Trabecular Meshwork Mitochondrial Function and Oxidative Stress: Clues to Racial Disparities of Glaucoma

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Purpose: To identify racial differences of oxidative damage and stress and mitochondrial function in human trabecular meshwork (TM).

Design: Experimental study.

Participants: One hundred seventy-three eyes of 173 patients undergoing intraocular surgery provided aqueous humor (AH) for analysis. Trabecular meshwork tissues from eye bank donors were used as healthy controls for primary cell culture.

Methods: Enzyme-linked immunosorbent assay methods were used to measure 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidative damage marker, in AH comparing Black and White Americans. Human TM primary cultured cells from Black and White donors were used for adenosine triphosphate (ATP) measurement under high and low oxygen culture conditions. Complex I activity was measured in mitochondrial fractions isolated from cultured TM cells. Mitochondrial quantification was performed by translocase of outer mitochondrial membrane 20 (TOMM20) Western blot. Intracellular reactive oxygen species (ROS) production was measured in live TM cells.

Main Outcome Measures: Oxidative damage in AH, ATP production, complex I activity, mitochondrial quantification, and intracellular ROS in cultured TM cells stratified by racial background.

Results: Aqueous humor samples (75 Black, 98 White) displayed significantly higher 8-OHdG levels ($P = 0.024$) in Black compared with White patients with severe stage glaucoma. Using cultured healthy donor TM cells, ATP production was higher in Black than White TM cells ($P = 0.002$) in low oxygen culture conditions. Complex I activity was not statistically different in Black compared with White TM cells, but TOMM20 expression was higher in Black versus White cells ($P = 0.001$). In response to hydrogen peroxide challenge, ROS production was significantly higher in Black compared to White TM cells ($P = 0.004$).

Conclusions: Significantly higher 8-OHdG levels in AH of Black compared with White patients with severe glaucoma indicated that oxidative damage may be a risk factor in glaucoma pathogenesis or the result of distinct pathologic features in the Black population. To identify potential origins or causes of this damage, our data showed that healthy Black cultured TM cells have higher ATP and ROS levels, with increased quantity of mitochondria, compared with White TM cells. These findings indicate that mitochondrial alterations and increased oxidative stress may influence racial disparities of glaucoma.
patients. Specifically, oxidative DNA damage, demonstrated by increased urinary and plasma levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), has been found in patients with glaucoma. Further studies showed the correlation between TM DNA oxidative damage and mean IOP as well as visual field defects in patients with glaucoma.

As the primary source of ROS, mitochondria have been linked to glaucoma pathogenesis. To further understand the racial disparities associated with oxidative damage in the aqueous outflow pathways, this study compared a marker of oxidative damage in AH and mitochondrial function and oxidative stress markers in TM cells cultured from healthy Black and White corneal donors.

Methods

Human Aqueous Humor Specimen Collection

This human study was approved by the institutional review board of the Washington University School of Medicine and complied with the tenets of the Declaration of Helsinki and Health Insurance Portability and Accountability Act guidelines. Informed consent was obtained from participants after the nature and possible consequences of the study were explained. This study was designed to collect AH to measure oxidative stress and antioxidants in patients undergoing cataract, glaucoma, or glaucoma combined with cataract surgery in an academic clinical practice, as reported previously. Patients were excluded from the study if they had a history of prior cataract surgery, pars plana vitrectomy, corneal endothelial dysfunction, ischemic ocular disease, diabetic retinopathy, inflammatory or traumatic ocular disease, ocular neoplasia, exudative macular degeneration, or monocular status. Only patients with POAG were included in the study, because all other types of glaucoma were excluded. Patients with cataract without a diagnosis of glaucoma were included as controls to compare with the patients with POAG. Racial background was based on self-report, as indicated on a standardized registration questionnaire. Additional patient information, including glaucoma medications, lens status, most recent IOP recorded before surgery, and stage of glaucoma severity (mild, moderate, and severe) based on visual field assessments were collected for the analysis. Patients were stratified by glaucoma severity, using Humphrey visual field mean deviations (MDs), as mild (<–6.0 dB), moderate (–6 to –12.0 dB), involving only 1 hemifield with sensitivity of <15 dB within 50 of fixation, and severe (>–12.0 dB) or at least 1 point in central 50 of fixation with sensitivity of 0 dB or points within the central 5° with sensitivity of <15 dB in both hemifields. Details of the surgical procedure are described in a previous report. In brief, at the beginning of the surgery, a 15° blade was used to enter the AC through the peripheral cornea. Aqueous humor (50–100 μl) was drawn into a 1-ml tuberculin syringe through a 30-gauge blunt cannula through the corneal entry, followed by rinsing of the AC volume with balanced salt solution. The AH specimen was immediately transferred to a sealed tube, placed on dry ice, and transported to storage in the gas phase of a liquid nitrogen tank until analysis.

DNA Oxidative Damage Marker in Human Aqueous Humor

To quantify 8-OHdG in the AH specimens, we used the Oxiselect Oxidative DNA Damage ELISA Kit (Cell Biolabs). All reagents were prepared and mixed thoroughly before use according to the manufacturer’s instructions. First, 100 μl of 1 μg/ml 8-OHdG conjugate was added to each well, was incubated at 4° C overnight, and then was washed with distilled water. Fifty microliters of AH sample or 8-OHdG standard were added to the wells of the 8-OHdG conjugate coated plate and were incubated at room temperature for 10 minutes. Next, 50 μl of the anti-8-OHdG antibody at 1:500 dilution were added to each well and incubated at room temperature for 1 hour. The wells were washed 3 times with wash buffer, and 100 μl of the diluted secondary antibody—enzyme conjugate was added to all wells. Finally, 100 μl of the substrate solution was added after washing 3 times and mixed with 100 μl of the stop solution into each well. The absorbance of each microwell was read on a spectrophotometer using 450 nm as the primary wavelength. The initial 35 AH samples and standards were assayed in duplicate without any significant differences. Because of limited volumes of AH specimens, only a single assay was performed from the rest of the patient AH specimens.

Human Trabecular Meshwork Cell Culture

Collection of Human Trabecular Meshwork Tissues. All donor eye tissues were examined and passed the standard quality criteria by our local eye bank (MidAmerica Transplant, Inc.). These tissues were used for TM cell culture and were designated as healthy (nonglaucomatous) controls. The donor corneas were first used for corneal transplantation surgery (keratoplasty) at Washington University Eye Center. As per published recommendations, residual corneoscleral tissue rings, including the TM region, were used for primary TM cell culture. To confirm typical cells in this region of the corneoscleral rings, fixed tissues were examined with cell markers alpha B crystallin antibody (zBCRY; mouse monoclonal; Abcam), aquaporin 1 (rabbit monoclonal; Abcam), fibulin-2 (rabbit polyclonal; Millipore/Sigma) as recommended by recent publication guidance. Meanwhile, potential contamination of cells was assessed by markers for desmin (muscle cell marker; mouse monoclonal; Santa Cruz) and keratin 5 (epithelial cell marker; rabbit polyclonal; Millipore/Sigma).

Primary Cell Culture. Strips of TM tissues from the donor corneoscleral rings were cut into 2- to 4-mm segments and placed into 35-mm collagen-coated dishes with 12-mm glass coverslips on top of the tissue. Three milliliters of Dulbecco’s Modified Eagle Medium containing 1 g/l glucose, 4 mM L-glutamine, 100 units/ml of penicillin/streptomycin, and 20% fetal bovine serum were added. Cultured TM tissue was maintained in a humidified 5% CO2-controlled incubator for 4 to 6 weeks. After growth of TM explant to near confluence, cells were passaged by trypsinization (first passage). The passaged cells were divided into dexamethasone (200 nM)-treated and nontreated groups for 7 days as per published guidelines. Trabecular meshwork cells were probed with myocilin (rabbit polyclonal; Abcam), aquaporin-1 (rabbit monoclonal; Abcam), and zBCRY (mouse monoclonal; Abcam), the recognized cell marker for juxtacanalicular TM cells, and fibulin-2, a marker of Schlemm’s canal cells. Meanwhile, potential contamination of cells was assessed by markers for desmin (muscle cell marker; mouse monoclonal; Santa Cruz) and keratin 5 (epithelial cell marker; rabbit polyclonal; Millipore/Sigma).

Additionally, the lysed cell samples from each group were examined by Western blot with an myocilin antibody. Trabecular meshwork cells from culture were limited to the use of up to 3 passages throughout this study. We also used conjunctival tissue cultured from the same donor with dexamethasone treatment as an internal negative control.
Adenosine Triphosphate Measurement in Trabecular Meshwork Cells (under Low and High Oxygen Culture Conditions)

We compared the adenosine triphosphate (ATP) level in TM cells cultured from Black and White healthy donors by following previously published procedures. In brief, primary TM cells were plated in 96-well dishes with the same cell density. When cells reached 80% confluence, they were cultured in low oxygen (2% O2, 5% CO2) and high oxygen (21% O2, 5% CO2) culture conditions for 1 week. The cellular ATP levels were measured by ATP bioluminescence assay Kit CLS II (catalog no., 11 699 695 001; Roche). The culture medium was removed, and 300 μl ice-cold phosphate-buffered saline was added to each well. After a rapid wash, all solution was removed and the TM cell dish was covered with dry ice. The cells were quickly frozen for more than 1 hour. One hundred microliters of phosphate-buffered saline was added to each well, and a small scraper was used to detach the cells. Ten microliters of cell plus phosphate-buffered saline mixture was removed from the well for protein measurement. Eight hundred ten microliters of lysate buffer containing 100 mM Tris and 4 mM edetic acid was added to each well. The solution was mixed gently and then aliquoted into 1.5-ml tubes. The tubes were centrifuged at 7000 g for 10 minutes. The supernatant was then mixed with 50 μl of luciferase reagent by automated injection. The luminescence intensity was detected by a FLUOSmart Optima microplate reader (BMG Labtech) and was integrated for 1 to 10 seconds.

Complex I Activity Assay

Mitochondrial fractions were isolated according to the method of Frezza et al. Briefly, TM cells were trypsinized and centrifuged. The cell pellets were suspended in 3 ml ice-cold mitochondria isolation buffer containing 0.2 M sucrose, 10 mM 3-(N-morpholino)propanesulfonic acid, 10 mM ethylene glycol-bis(β-aminoethy1 ether)-N,N,N′,N′-tetraacetic acid, and 10 mM Tris HCl (pH 7.4) and were homogenized using a glass homogenizer, followed by centrifugation at 600 g for 10 minutes. The supernatant was saved and centrifuged at 7000 g for 10 minutes. Pellets were collected and washed with 200 μl of isolation buffer followed by centrifugation at 7000 g for another 10 minutes. The final fraction enriched in mitochondria was resuspended in an isolation buffer and immediately used for measuring complex I activities. The enzymatic activity of complex I was assayed using the mitochondrial complex I activity assay kit (catalog no., MAK359; Sigma-Aldrich). In brief, 2 μg of mitochondrial was mixed with 66 μl of reaction mixture containing 20 mM monopotassium phosphate, 3.5 mg/ml Bovine serum albumin, 60 μM 2,6-dichlorophenolindophenol (DCIP), 70 μM decylubiquinone (prepared in dimethyl sulfoxide), 1 μM antimycin A at 30 °C, and 10 mM nicotinamide adenine dinucleotide (NADH). The absorbance was measured at 30-second intervals for 5 minutes at room temperature. Subsequently, 1.0 μl of rotenone (1 mM in dimethyl sulfoxide) was added, and the absorbance was measured at 30-second intervals for an additional 5 minutes to confirm rotenone-sensitive complex I activity measurements. The enzyme activity was expressed as nanomoles of DCIP reduced per minute per milligram of protein. The substrate DCIP, a water-soluble final electron acceptor, is specific for complex I because DCIP cannot receive electrons from other nonmitochondrial NADH dehydrogenases.

Mitochondrial Quantification by MitoTracker Red and Translocase of Outer Mitochondrial Membrane 20

To determine mitochondrial differences in cultured TM cells from Black and White patients, we subsequently used MitoTracker Red (M7512; Thermo Fisher), which selectively stains mitochondria in live cells with its accumulation dependent on membrane potential for labeling mitochondria. TM cells from Black and White patients, nearly 70% in confluence, were incubated with prewarmed (37 °C) non serum-cultured medium containing MitoTracker at final concentration 50 nM incubation for 30 minutes. The cells were immediately observed by a fluorescence microscope attached to 37 °C incubated chamber.

To quantify apparent differences more precisely and objectively, we performed Western blot with TOMM20 antibody at 1:1000 dilution and β-actin (mouse monoclonal antibody; Abcam), a central component marker of the mitochondrial outer membrane protein. The lysated cultured TM cells from individual donors loading at 10 μg/lane were run with 10% Bis-Tris gel. The transferred membrane was probed with both TOMM20 antibody at 1:1000 dilution and β-actin (mouse monoclonal antibody; Abcam) at 1:2000 dilution. Secondary antibodies used were IRDye 800CW and 680RD at 1:20 000 dilution. The blot was detected and quantified by Near-Infrared Odyssey CLXs (LI-COR).

Measurement of Reactive Oxygen Species Production Induced by Hydrogen Peroxide in Trabecular Meshwork Cells from Black and White Patients

To determine if mitochondrial alterations are associated with ROS overproduction, cultured TM cells (passages 1–3) were treated with CellROX Orange (Invitrogen) to evaluate the resulting ROS-sensitive fluorescent product. Briefly, the cells were incubated with 5 μM CellROX Orange for 30 minutes and then examined by an inverted phase contrast light and fluorescence microscope attached to a 37 °C chamber. Live-cell imaging (epifluorescent microscope interfaced with imaging software LAS X; Leica) was performed using a ×20 objective at 545 or 565 nm to detect the baseline. Another group of TM cells was challenged with 200 μM hydrogen peroxide (H2O2) for 30 minutes. After assessment of H2O2 concentrations (50–300 μM), we determined that lower concentrations of H2O2 required extended exposure times (2–4 hours), which were poorly tolerated by the sensitive TM cells in culture with fetal bovine serum-free medium. Reactive oxygen species accumulation was measured and compared with the baseline. All experiments were performed in triplicate, and the fluorescence intensity was quantified by ImageJ version 1.51a (National Institute of Health).

Statistical Analysis

Statistical analysis was performed by GraphPad Prism software version 9.0 (GraphPad). Demographic data comparing racial backgrounds were analyzed by the 2-tailed Student t test or chi-square test. Multiple linear regression analyses were performed with adjustment for potential biological confounding variables, including age, race, glaucoma severity, and IOP. For 8-OHdG data analysis, the outlier data were identified by the robust regression and outlier removal method (Q = 1%). To compare ATP levels and ROS production stratified by races, we applied Tukey’s multiple comparisons test (ordinary 1-way analysis of variance) for the data analysis. A P value of < 0.05 was considered statistically significant.
**Results**

8-Hydroxy-2'-Deoxyguanosine Levels in the Aqueous humor of Patients with Glaucoma

We compared 8-OHdG levels in AH collected from 173 eyes of 173 patients who had undergone surgery either for cataract, POAG, or combination POAG with cataract extraction (Table 1). The patients with cataract diagnosis only were considered the control group and the patients with POAG were further divided into 3 groups of glaucoma severity as described previously (mild, moderate, and severe). All patients were stratified into 2 groups based on their self-reported race. The 75 Black patients and 98 White patients had mean ages of 69.1 ± 10.1 years and 72.4 ± 9.5 years, respectively. Overall, the White group was significantly older than the Black group (P = 0.028). No difference was found between the Black and White groups in terms of 8-OHdG levels based on sex. Further subgroup analyses of age differences between the Black and White groups identified a significant difference only in patients with severe glaucoma. The Black group was significantly younger than the White group in this subgroup (P = 0.045). Black patients showed significantly higher IOP than White patients (18.86 ± 5.53 mmHg vs. 16.86 ± 5.21 mmHg; P = 0.019). Multiple linear regression was performed to assess the impact of variables including age, race, glaucoma diagnosis and severity, and IOP on 8-OHdG levels. Because no significant differences were found in sex or number of glaucoma medications, these variables were excluded from the regression model. In the regression analysis, glaucoma severity was correlated most positively to 8-OHdG (P < 0.001; Table 2). Race did not show a statistically significant effect on 8-OHdG (P = 0.079). However, further detailed subgroup analysis based on race indicated that 8-OHdG levels were significantly higher (P = 0.024) in Black patients (1.59 ± 0.75 ng/ml) compared with White patients (1.17 ± 0.55 ng/ml; Fig 1) only in patients with severe glaucoma. There was no significant racial difference in 8-OHdG measurements in the cataract control group (P = 0.808), mild glaucoma group (P = 0.578), and moderate glaucoma group (P = 0.788; Fig 1). Comparing all cataract control participants of either racial group with all patients with POAG, higher levels of 8-OHdG were found in the glaucoma group (P = 0.010; Fig 1). A total of 12 outliers were identified using the robust regression and outlier removal method and were excluded in the statistical analyses.

**Primary Trabecular Meshwork Cell Culture**

Primary TM cells derived from Black and White donor eyes were used as healthy control TM cells for this study. We first confirmed that the fresh TM tissue obtained from residual corneoscleral rims was identified with generally acknowledged appropriate cell markers aquaporin-1, αBCRY, and fibulin-2 positive staining results at the TM, juxtacanalicular tissue, and Schlemm’s canal, respectively (Fig 2, top row). These cell markers were also expressed in the first passage of cultured TM cells from this tissue source (Fig 2, middle row). We further identified minimal contamination by adjacent epithelial cell marker (keratin 5) and negative staining for ciliary muscle (desmin; Fig 2,

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**Table 1. Patient Characteristics**

| Characteristic          | Black American | White American | P Value |
|-------------------------|----------------|----------------|---------|
| No.                     | 75             | 98             |         |
| Age (years)*            | 69.05 ± 10.10  | 72.42 ± 9.52   | 0.028   |
| Sex                     |                |                |         |
| Male                    | 45             | 54             |         |
| Female                  | 30             | 44             |         |
| No. of glaucoma medications* | 2.35 ± 0.79 | 2.14 ± 0.84 | 0.197   |
| IOP (mmHg)*             | 18.86 ± 5.53   | 16.86 ± 5.21   | 0.019   |
| Control                 |                |                |         |
| Cataract                | 22             | 25             |         |
| Age (yrs)*              | 69.91 ± 8.25   | 73.64 ± 11.91  | 0.215   |
| Glaucoma severity       |                |                |         |
| Mild                    | 10             | 17             |         |
| Age (yrs)*              | 72.90 ± 9.26   | 69.59 ± 6.04   | 0.329   |
| Moderate                | 17             | 30             |         |
| Age (yrs)*              | 69.24 ± 8.30   | 72.17 ± 7.97   | 0.246   |
| Severe                  | 26             | 26             |         |
| Age (yrs)*              | 66.73 ± 12.56  | 73.38 ± 10.66  | 0.045   |

IOP = intraocular pressure.

Boldface values indicate statistical significance.

*Data are presented as no. or mean ± standard deviation, unless otherwise indicated.

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**Table 2. Results of Multiple Regression Analysis for Aqueous Humor 8-Hydroxy-2'-Deoxyguanosine**

| Variable              | β Coefficient | 95% Confidence Interval | P Value |
|-----------------------|---------------|-------------------------|---------|
| Age                   | −0.002        | −0.009 to 0.005         | 0.603   |
| Race                  | −0.132        | −0.281 to 0.016         | 0.079   |
| Glaucoma severity     | 0.113         | 0.051 to 0.175          | <0.001  |
| Intraocular pressure  | 0.008         | −0.006 to 0.021         | 0.270   |
We used 6 donor corneal rims (mean ± SD age, 47.5 ± 12.6 years; 4 male and 2 female donors; 2 Black and 4 White donors) for the primary cell culture, confirming identification of TM cells as described in recently published guidance.35 For donor tissue, the time from death to harvest (Optisol-GS corneal preservation medium) was 8.1 ± 2.3 hours.

After these consensus recommendations on TM cells,35 we successfully established primary TM cell cultures confirmed by steroid-induced specific cell marker myocilin (Fig 3A). Dexamethasone-treated (200 nM) TM cells showed significant induction of myocilin expression compared with untreated cells (Fig 3C). Western blot further confirmed this specific induction of myocilin (Fig 3B) as reported previously.36 The juxtacanalicular cell marker35 αBCRY was also expressed in the cultured TM cells, unaffected by steroid treatment (Fig 3C). Primary conjunctival cell cultures sourced from each donor were used as an internal negative control. This TM cell model mimics TM tissue with mixed TM cell cultures, including cells from juxtacanalicular tissue, Schlemm’s canal, uveoscleral TM, and corneoscleral TM for this study comparing effects of racial background.

**Adenosine Triphosphate Level and Complex I Activity in Black Trabecular Meshwork Cells**

We compared ATP levels in healthy control TM cells cultured from Black and White donor TM tissues to evaluate mitochondrial function. In addition to the traditional culture condition (21% pO2), we also used a culture system with 2% pO2 to mimic the physiologically low-oxygen environment in the eye, as reported in our previous human study.22 Twelve donors were used to establish TM cell cultures in this experiment (mean ± SD age, 50.09 ± 11.71 years; 9 male and 3 female donors; 6 Black and 6 White donors). We found that ATP production was higher in Black TM cells compared with White cells at the low-oxygen (2% pO2) condition (Fig 4A; Black, 181.5 ± 72.6 pmol/mg vs. White, 65.2 ± 25.8 pmol/mg total protein; P = 0.002), but did not reach significant differences in the high-oxygen (21% pO2) condition (P = 0.443). Both Black and White tissues at high-oxygen conditions produced significantly higher ATP (188.1 ± 47.2 pmol/mg and 147.2 ± 22.4 pmol/mg total protein) than White TM cells at the low-oxygen condition (2% pO2; P = 0.029 and P = 0.001, respectively; Fig 4A). However, the oxygen conditions did not affect Black TM cells (P = 0.995, Tukey’s multiple comparisons test with adjusted P value). Each donor TM cell sample was run in triplicate.

Adenosine triphosphate production occurs via the proton gradient created by the electron transport chain (ETC) complexes within the inner membrane of mitochondria. Mitochondrial complex I is the entry point and one of the largest catalytic complexes of the ETC. To explore why ATP level is higher in Black TM cells, we compared the complex I activity in mitochondria isolated from 10 donors (mean ± SD age, 48.1 ± 16.1 years; 6 male and 4 female donors; 5 Black and 5 White donors; passages 2–3). As shown in Figure 4B, complex I activity was higher, but not of statistical significance (P = 0.258), in Black TM cells (31.0 ± 13.9 mU/mg mitochondrial protein) than in White TM cells (20.58 ± 11.1 mU/mg mitochondrial protein).

**Mitochondrial Quantification by MitoTracker Red and Translocase of Outer Mitochondrial Membrane 20**

MitoTracker Red staining indicated that the live TM cells were abundant with mitochondria in the cytoplasm (5 Black and 5 White donors; Fig 5, top row). Western blot of TOMM20 showed significantly higher expression in Black TM cells (n = 11; mean ± SD age, 48.18 ± 16.90 years; 7 male and 4 female donors) than in White TM cells (n =
15; mean ± SD age, 39.14 ± 16.75 years; 9 male and 6 female donors; \( P = 0.001 \), with protein expression normalized by β-actin (Fig 5, bottom row).

**Increased Oxidative Stress in Black Trabecular Meshwork Cells**

 Reactive oxygen species overproduction was measured in healthy control TM cells in culture treated with and without \( \text{H}_2\text{O}_2 \) from 9 donors (mean ± SD age, 47.0 ± 16.6 years; 4 Black and 5 White donors; passages 1–3). As shown in Figure 6, the fluorescence intensity of ROS was slightly higher in Black TM cells compared with White TM cells (live cell images), but not statistically significant (Black, 148.2 ± 31.8 vs. White, 120.8 ± 30.9; \( P = 0.232 \), Tukey’s multiple comparisons test with adjusted \( P \) value). However, when TM cells were challenged with 200 μM \( \text{H}_2\text{O}_2 \), the fluorescence in Black TM cells was significantly higher than that of White TM cells (Black, 775.3 ± 135.8 vs. White, 493.6 ± 73.9; \( P = 0.004 \)).

**Discussion**

 This study provided evidence of greater oxidative damage as reflected in the higher level of 8-OHdG in the AH of Black patients with severe-stage POAG compared with White patients. 8-Hydroxy-2′-deoxyguanosine is a widely recognized, sensitive biomarker of DNA oxidative damage.\(^\text{40}\) 8-Hydroxy-2′-deoxyguanosine has been identified in various
body tissues and fluids, including AH, cerebrospinal fluid, serum, and urine.41,42 This modified DNA is excised and exported into extracellular fluids and may reflect the severity and duration of neurodegenerative disease.43,44 Because TM cells are bathed in AH, which serves as its source of nutrients and transporter of byproducts, AH may also reflect the oxidative “fingerprint” of its surrounding tissues.40 Although our data indicated that 8-OHdG was significantly higher in the AH of Black patients with severe glaucoma compared to White patients with severe glaucoma (Fig 1), the fact that this finding was demonstrated only in severe disease is very interesting. Notably, 8-OHdG has been identified in TM of patients with glaucoma,46 but previous studies of 8-OHdG in AH of patients with glaucoma were not stratified by disease severity or racial background, and instead compared patients with glaucoma only with control participants with cataract.51 Because most of these studies recruited patients undergoing glaucoma filtration surgery, a procedure traditionally reserved for more severe disease, this may explain differences in our data, which included all stages of disease. Our data support these previous findings in a comparison of all stages of glaucoma severity, indicating higher levels of 8-OHdG than in control participants with cataract.23 Both of these AH collections shared significantly higher pO2 levels in the AC, including the AC angle adjacent to the TM, compared with White patients and phakic or

Figure 3. Demonstration of trabecular meshwork (TM) cell primary cultures. A, Phase-contrast micrograph of TM cell outgrowth from corneoscleral rim donor (a 60-year-old White man). B, Western blot showing myocilin (MYOC) induction by dexamethasone (DEX) treatment (n = 6). β-actin serves as loading control. C, Immunocytochemistry staining of (top row) myocilin confirming significant induction by dexamethasone (red; n = 8), (middle row) partial expression of αB crystallin (αBCRY; green) in TM cells with no response to dexamethasone, and (bottom row) combined staining DAPI (blue). DAPI = 4′,6-diamidino-2-phenylindole. Bar = 50 μm. Right column shows high-magnification images.
nonvitrectomized monkeys in our previous studies, respectively.\textsuperscript{14,23} This is an essential finding in revealing a potential biological foundation for racial disparities in glaucoma; however, 8-OHdG in AH may only indirectly reflect the oxidative status in the AC tissues because its precise source cannot be determined definitively and may be affected by other unknown factors, such as other ocular or systemic medications. Importantly, we excluded other ocular conditions that may affect AH levels of 8-OHdG, including proliferative diabetic retinopathy,\textsuperscript{45} exudative macular degeneration,\textsuperscript{46} corneal endothelial dysfunction, and a history of pars plana vitrectomy.\textsuperscript{23} In addition, we excluded other secondary forms of glaucoma and evaluated only patients with POAG. Notably, a significant difference was found in the age of patients with severe glaucoma, with Black patients being younger than White patients. One might surmise that aging would be correlated with oxidative damage, but this was not the case.

Figure 4. \textbf{A}, Graph showing trabecular meshwork (TM) cell adenosine triphosphate (ATP) production and complex I activity. Blue, White TM cells; red, Black TM cells. (Black, \(n = 6\); White, \(n = 6\); passages 1–2, Tukey’s multiple comparisons test with adjusted \(P\) values). \textbf{B}, Complex I activity in mitochondria isolated proteins from 10 donors (Black, \(n = 5\); White, \(n = 5\); passages 2–3; 2-tailed Student \(t\) test). Red dots, Black; blue dots, White. Bar = mean ± standard deviation.

Figure 5. Mitochondrial quantification. \textbf{Top row}, MitoTracker Red staining of live trabecular meshwork cells with abundant mitochondria. \textbf{Bottom row}, Western blot quantification of translocase of outer mitochondrial membrane 20 (TOMM20) showing significantly higher expression in Black (\(n = 11\)) than White (\(n = 15\)) cells. Red dots, Black; Blue dots, White. Error bar = mean ± standard deviation.
case in this cohort of patients with severe glaucoma. Thus, further investigation of the role of oxidative stress specifically in the TM is critical in this study.

Using AH from patients with POAG, our 8-OHdG data clearly indicated that increased oxidative stress may be associated with disparities in Black patients with glaucoma. However, whether oxidative stress is an underlying cause or a direct consequence of disease remains unclear. To answer this critical question of causation versus correlation, we used TM cells cultured from healthy Black and White donors. This allowed us to explore possible biological disparities in glaucoma and to build a fundamental basis regarding aspects of oxidative stress and damage. Notably, these donors were highly significantly younger than the patients with POAG and cataract recruited for the intraoperative AH sample collection (mean ± SD age, 46.1 ± 15.4 years vs. 71.1 ± 10.0 years; \( P < 0.0001 \)). We emphasize that no suitable animal model or cell line is available to study racial disparities; thus, human eye tissue is the only acceptable source for this study. Acknowledging the limited supply of human TM tissues obtained directly from patients with confirmed POAG and donors, primary cultured TM cells are a precious model system to study various cellular mechanisms.36

Mitochondria are the primary site of energy production in TM cells, consuming molecular oxygen and producing ATP via the tricarboxylic acid cycle and oxidative phosphorylation (OXPHOS).47 The tricarboxylic acid cycle, also known as the Krebs cycle, is a series of catabolic reactions that generates both energy and reducing power. For each completed cycle, 1 molecule of flavin adenine dinucleotide (quinone form) is reduced to flavin adenine dinucleotide (hydroquinone form) and 3 molecules of NAD\(^+\) (oxidized form of NAD) are reduced to NADH. These molecules then transfer their energy to the mitochondrial respiratory chain, also known as the ETC, to produce ATP through OXPHOS. In this study, ATP levels in TM cells cultured from healthy Black TM tissues under high-oxygen conditions (21% pO\(_2\)) and a low-oxygen environment (2%) measured in human eyes in vivo\(^{22}\) are more significant compared with levels in healthy donor White TM cells. Our data were further supported by Krzywanski et al.,\(^{48}\) who demonstrated that primary human umbilical vein endothelial cells isolated from healthy Black donors tend to have higher intracellular ATP levels than White donors. Elevated ATP levels suggest that Black TM cells compared with White TM cells may have enhanced OXPHOS, and thus generate additional mitochondrial-associated ROS as the byproduct of ATP production. This result is consistent with the finding that a higher pO\(_2\) environment would be a source of oxidative stress and damage to porcine TM cells.\(^{49}\) Specific African mitochondrial DNA haplogroups have been identified as risk factors for POAG, although the biological mechanism currently is unknown.\(^{50,51}\) These haplogroups of various geographic origins may play a role in OXPHOS efficiency and disease susceptibility.\(^{52}\) Interestingly, genes associated with enhanced OXPHOS and mitochondrial biogenesis may also underlie the increased incidence and worse outcomes of several types of cancer in Black versus White patients.\(^{53}\)

In addition to being a powerhouse of ATP, mitochondria are also the major source of ROS in most mammalian cells. Under nonstressed conditions, ROS are produced as the byproduct for ATP generation. For each tricarboxylic acid cycle, 0.2% to 2% of the electrons may leak out from the ETC and react directly with oxygen to produce ROS such as superoxide and H\(_2\)O\(_2\).\(^{47,54}\) Currently, 11 sites in the ETC have been identified as the source of superoxide or H\(_2\)O\(_2\) production, or both. Among these targets, the flavin mononucleotide group, iron-sulfur clusters, Coenzyme Q binding sites in complex I, and the outer Coenzyme Q-binding sites in complex III have a significant role in ROS production.\(^{47,55}\) In the present study, we detected higher complex I activity in Black TM cells compared with
White TM cells. Our findings are consistent with other reports indicating that the cells transfected with African origin L haplogroup showed much higher expression levels of 5 mitochondrial DNA-encoded mitochondrial complex I subunits, including NADH dehydrogenase subunits 1 through 3 and NADH dehydrogenase subunits 5 and 6. Collectively, our data showed a trend of elevated complex I activity that may partially explain the higher levels of ATP and elevated ROS production in Black TM cells. Because we showed that Black cells contain more mitochondrial protein, this alters the calculation of mitochondrial complex I activity based on milliunits per milligram of mitochondrial protein. Consequently, Black TM cells would be expected to have greater complex I activity. Other than complex I activity, complex III activity may also be increased in Black compared with White TM cells. Because we showed that Black cells contain more mitochondrial protein, this alters the calculation of mitochondrial complex I activity based on milliunits per milligram of mitochondrial protein. Consequently, Black TM cells would be expected to have greater complex I activity. Other than complex I activity, complex III activity may also be increased in Black compared with White TM cells.

The summation of elevated complex I activity, elevated complex III activity, and higher mitochondrial content in Black TM cells may lead to increased ATP production, resulting in higher ROS levels. In future research, we will increase specimen numbers and explore more detailed analysis of complex I (e.g., gene and protein expression levels of subunits), as well as mitochondrial complex III activity, another critical factor for ATP production and ROS production.

Several studies have examined racial differences in oxidative metabolism that reflect differences in systemic physiology, including lower maximum oxygen consumption in Black individuals compared with White individuals. These racial differences were present in participants across the age spectrum from prepubertal children and adolescent girls, and from sedentary, premenopausal women to similarly trained male distance runners. In one study, physiologic analysis revealed that mitochondrial oxidative capacity and oxygen delivery capability accounted for most of the measured differences in maximum oxygen consumption. It has not been demonstrated that similar biochemical differences account for the racial variation in oxygen metabolism in the eye. However, the ocular anterior segment offers an excellent opportunity to study the biochemical factors influencing oxygen use and mitochondrial function. The oxygen source for these tissues in AH is not affected by changes in local or systemic blood flow, providing a unique environment for these physiologic studies. Notably, Black patients also have increased levels of systemic oxidative stress compared with White patients, after adjustment for differences in cardiovascular disease risk factors and inflammation. In addition, significant correlates of systemic antioxidant status and oxidative DNA damage differ for Black and White individuals.

Alvarado et al first suggested that aging and oxidative stress underlie the degeneration of TM cells in patients with glaucoma. Subsequent studies provide strong evidence for oxidative damage in the outflow pathway and mitochondrial dysfunction of TM cells. Our data indicated that, compared with White patients, Black patients undergoing intraocular surgery showed significantly higher intraocular pO2 in the AC angle, the region of AH outflow and TM. Notably, our clinical data also showed significantly higher levels of 8-OHdG in AH of Black patients compared with White patients (Fig 1) with severe POAG. The lack of differences found in earlier disease stages is not surprising because 8-OHdG reflects an advanced level of oxidative damage consistent with advanced disease. In vitro study of TM cells further confirmed that Black TM cells showed lower resistance in response to H2O2 challenge, producing significantly higher ROS levels than White TM cells from healthy donors (Fig 5). These fundamental physiologic differences of measured oxidative damage, increased pO2 in the surrounding AH (potential ROS source), and greater vulnerability to H2O2 challenge identified in Black versus White TM cells may provide significant evidence for mechanisms of increased risk and severity of POAG in Black individuals.

As mentioned above, besides functioning as a powerhouse, mitochondria are also the major source of ROS within most mammalian cells, including TM cells. Elevated pO2 in AH of Black patients may provide a pro-oxidant environment favoring ROS production in TM cells. When we compared the ROS level in TM cells under nonstressed conditions, Black TM cells trended toward slightly increased intracellular ROS, but the difference was not statistically significant. Strikingly, when cells were exposed to H2O2, the ROS production was almost doubled in Black TM cells compared with White TM cells. This finding is important because long-term exposure to higher ROS levels leads to sustained stress response and eventually may lead to cellular damage in Black TM cells. One possible explanation is that the antioxidant defense system of Black TM cells may be weaker than that of White TM cells, which is supported by our data that indicated higher ROS levels after a challenge with H2O2. The major antioxidant enzymes that directly detoxify ROS include superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins, whereas small-molecule antioxidants include glutathione, ascorbic acid, and α-tocopherol. In addition, thiol-based antioxidant enzymes including thioredoxin, glutaredoxin, and methionine sulfoxide reductases are also crucial for oxidative damage. Additional evidence supporting the role of oxidative damage in TM degeneration includes significantly decreased total reactive antioxidant potential and increased activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase in AH from patients with glaucoma. These findings highlight the importance of oxidative stress in the pathogenic cascade of the disease. To investigate the possible origin of these excessive ROS, a future study is needed to test whether the aforementioned antioxidant defense systems are compromised in Black TM cells.

Some limitations in this initial study need to be addressed in future research. Unknown ocular or systemic disease history, including smoking history and body mass index from both patients and donor tissues, and possible inaccuracy of self-reported race, may influence our findings. Given the heterogeneous genetic background of mixed ancestry in the United States, this represents a significant challenge for binary classification. To overcome these challenges, a more comprehensive racial stratification using genomics and...
mitochondrial DNA haplogroup analyses will be used in future studies. Importantly, we plan to challenge TM cells cultured from fresh POAG TM surgical specimens to compare with healthy control TM, as in this study. Future research is also needed to decode the mitochondria—ROS crosstalk and to clarify how oxidative stress and mitochondrial alterations in TM cells contribute to the racial disparity of glaucoma.

This is a key study to compare racial differences in oxygen metabolism and mitochondrial function by using primary TM cells as a model. We found that Black TM cells have a higher ATP level compared with White TM cells, indicating that race-associated mitochondrial alterations may play a role in glaucoma disparity. In addition, we also found that ROS detoxification function was compromised in Black patients. These findings are important because elevated pO₂ in AH compounded with weaker antioxidative defenses in TM cells may contribute significantly to glaucoma risk in individuals of African descent. Thus, the primary TM culture systems from healthy donor eyes offer a powerful tool to unravel the biological cause of racial disparities. Our study provides initial insights to support the hypothesis that racial differences in oxygen and mitochondrial metabolism and oxidative stress may play an important role in glaucoma disparities. This research may also aid in the development of novel targeted therapies and individualized treatment for Black patients with glaucoma.

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Data collection: Wu, Shui, Y.Liu, X.Liu, Siegfried
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Abbreviations and Acronyms:

\( \alpha \text{BCRY} \) = alpha B crystallin;
\( \text{AC} \) = anterior chamber;
\( \text{AH} \) = aqueous humor;
\( \text{ATP} \) = adenosine triphosphate;
\( \text{DCIP} \) = 2,6-dichlorophenolindophenol;
\( \text{ETC} \) = electron transport chain;
\( \text{H}_2\text{O}_2 \) = hydrogen peroxide;
\( \text{IOP} \) = intraocular pressure;
\( \text{NADH} \) = nicotinamide adenine dinucleotide;
\( \text{OXPHOS} \) = oxidative phosphorylation;
\( \text{pO}_2 \) = partial pressure of oxygen;
\( \text{POAG} \) = primary open-angle glaucoma;
\( \text{ROS} \) = reactive oxygen species;
\( \text{TM} \) = trabecular meshwork;
\( \text{TOMM20} \) = translocase of outer mitochondrial membrane 20;
\( \text{8-OHG} \) = 8-hydroxy-2'-deoxyguanosine.

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