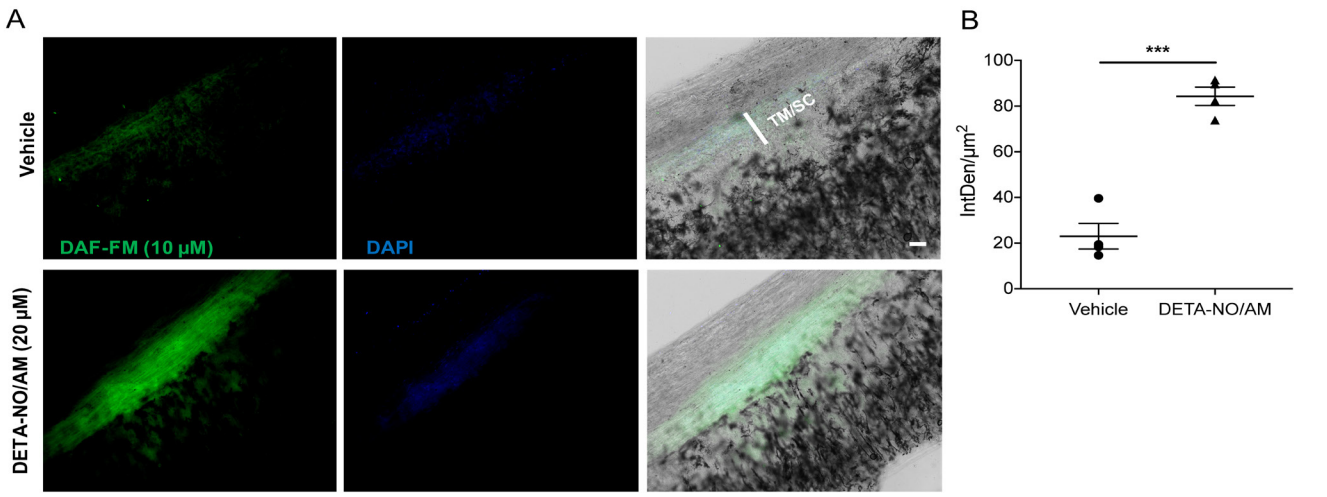

Figure 4. Detection of exogenous NO released from DETA-NO/AM in human corneoscleral segments using a fluorescent NO-indicator. **A:** Increase in DAF-FM fluorescence intensity in quadrants of human corneoscleral segments after treatment with DETA-NONOate/AM (exogenous nitric oxide [NO]-donor) compared with vehicle-treated controls. Quadrants of human donor corneoscleral segments from each eye (n = 4 per group) were pretreated with intracellular NO-indicator DAF-FM dye (10 µM) and then treated with DETA-NONOate/AM (20 µM) or vehicle (0.1% dimethyl sulfoxide [DMSO]) at 37 °C for 30 min. Images were taken using fluorescence microscopy at 100X magnification (Scale bar = 50 µm). **B:** Quantification of DAF-FM fluorescence intensity per unit area (IntDen/µm^2) in DETA-NO/AM and vehicle-treated corneoscleral segments using ImageJ analysis. Data are expressed as means ± standard error of the mean (SEM); n = 4 for each group; *** p<0.001; Two-tailed unpaired Student *t* test.

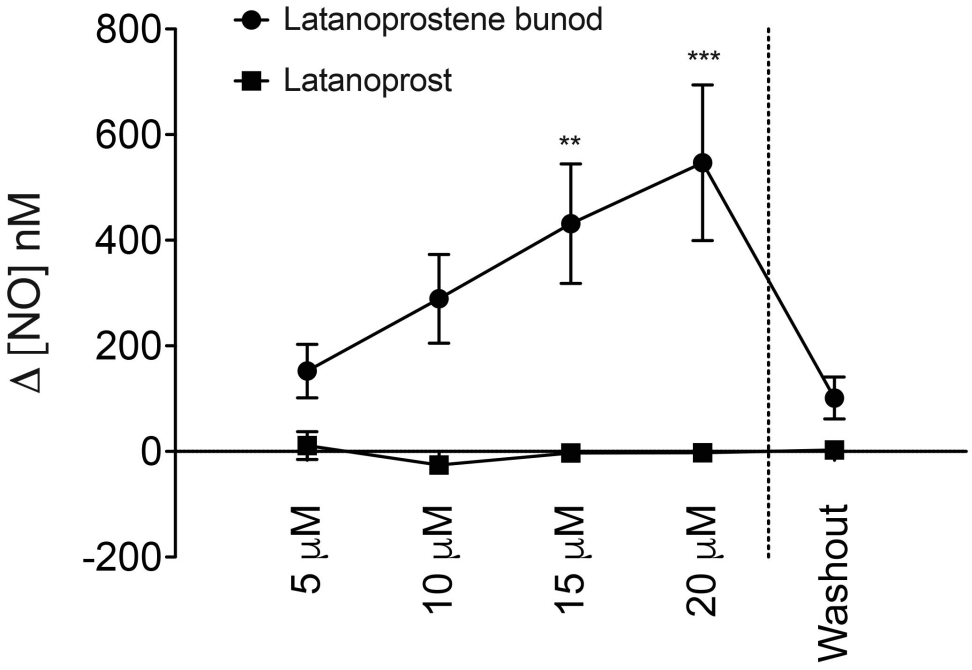

Figure 5. Dose-dependent increase in NO levels in human corneoscleral segments after treatment with latanoprostene bunod compared with latanoprost vehicle control. Cultured human donor corneoscleral segments were treated with increasing doses of latanoprostene bunod (5–20 µM) or equivalent volume of latanoprost (vehicle), and changes in the levels of NO from baseline were recorded. Each subsequent bolus dose was given after the signal plateaued (~10 min). Washout readings were recorded after a 30-min period. Data are expressed as means ± standard error of the mean (SEM); n = 4 for each group; ** p<0.01, *** p<0.001; two-way analysis of variance (ANOVA).

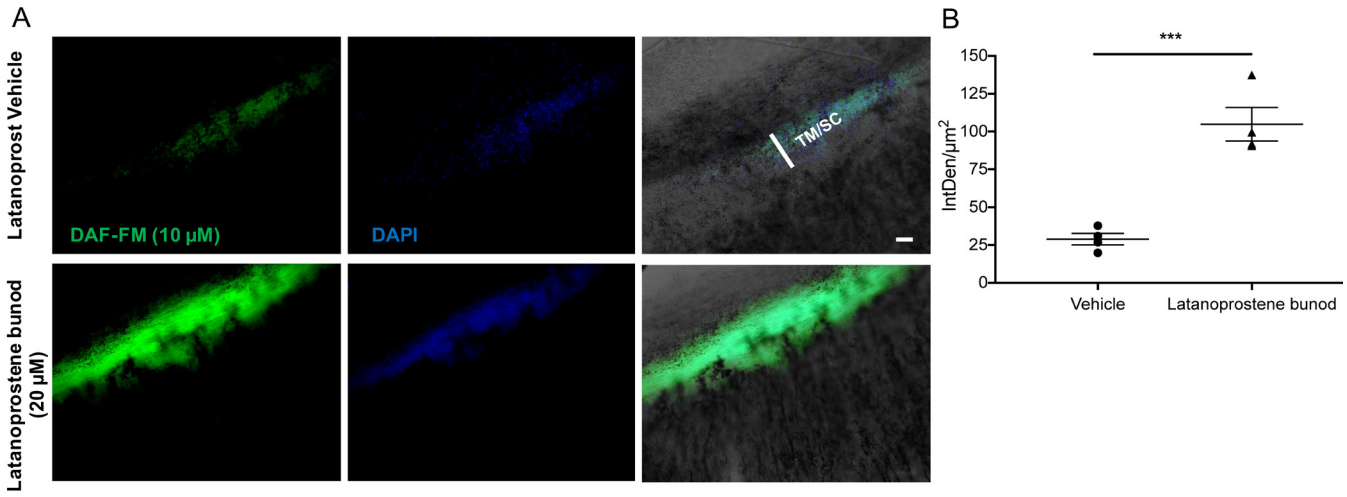

Figure 6. Detection of exogenous NO released from Latanoprostene bunod in human corneoscleral segments using a fluorescent NO-indicator. **A:** Increase in DAF-FM fluorescence intensity in quadrants of human corneoscleral segments after treatment with latanoprostene bunod compared to controls treated with vehicle latanoprost. Quadrants of human donor corneoscleral segments from each eye (n = 4 per group) were pretreated with intracellular nitric oxide (NO)-indicator dye DAF-FM dye (10 µM) and then treated with latanoprostene bunod (20 µM) or a latanoprost vehicle. Quadrants treated with latanoprostene bunod showed increased DAF-FM fluorescence intensity compared with vehicle-treated controls. Images were taken using fluorescence microscopy at 100X magnification (Scale bar = 50 µm). **B:** Quantification of DAF-FM fluorescence intensity per unit area (IntDen/µm^2) in latanoprostene bunod and latanoprost vehicle treated corneoscleral segments using ImageJ analysis. Data are expressed as means ± standard error of the mean (SEM); n = 4 for each group; *** p<0.001; two-tailed unpaired Student *t* test.

DISCUSSION

The cornea is the most frequently donated human tissue in the world. In the United States alone, 116,990 corneas were donated in 2016; of these, only 63,596 corneas were used for transplantation [38]. Due to low specular cell counts (<2000 cells), accidental damage, or the abnormal medical history of the patient (suspected cases of dementia or risk of prions disease) most donated corneas are deemed ineligible for transplant. These ineligible corneoscleral explants with intact conventional outflow pathway tissue are made available for research with lower processing cost compared with intact whole globes. Cost and tissue availability are the two barriers that prevent wider use of human tissue in biomedical research. Vision scientists who collaborate with eye banks are in a unique position to overcome these barriers and study disease pathophysiology in an ex vivo system that closely mimics the in vivo state. We previously reported on the versatility of ex vivo cultured human donor corneoscleral segments in modeling glaucomatous pathology (manuscript in review). We showed that treatment of human corneoscleral segments with glaucoma-associated factors like dexamethasone and recombinant transforming growth factor (TGF) β2 results in glaucoma-related phenotypes, which include increased extracellular matrix (ECM) production and endoplasmic reticulum (ER) stress in the AH outflow pathway. In this study, we aimed to determine the feasibility of using donated human corneoscleral segments for directly measuring NO production in real time.

In POAG, dysfunction in the NO signaling pathway and reduced bioavailability of NO have often been associated with disease pathology [39-41]. As a result, enhancement of NO signaling using exogenous NO-donor compounds has emerged as a promising new therapeutic avenue. Furthermore, the recent push in the development of IOP-lowering NO-donor compounds has uncovered an unmet need for direct measurement of NO in ocular tissues. However, the high reactivity, rapid diffusion, and short half-life of biological tissues make it challenging to accurately measure their NO level. Currently, electrochemical measurement is the sole method of directly quantifying physiologically relevant amounts of NO in real time. A previous study used a derivative of the Clark-type electrode setup for direct electrochemical measurement of NO in bovine eyes [33]. Since then, electrode technology has seen considerable progress, with the development of smaller, more flexible, and more sensitive electrodes for NO measurement in tissues. We used a commercially available NO-sensing electrode with high selectivity for NO to measure real-time NO production in

human tissues. To our knowledge, this is the first study using ex vivo cultured human corneoscleral segments to directly measure NO generated from donor compounds in real time. We electrochemically measured NO produced in the AH outflow pathway after treatment of human corneoscleral segment tissues with two structurally different exogenous NO-donor compounds, both having esterase-dependent intracellular NO release mechanisms. Each pair of human corneoscleral segments was treated with either the NO-donor compound DETA-NONOate/AM (20 µM) or latanoprostene bunod (5–20 µM) against the equivalent vehicle control. The detected NO currents that corresponded to the amount of NO produced were significantly higher in the tissues treated with NO-donors compared with the equivalent vehicle-treated controls (Figure 3A-B, Figure 5). Furthermore, we used the DAF-FM assay as a secondary technique for validation of the electrochemical data. The fluorescence intensity of NO-binding dye DAF-FM was higher in the quadrants of corneoscleral segment tissues treated with the NO-donors compared to controls (Figure 4A-B and Figure 6A-B). These results indicate that the NO-sensing electrode can successfully detect the exogenous NO produced by NO donors in human tissues. We also examined whether there were differences in NO-donor compounds in terms of NO release. We observed relatively robust NO currents and higher DAF-FM fluorescence intensity in segments treated with latanoprostene bunod (20 µM) compared with DETA-NONOate/AM (20 µM)-treated segments. Latanoprostene bunod appears to be superior in terms of the tissue penetration, metabolization rate, and NO production. This was surprising because the stoichiometric ratio for NO release from latanoprostene bunod is lower (1 mol of NO/mol) than that of DETA-NONOate/AM (1.83 mol of NO/mol). A possible explanation for this is that latanoprostene bunod is an aqueous-soluble compound compared with DETA-NONOate/AM. Furthermore, the latanoprostene bunod formulation used in this study is clinically approved and refined for superiority compared with the relatively untested DETA-NONOate/AM.

Ocular tissues like the TM, corneal endothelium, and Schlemm's canal endothelium can metabolize donor compounds and produce NO currents. It is important to note that both the NO donors used in the study can indiscriminately

Table 1. Troubleshooting solutions for real-time electrochemical measurement of NO in human corneoscleral segments (adapted from World Precision Instruments).

Issue	Possible cause	Solution
Baseline current below specified range	Incorrect setting selected on the TBR1025 analyzer.	Set the poise voltage to 865 mV (NO setting) on TBR1025. Set the range to 100 nA
	Sensor may be nearing the end of its usable life.	Perform calibration using fresh standard solutions. If problem persists, change NO sensor.
Unstable baseline	Interference from chemical contaminants from growth media.	Wash corneoscleral segments with PBS at least 5 times prior to initiating measurement.
	The polarizing solution may be contaminated.	Prepare fresh polarizing solution (0.1M $CuCl_2$). After polarization, we recommend using PBS to stabilize the electrode.
	External electrical interferences may be a problem.	Identify and isolate electrical interference.
	Interference from external heat sources.	Identify external heat sources and isolate equipment. We recommend using a laminar airflow hood.
Non-linear calibration	Stock solutions have deteriorated.	Prepare fresh SNAP standard solution and repeat calibration.
	Chemical contaminants in water or on glassware.	Use ultrapure milliQ water in preparing solutions. Wash glassware with milliQ water prior to use.
	Uneven aliquots may have been used.	Check pipette calibration.
Low sensitivity	Probe not in contact with the trabecular meshwork (TM) rim.	Secure the sensor in a manner that it is in constant contact with the TM rim.
	Use of disintegrated TM rim tissue corneoscleral segment tissue.	Use corneoscleral segments with intact TM rim and overall tissue morphology for a robust response.
	Foreign material adsorbed on the sensor surface.	Wash the sensor with detergent if the material is protein from growth media or Ultrapure milliQ water if it is salt from PBS.
	Sensor has reached the end of its usable life.	Replace the sensor.

enter cells of any tissue and produce exogenous NO currents. Although this makes it difficult to determine the exact tissue source for the detected NO currents, we consider that the majority of detected NO is produced at the conventional outflow pathway tissues. Given the short half-life and rapid reactivity of NO, proximity of the electrode to the tissue is important for capturing the NO and generating a robust electrical signal. Like in the previous study by Millar, we placed the electrode on the TM to maximize the capture of local NO released from the TM [33]. Therefore, close proximity of the electrode to the TM and Schlemm's canal ensures maximum contribution from the conventional AH outflow pathway tissues.

In this study, we electrochemically measured the levels of exogenous NO released in the outflow pathway tissues. However, we recognize that this setup may also be able to detect endogenous NO levels from ex vivo cultured human corneoscleral segments. This is evident in that we could detect baseline NO currents in vehicle-treated corneoscleral segments (Figure 3A). Endogenous NO in cells is produced by the constitutive and induced NOS enzymes, as well as via reduction of nitrates/nitrites by cellular reductases [42]. Future work will focus on refining this system further to improve detection of endogenous NO in ocular tissues.

Although the electrochemical method for quantifying NO holds much promise and utility in studying the pharmacodynamic properties of glaucoma medications in real time, it is also important to understand the limitations of this technique. Electrodes used for detecting NO are inherently sensitive to environmental influences. The current iteration of this setup is an open system that is sensitive to temperature and pH changes. Our setup utilizes a laminar airflow hood for electrical and thermal isolation to reduce the effect of temperature fluctuations on the system. However, development of a closed system is necessary to control for the confounding effects of temperature and pH. Future iterations will need integration of temperature and pH sensors within a closed measurement chamber. This closed measurement chamber can be placed in an incubator for quantifying endogenously produced NO at physiologically relevant temperatures, which will increase tissue viability for longer measurement time points.

APPENDIX 1. DEIDENTIFIED DONOR INFORMATION FOR THE POST-MORTEM HUMAN CORNEOSCLERAL SEGMENT TISSUES USED IN THE STUDY.

To access the data, click or select the words "Appendix 1."

ACKNOWLEDGMENTS

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