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# Intraocular Oxygen and Antioxidant Status: New Insights on the Effect of Vitrectomy and Glaucoma Pathogenesis



CARLA J. SIEGFRIED AND YING-BO SHUI

• **PURPOSE:** The purpose of this study was to investigate correlations of partial pressure of oxygen ( $pO_2$ ) in the ocular anterior segment of human eyes and aqueous humor antioxidant levels of ascorbate (AsA) and total reactive antioxidant potential (TRAP) with glaucoma and vitreous status.

• **METHODS:** This prospective, cross-sectional study stratified patients ( $n = 288$  eyes) by lens and vitreous status and the presence of primary open-angle glaucoma for statistical analyses. Intraocular  $pO_2$  concentrations were measured using a fiberoptic probe in patients at the beginning of planned glaucoma and/or cataract surgery. Aqueous humor specimens were obtained for antioxidant analysis of AsA and TRAP.

• **RESULTS:** Following prior pars plana vitrectomy,  $pO_2$  levels were significantly higher than in the reference group of cataract surgery in the anterior chamber angle ( $16.2 \pm 5.0$  vs.  $13.0 \pm 3.9$  mm Hg;  $P = .0171$ ) and in the posterior chamber ( $7.6 \pm 3.1$  vs.  $3.9 \pm 2.7$  mm Hg;  $P < .0001$ ). AsA and TRAP levels were significantly lower ( $1.1 \pm 0.4$  vs.  $1.4 \pm 0.5$  mM, respectively;  $403.3 \pm 116.5$  vs.  $479.0 \pm 146.7$  Trolox units, respectively;  $P = .004$  and  $P = .024$ , respectively) in patients after vitrectomy. In patients with an intact vitreous, neither  $pO_2$  nor antioxidant status correlated with lens status or glaucoma.

• **CONCLUSIONS:** Increased  $pO_2$  and antioxidant depletion following vitrectomy suggests an alteration of the intraocular oxidant-antioxidant balance. Our study links physiologic factors such as increased  $pO_2$  in the anterior chamber angle and the posterior chamber to decreased antioxidant levels in aqueous humor following vitrectomy. Oxidative stress/damage to the trabecular meshwork in such post-vitrectomy cases may contribute to intraocular pressure elevation and increased risk of glaucoma. **NOTE:** Publication of this article is sponsored by the American Ophthalmological Society. (Am J Ophthalmol 2019;203:12–25. © 2019 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).)

**T**HE PRECISE PATHOGENESIS OF PRIMARY OPEN ANGLE glaucoma has not been fully elucidated. It likely represents a variety of different pathologies, genetic predispositions, and contributing environmental factors. Alterations in the local environment of the trabecular meshwork (TM), the main pathway for the conventional outflow of aqueous humor, may also affect its function, leading to increased intraocular pressure (IOP), an important risk factor for glaucoma. Understanding that ocular structures are “interconnected” is not a new idea. For example, feedback mechanisms of IOP regulation exist through nitric oxide synthesis in the TM,<sup>1</sup> cyclic mechanical stress yields alterations in conventional outflow facility,<sup>2</sup> and TM cells undergo contractile changes, potentially through rho-kinase-mediated signaling.<sup>3</sup> In addition to these physiologic mechanisms, the concept of intraocular surgical procedures modifying this environment in a potentially deleterious manner has been previously noted.<sup>4,5</sup>

Pars plana vitrectomy (PPV) now represents the third most common intraocular procedure performed in the United States, with overall rates in the Medicare population increasing 31% from 2001 to 2012.<sup>6</sup> This increase is likely related to improvements in the safety profile of the procedure, improved success of vision-saving surgical interventions, decreased use of alternative surgical therapies, and earlier intervention for non-vision-threatening pathologies (eg, “floaterectomy”). Evaluation of adverse events of this procedure between 1994 and 2005 indicated, however, that rates of severe complications such as endophthalmitis, suprachoroidal hemorrhage, and retinal detachment remained stable, but rates of less-severe complications such as glaucoma increased with the prevalence of vitrectomy.<sup>7</sup>

The vitreous humor is an important ocular structure that plays a prominent role in maintaining biochemical homeostasis, consuming molecular oxygen ( $pO_2$ ) and protecting the lens from oxidative damage.<sup>8</sup> Shui and colleagues<sup>8</sup> found that vitreous gel, in comparison to liquefied vitreous due to myopia, aging, or surgical removal, has a higher concentration of ascorbate (AsA) and consumes  $pO_2$  at a faster rate. Previous studies indicated that antioxidant levels, specifically AsA and glutathione, are present in high concentrations in the vitreous humor.<sup>9,10</sup> The discovery of this role of vitreous gel to maintain the physiologic hypoxic environment around the lens is important. Initial studies of patients undergoing long-term hyperbaric oxygen therapy noted a 50% incidence of nuclear cataract

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development within 1 to 3 years.<sup>11</sup> In patients undergoing PPV, it has been observed that a nuclear sclerotic cataract develops and progresses rapidly in the ensuing 12 to 18 months following vitrectomy, with 37% to 95% of patients requiring cataract extraction within 2 years.<sup>12–18</sup> Vitrector gauge size did not influence these results.<sup>19</sup> Increased vitreous liquefaction increases the risk of nuclear cataract development.<sup>20</sup> Identification of increased pO<sub>2</sub> levels within the vitreous cavity and at the posterior surface of the lens following vitrectomy led to the proposal that increased pO<sub>2</sub> exposure leads to oxidative damage to the lens and nuclear cataract formation.<sup>21</sup>

Besides the lens, other ocular structures are continuously exposed to a broad spectrum of pO<sub>2</sub> levels, ranging from hyperoxic to markedly hypoxic. Cells exposed to high pO<sub>2</sub> levels as well as ultraviolet light (eg, corneal epithelium) contain nuclear ferritin,<sup>22,23</sup> AsA,<sup>24</sup> glutathione,<sup>25</sup> superoxide dismutase, and catalase and other antioxidants.<sup>26,27</sup> Ocular cells that function at low physiologic levels of pO<sub>2</sub> are unlikely to adapt to altered (ie, higher) levels of pO<sub>2</sub> exposure. Oxygen either is consumed by functioning cells and/or antioxidants, remains in its molecular form, or is transformed into other potentially unstable reactive oxygen species (ROS), capable of causing damage to RNA, DNA, and proteins. “Oxidative stress” is defined as an increase over physiologic values in the intracellular concentrations of ROS, which include superoxide anions, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals, peroxy radicals, and singlet oxygen. Such ROS may be detrimental to cellular structures, destroying membrane lipids as well as structural and enzymatic proteins and DNA, contributing to cell senescence and potentially genetically programmed cell death or apoptosis. Cellular dysfunction results from decreased mitochondrial respiratory function and protein degradation.<sup>28</sup> Increases in intracellular ROS may be the result of increased endogenous production by mitochondrial respiration or decreased antioxidant capacity. Increased oxidative stress has been identified as a contributing factor to the pathogenesis of several age-related ocular diseases including glaucoma.<sup>29,30</sup>

Evidence supporting such oxidative damage to the TM was initially reported by Alvarado and colleagues<sup>31</sup> in 1981, as they first suggested that aging and oxidative stress underlay the degeneration of TM cells in patients with glaucoma. Cell senescence has been shown to increase generation of ROS, leading to reduced numbers and function of mitochondria.<sup>32</sup> As a result of this exposure to oxidative stress, changes occur in TM protein expression that affect extracellular matrix turnover. For example, in vivo perfusion of calf anterior segments with H<sub>2</sub>O<sub>2</sub> following depletion of glutathione in the TM increases outflow resistance.<sup>33</sup> TM tissue, compared to corneal and iris tissues, was found to be most sensitive to oxidative damage induced by exposure to H<sub>2</sub>O<sub>2</sub>.<sup>34</sup> Subsequent studies provide evidence of oxidative damage and reduced

resistance to oxidative stress in the outflow pathway.<sup>35,36</sup> Oxidative damage to DNA is greater in TM cells in glaucoma patients than in controls<sup>37</sup> and correlates with IOP level and visual field loss.<sup>38</sup> Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an established biomarker of oxidative DNA damage, are significantly higher in both aqueous humor and sera in patients with glaucoma than in controls.<sup>39</sup> Increased levels of 8-OHdG have also been found in TM specimens from primary open-angle glaucoma (POAG) patients.<sup>38</sup>

Importantly, PPV has also been associated with increased pO<sub>2</sub> exposure to the microenvironment of the TM and outflow pathways<sup>40</sup> and increased risk of developing open-angle glaucoma in several retrospective clinical studies<sup>41–46</sup> and a recent population-based study.<sup>47</sup> The present study was undertaken to provide further understanding of the impact of exposure to increased pO<sub>2</sub> and/or its metabolites in the local environment of the aqueous outflow pathways following vitrectomy. We hypothesized that increased pO<sub>2</sub> in these cases may contribute to alterations of oxidant-antioxidant balance leading to increased oxidative stress and damage of the TM. To assess these conditions in human subjects, we measured in vivo levels of pO<sub>2</sub>, total reactive antioxidant potential (TRAP) activity, and AsA levels in aqueous humor in eyes of patients undergoing glaucoma and/or cataract surgery to determine associations with glaucoma and vitreous status.

## METHODS

• **STUDY DESIGN:** This prospective, cross-sectional study was approved by the Institutional Review Board of the Washington University School of Medicine, in compliance with the tenets of the Declaration of Helsinki and Health Insurance Portability and Accountability Act guidelines. Informed consent was obtained from subjects after the nature and possible consequences of the study were explained to them. This study was designed to measure pO<sub>2</sub> distribution within the anterior segment of the eye and to collect aqueous humor for measurement of antioxidants in patients undergoing cataract and/or glaucoma surgery in an academic clinical practice. Patients were excluded from the study if there was evidence of corneal endothelial dysfunction; ischemic ocular disease, including diabetic retinopathy, anterior chamber angle closure, inflammatory or traumatic ocular disease, ocular neoplasia, requirement for general anesthesia; or monocular status.

• **PATIENTS AND PO<sub>2</sub> MEASUREMENTS:** A complete general medical and ophthalmic history and comprehensive ophthalmic examinations, including Lens Opacities Classification System III (LOCS III) analysis for quantitative and qualitative assessment of lens opacities, were performed prior to surgical intervention. The use of topical

glaucoma medications within 1 month of the surgical procedure was verified preoperatively. Patients with a diagnosis of POAG (based on optic nerve and visual field criteria) were classified by glaucoma severity as mild, moderate, or severe (using Hodapp Parrish Anderson criteria).<sup>48</sup> Central corneal thickness was measured by ultrasonography (DGH 55 Pachmate, DGH Technology Inc., Exton, Pennsylvania), and axial length measurements were recorded (IOLMaster 500, Carl Zeiss Meditec, Inc., Jena, Germany) for patients undergoing cataract extraction. Racial background was based on self-reports, as indicated on a standardized registration questionnaire.

According to our routine surgical protocol, the patient was placed in a supine position, intravenous sedation was administered, the eye was prepared and draped, and a lid speculum was placed. Supplemental pO<sub>2</sub> (21%–30%) was provided through nasal cannulation and separated from the ocular region by adhesive sterile drape to avoid any additional pO<sub>2</sub> exposure. This technique did not impact intraocular pO<sub>2</sub> measurements as previously reported.<sup>21</sup> Blood oxygen saturation was monitored by continuous pulse oximetry and maintained between 95% and 100%. Topical lidocaine hydrochloride jelly, 2%, was placed on the ocular surface in the preoperative area. A sub-Tenons injection of 1 to 3 ml of 2% lidocaine and 0.375% bupivacaine mixture (50/50) was performed to provide additional local anesthesia as indicated. At the beginning of the planned surgical procedure, a 30-gauge needle was used for entry through the periphery of a clear cornea into the anterior chamber (AC) and an Oxylab pO<sub>2</sub> optical oxygen sensor probe (Optode; Oxford Optronix, Oxford, United Kingdom) was then carefully introduced into the AC without aqueous humor leakage. The instrumentation was calibrated prior to each set of measurements. Under direct visualization with an operating microscope, the tip of the flexible fiberoptic probe was positioned for 3 measurements in all patients as described in our previous studies: 1) underneath the central corneal endothelium, 2) in the mid-AC, and 3) in the AC angle.<sup>49,50</sup> In pseudophakic patients or those scheduled to undergo cataract extraction, 2 additional measurements were obtained, 4) at the central anterior lens surface and 5) in the posterior chamber just behind the iris. These latter 2 measurements were not made in patients remaining phakic in order to avoid risk of lens damage. Approximately 46 seconds (total: <5 minutes) was required for each set of measurements. In order to confirm precise and consistent probe positioning and stabilization of the pO<sub>2</sub> level, duplicate testing in the same locations were performed for verification.

• **AQUEOUS HUMOR SPECIMEN COLLECTION:** After pO<sub>2</sub> levels were measured, the needle entry site in the cornea was slightly enlarged with a 15-degree blade or side port

instrument, and a sample of aqueous humor (50–100 µL) was drawn into a 1-mL tuberculin syringe through a 30-gauge blunt cannula, followed by re-inflation of the AC volume with balanced salt solution. Care was taken to avoid contamination of the specimen with blood. The aqueous humor 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. The scheduled surgical procedure was subsequently performed with standard postoperative management, and the patients were monitored for any complications.

• **AQUEOUS HUMOR ANALYSIS OF ASCORBATE:** AsA concentration was quantified in triplicate, based on its ability to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup> and the resulting change in the A<sub>525</sub> of complexes of Fe<sup>2+</sup> with 2,2'-dipyridyl.<sup>51</sup> Assay modifications enabled the analysis of 10-µL samples, and a standard curve was used for all measurements, as in our previous report.<sup>8</sup> Gas chromatography-mass spectrometry studies confirmed the specificity of this colorimetric assay. Samples of aqueous humor were mixed with a known amount of carbon 13-labeled ascorbic acid (Omicron Biochemicals, South Bend, Indiana), dried, and then reacted with N,O-bis(trimethylsilyl)trifluoroacetamide. The sample was separated on a gas chromatograph (Varian Inc., Palo Alto, California) using a 30-m, 0.25-mm-internal gas chromatography column with a 0.25-µm film (DB-5 ms column; PJ Cobert Associates Inc., St. Louis, Missouri), maintained at 80°C for 1 minute and then eluted with a temperature gradient of 80°C to 300°C at 15°C/minute. The injection port and transfer line were at 250°C and the source temperature at 200°C of a mass spectrometer (Finnigan MS SSQ7000; Thermo Electron Corp, Waltham, Massachusetts) operated in the electron ionization mode at 70 eV. AsA concentration was calculated from the [<sup>13</sup>C]AsA-to-[<sup>12</sup>C]AsA ratio. In order to confirm the specificity of the AsA assay, 2 units of ascorbate oxidase (AO; A 0157, Sigma Chemical, St. Louis, Missouri) were added to each of the 10-µL samples and mixed well at room temperature, and AsA measurements were repeated.

• **TOTAL REACTIVE ANTIOXIDANT POTENTIAL:** The TRAP assay is a means to determine the ability of a sample to destroy chemically generated free radicals. A sample is added to a solution containing 2,2'-azobis(2-amidinopropane) (ABAP) (Sigma Aldrich, St. Louis, Missouri) and 40 µM luminol (3-aminophthalhydrazide; Sigma Aldrich). ABAP combines with pO<sub>2</sub> to produce alkyl peroxy radicals at a constant rate. In the absence of antioxidant, these radicals react with luminol to produce light, which is measured in a scintillation counter. Antioxidants quench luminescence by reacting with the peroxy radicals. Because ABAP produces radicals at a constant rate, antioxidant activity is measured by the length of time required to quench luminescence. The assay is standardized using Trolox, a water-soluble

**TABLE 1. Patient Demographic Information and Group Descriptions**

Groups	Group Description	Eyes (n)	Age (y)	F/M	Type of Surgery			Race		
					CAT	GL	Combined	AA	CC	Other
CAT	No prior history of eye surgery or POAG (reference group)	72	68.0 ± 11.4	48/24	72	0	0	26	44	2
GL/CAT	POAG undergoing glaucoma surgery or combined cataract/glaucoma surgery	143	70.4 ± 10.8	80/63	35	30	78	39	102	2
GL/IOL	Pseudophakic POAG patients undergoing glaucoma surgery	39	73.8 ± 9.0	32/7	0	39	0	10	29	0
VIT	Patients who had undergone previous pars plana vitrectomy	34	63.1 ± 13.5	18/16	24	10	0	6	27	1
Total		288		178/110	131	79	78	81	202	5

AA = African American; CAT = cataract; CC = caucasian; F = female; GL = glaucoma; IOL = intraocular lens; M = male; POAG = primary open-angle glaucoma; VIT = prior vitrectomy.

vitamin E analog, and reported as “Trolox units,” with 1 unit equal to the amount of time required to quench luminescence by a sample containing 1  $\mu$ M Trolox. TRAP was measured in the aqueous humor samples, and samples were treated with ascorbate oxidase to remove AsA as described above. Measurements were then repeated in order to differentiate AsA- from non-AsA-dependent effects on the composite TRAP value.

• **STATISTICAL ANALYSIS:** Multivariate regression analyses were performed with adjustment for all potential confounding variables ( $P < .1$ ) including age, sex, race, medications, and lens status by using SPSS version 24.0 software (Chicago, Illinois). The  $t$ -test, one-way ANOVA with multiple comparison analysis (Bonferroni correction), and Spearman correlation analyses were performed using Prism version 8.0 software (GraphPad, La Jolla, California). Results are expressed as means  $\pm$  SD.  $P$  values less than .05 were defined as statistically significant.

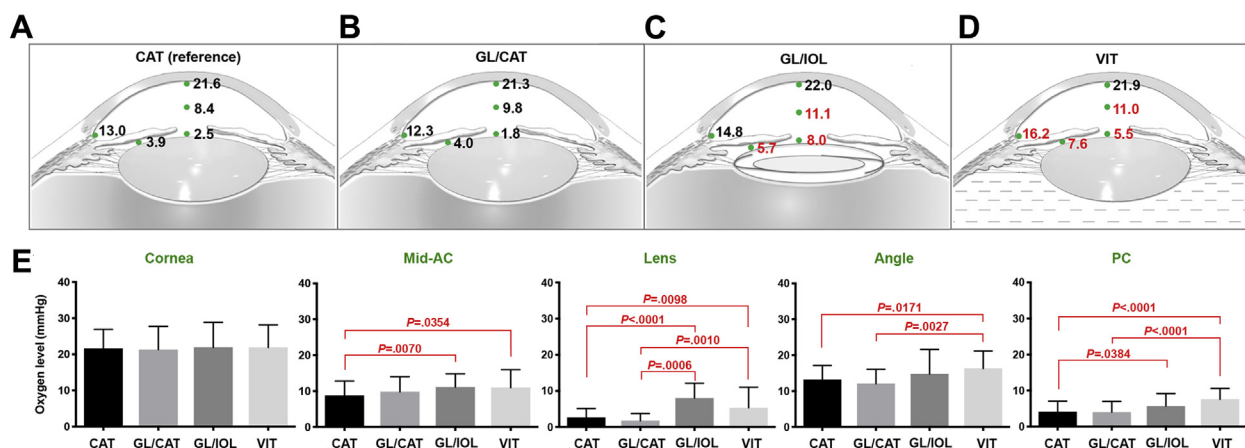
## RESULTS

• **PATIENT RECRUITMENT AND GROUP ANALYSIS:** A total of 288 eyes of 288 patients participated in the study between July 2007 and August 2015. Our initial cohort (July 2007-July 2010) of 112 eyes of 112 patients were included from a previously published study evaluating intraocular pO<sub>2</sub> measurements.<sup>49</sup> We extended that work to study a total 288 eyes for pO<sub>2</sub>, AsA, and TRAP measurements after exclusion of 24 eyes due to inadequate specimen collection. Patients with secondary open-angle glaucoma (ie, pseudoexfoliative or pigmentary) and low-tension glaucoma were excluded from this study. Patient characteristics (Table 1) indicate a greater number of females and caucasian patients in the study. The cataract

group (CAT) had no history of ocular surgery, glaucoma, or exposure to ocular glaucoma medications. That group served as the reference or control group for select statistical analyses. Consistent with our previously published data,<sup>49</sup> patients with a diagnosis of POAG (GL) were subdivided into patients undergoing glaucoma surgery or combined cataract and glaucoma surgery (GL/CAT) and pseudophakic patients undergoing glaucoma surgery (GL/IOL). Patients with a history of vitrectomy who had previously undergone PPV for vitreoretinal conditions including rhegmatogenous retinal detachment, epiretinal membrane, and macular hole but excluding those with proliferative retinopathy made up the VIT group. All these patients were either pseudophakic or were scheduled to undergo cataract extraction or glaucoma surgery. Patients in the GL/CAT and GL/IOL groups were older than those in the VIT group ( $P = .0005$  and  $P = .0001$ , respectively). The GL/IOL patients were also older than the CAT reference group patients ( $P = .005$ ). Subgroup analyses were performed to identify correlations with race, age, sex, lens, vitreous status, and ocular medications with pO<sub>2</sub> levels and antioxidant status. We randomly selected 1 eye for the final data analysis in patients who had measurements and specimens taken from both eyes.

• **INTRAOCULAR PO<sub>2</sub> MEASUREMENTS:** Oxygen measurements at five intraocular locations were analyzed by multiple comparison analysis with a Bonferroni correction (Figure 1). Intraocular pO<sub>2</sub> measurements were significantly higher after vitrectomy (VIT) than those in the reference (CAT) group in the AC angle ( $16.2 \pm 5.0$  mm Hg vs.  $13.0 \pm 3.9$  mm Hg, respectively;  $P = .0171$ ) and posterior chamber ( $7.6 \pm 3.1$  mm Hg vs.  $3.9 \pm 2.7$  mm Hg, respectively;  $P < .0001$ ). In the GL/IOL (pseudophakic) group, there were significantly higher levels of pO<sub>2</sub> at the anterior lens surface than in the reference group ( $8.0 \pm 4.1$  mm Hg vs.  $2.5 \pm 2.4$  mm Hg, respectively;





**FIGURE 1.** Intraocular pO<sub>2</sub> measurements (mm Hg) were made at the indicated locations (green dots). (A) CAT groups were used as references for comparison with the other groups. Red numbers indicate P values < .05. (B) GL/CAT indicates diagnosis with cataract (C) GL/IOL shows glaucoma diagnosis with history of prior cataract surgery. (D) History of prior pars plana vitrectomy. (E) Comparison among pO<sub>2</sub> levels at intraocular locations. Multiple comparisons were made using ANOVA and Bonferroni correction; P values < .05; error bar indicates mean mm Hg ± SD. ANOVA = analysis of variance; CAT = cataract; GL = glaucoma; IOL = intraocular lens; VIT = vitrectomy.

$P < .0001$ ), in the mid-AC ( $11.1 \pm 3.8$  mm Hg vs.  $8.4 \pm 3.9$  mm Hg, respectively;  $P = .007$ ), and in the posterior chamber ( $5.7 \pm 3.4$  mm Hg vs.  $3.9 \pm 2.7$  mm Hg, respectively;  $P = .0384$ ).

- **ASCORBATE MEASUREMENTS:** AsA levels were significantly lower in the VIT group ( $1.1 \pm 0.4$  mM;  $P = .004$ ) than in the CAT reference group ( $1.4 \pm 0.5$  mM). We further confirmed the fact that AsA is correlated with prior vitrectomy in a multivariate regression model ( $\beta = -.198$ ;  $P = .004$ ). AsA levels were increased in phakic patients with POAG diagnosis (GL/CAT:  $1.8 \pm 0.7$  mM;  $P = .002$ ) as shown in Figures 2A and 2D. Multivariate regression analyses did not identify any correlations between pO<sub>2</sub> and AsA following adjustment for race, age, sex, lens status, and presence of glaucoma.

- **TRAP AND NON-ASA-DEPENDENT TRAP:** TRAP and its component AsA are highly correlated in all human aqueous humor specimens, confirmed by the marked reduction of TRAP values in specimens treated with ascorbate oxidase. We designated the calculated remainder TRAP value non-AsA-dependent TRAP (non-AsA TRAP) in our subsequent analyses. As shown in Figures 2B, 2C and 2D, there were significantly lower levels of TRAP after vitrectomy (VIT:  $403.3 \pm 116.5$  Trolox units;  $P = .024$ ) than in the reference CAT group ( $479.0 \pm 146.7$  Trolox units). Multivariate regression analysis confirmed the correlation between TRAP and post-vitrectomy status ( $\beta = -.186$ ;  $P = .007$ ). TRAP is significantly directly correlated with age ( $r_s = .175$ ;  $P = .01$ ) as indicated in Figure 3. The non-AsA TRAP component percentage was significantly greater in the VIT group ( $149.9 \pm 51.9$

Trolox units [41.4%]) than in the CAT group ( $114.5 \pm 61.9$  Trolox units [24.3%];  $P = .014$ ). Multivariate regression also showed correlations between non-AsA TRAP and vitreous status ( $\beta = .135$ ;  $P = .05$ ). There were no differences between the CAT group and the TRAP activity in both the GL/CAT and the GL/IOL groups. Figure 4 illustrates the comparative contributions of the components of TRAP in each group. AsA contributed 76% of TRAP in the CAT group whereas AsA in the VIT group contributed only 58%. Multivariate regression analyses did not indicate any correlations between pO<sub>2</sub> and TRAP in the anterior segment following adjustment for race, age, sex, lens status, and medication use.

- **TOPICAL GLAUCOMA MEDICATIONS:** Medications were classified as beta-blockers (timolol, betaxolol), carbonic anhydrase inhibitors (dorzolamide, brinzolamide), alpha-2 agonist agents (brimonidine), or prostaglandin analogs (bimatoprost, latanoprost, travoprost). Fixed combination agents (brimonidine tartrate/timolol maleate ophthalmic solution [Combigan], dorzolamide hydrochloride-timolol maleate ophthalmic solution [Cosopt], and brinzolamide/brimonidine tartrate ophthalmic suspension [Simbrinza]) were categorized by their individual medication components. Because most patients were taking a combination of medications, each of the agents was analyzed individually. There was a significant correlation ( $\beta = 0.274$ ;  $P = .004$ ) between the use of topical carbonic anhydrase inhibitors (CAIs) and levels of AsA in the aqueous humor of all glaucoma patients (GL/CAT, GL/IOL). Notably, 69 of 146 patients (47.2%) in this group were taking topical CAIs as a component of their medical regimen. Four of 35 patients (11.4%) in the VIT group were taking CAI

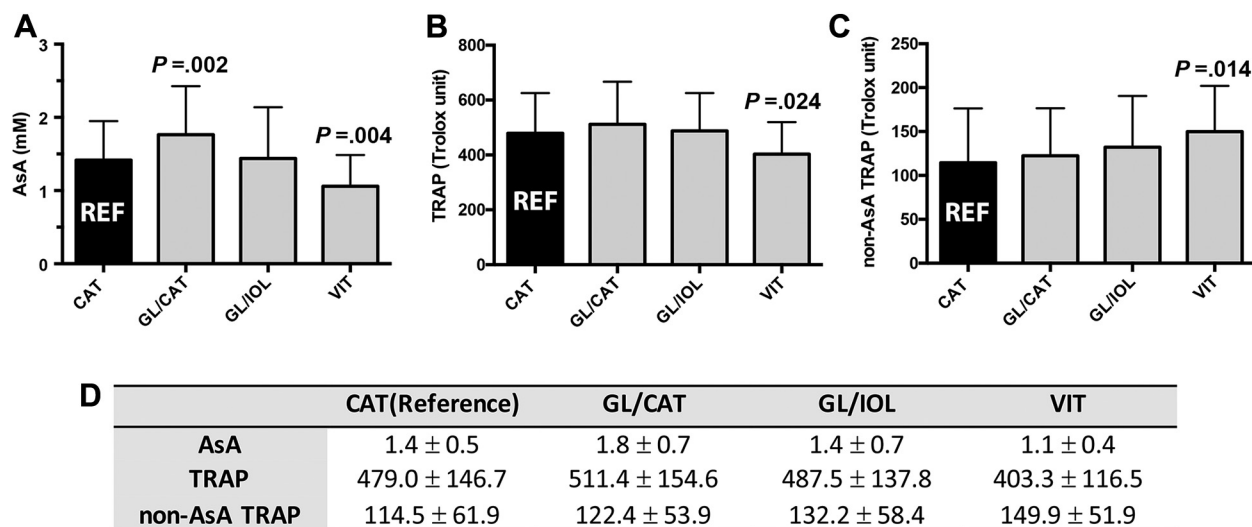


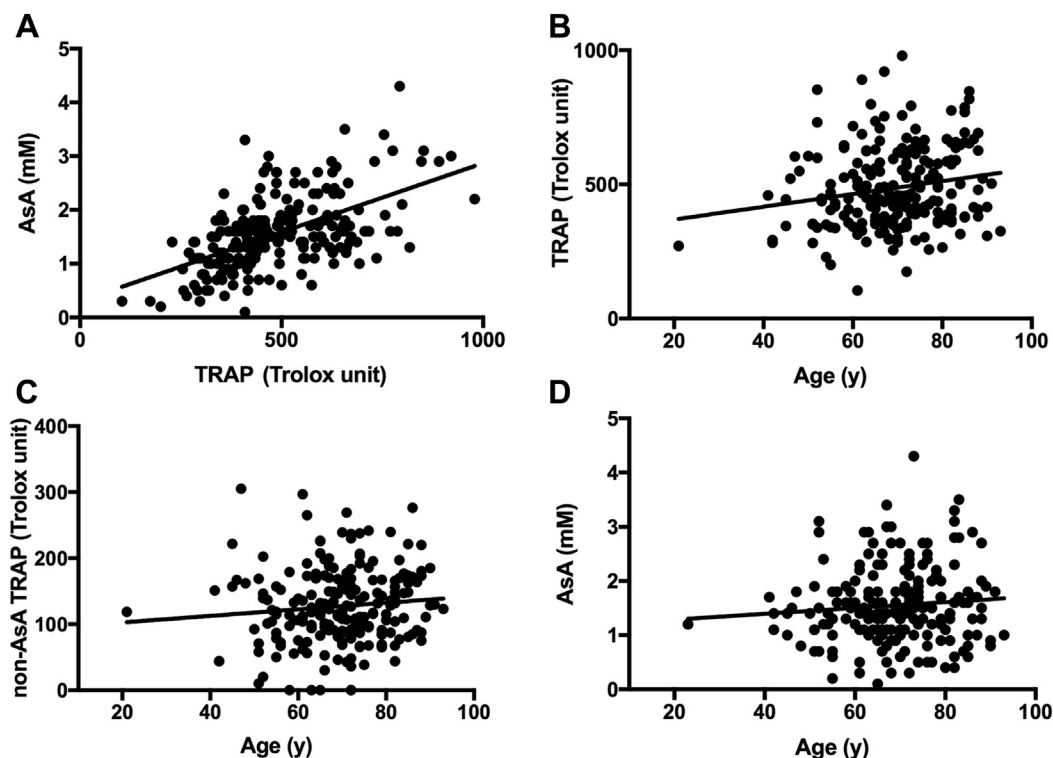
FIGURE 2. Comparison between aqueous humor antioxidant levels and reference groups (CAT black bars). (A) AsA; (B) TRAP; (C) Non-AsA TRAP; and (D) Mean value ± SD. *P* values were calculated from unpaired *t*-tests. Bars are standard deviations. AsA = ascorbate; CAT = cataract; GL = glaucoma; IOL = intraocular lens; REF = reference; TRAP = total reactive antioxidant potential; VIT = vitrectomy.

agents. After adjustments were made for race, age, sex, and lens status, no other medication class was correlated with AsA or TRAP levels. Multivariate regression analysis correcting for this variable resulted in demonstration of this drug's significant impact on AsA levels (Table 2).

## DISCUSSION

• **OXYGEN MEASUREMENTS AND HOMEOSTASIS:** This prospective, cross-sectional study represents the largest reported cohort of patients undergoing cataract and/or glaucoma surgery in which assessments of both intraocular  $pO_2$  levels and aqueous humor antioxidant status were obtained. Precise in vivo measurements of  $pO_2$  made by our colleagues in rabbit and in human vitreous led to these studies of the anterior segment of the human eye, revealing consistent  $pO_2$  gradients.<sup>21,52,53</sup> Our studies of how  $pO_2$  homeostasis is altered by surgical intervention, aging, and disease may reveal important insights of physiology and pathology. Because the ocular anterior segment represents an environment “protected” from direct blood flow, it provides an ideal site in which to study homeostatic mechanisms of oxygen metabolism in addition to oxidant-antioxidant balance. Additionally, by excluding patients with ischemic retinal disease and the use of general anesthesia, we separated effects of decreased retinal blood flow and hyperoxic conditions on intraocular  $pO_2$  levels, respectively.

Increased  $pO_2$  in the AC angle of post-vitrectomy patients (VIT) compared to reference CAT patients may provide an important source of pro-oxidants leading to increased oxidative stress in the TM. Elevated  $pO_2$  levels in the TM region and in the posterior chamber may increase ROS in the aqueous outflow pathway by diffusion from the ciliary body stroma into the aqueous humor at the root of the iris. This movement is consistent with the mechanism of this pathway facilitating movement of plasma proteins through the TM, as described by Fredde and colleagues,<sup>54</sup> and our previously published hypothesis regarding the correlation of  $pO_2$  levels in the AC and posterior chamber.<sup>49</sup> Other body tissues exposed to excess levels of molecular  $pO_2$  have been shown to accumulate ROS. For example, pulmonary epithelial cells are adapted to much higher  $pO_2$  levels than other cells in the body (21%  $O_2$  or 160 mm Hg), but during prolonged exposure to levels as high as 40%  $O_2$  or greater, increased intracellular ROS leads to pulmonary  $pO_2$  toxicity.<sup>55,56</sup> Physiologic conditions for TM cells are relatively hypoxic as we discovered in the rabbit, monkey, and human.<sup>40,49,53</sup> Exposure of these specialized cells to elevated  $pO_2$  may be “toxic,” leading to decreased TM cellularity, altered extracellular matrix formation, and ultimately decreased outflow facility and increased IOP. If the protective mechanisms of the aqueous humor are overwhelmed, then oxidative stress and damage may result. Notably, however, in POAG patients with an intact vitreous, we did not find increased intraocular  $pO_2$  in the TM region or posterior chamber, suggesting this may not be an important factor in all glaucoma subtypes.



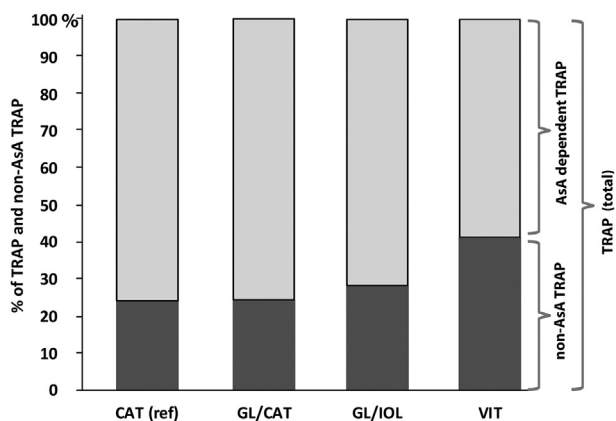
Variables	Spearman R	95% Confidence interval	p value
AsA and TRAP	0.546	0.4425 to 0.6359	<b>0.0001</b>
TRAP and Age	0.175	0.0388 to 0.3047	<b>0.0001</b>
AsA and Age	0.095	-0.0426 to 0.2291	0.1860
Non-AsA TRAP and Age	0.105	-0.0326 to 0.2386	0.1232

FIGURE 3. Scatterplot diagrams show relationships between (A) AsA and TRAP; (B) TRAP and age; (C) non-AsA TRAP and age; and (D) AsA and age in all cases demonstrating the best linear fit to the data. Analysis used Spearman's rank-order correlations and confidence intervals. AsA = ascorbate; TRAP = total reactive antioxidant potential.

Adaptation of ocular structures to specific levels of  $pO_2$  is revealed in studies of oxidative damage and defense. For example, the basal layer of corneal epithelium is accustomed to high levels of  $pO_2$ , essentially equivalent to air with  $pO_2$  of 160 mm Hg (21%  $pO_2$ ). In contrast,  $pO_2$  in inner retinal tissue and the vitreous adjacent to retinal blood vessels is approximately 20 mm Hg, consistent with other body tissues.<sup>21,57,58</sup> The environment surrounding the lens is notably hypoxic under normal conditions, measuring approximately 7 mm Hg at the posterior surface<sup>21</sup> and 3 mm Hg at the anterior and lateral surfaces of the lens,<sup>49</sup> with oxygen consumption by lens cells further decreasing  $pO_2$  within the lens nucleus.<sup>59,60</sup> Extraction of the natural lens and replacement with an IOL removes the contributing factor of oxygen consumption by the lens epithelium, thereby increasing  $pO_2$  around the lens, including the posterior chamber. This  $pO_2$  elevation does not reach the  $pO_2$  levels following vitrectomy.

• **OCULAR ANTIOXIDANT STATUS: ASCORBATE AND TRAP:** We identified significantly decreased levels of both AsA and TRAP in aqueous humor of patients who had undergone vitrectomy (VIT) compared to those in the reference group (Figure 2). Vitrectomy, independent of lens status, results in decreased TRAP in comparison to all other groups analyzed. Vitrectomized eyes also displayed an increase in non-AsA TRAP compared to that in the reference group (Figure 4). Interestingly, we found that POAG patients (GL/CAT, GL/IOL) had higher levels of AsA and no differences in TRAP than in the reference group (Figure 2). Lee and colleagues<sup>61</sup> also noted increased aqueous humor AsA in glaucoma patients compared to cataract surgery controls, whereas Ferreira and colleagues<sup>62</sup> found that AsA levels were decreased in patients with both POAG and exfoliation syndrome and that TRAP levels from glaucoma patients were significantly lower than those in the cataract group.<sup>63</sup>





**FIGURE 4.** Comparison between AsA and non-AsA components of TRAP. TRAP was designated as 100%, and the AsA component (light gray) and the non-AsA-dependent component (dark gray) are shown as percentages of TRAP values in each patient group. AsA = ascorbate; CAT = cataract; GL = glaucoma; IOL = intraocular lens; REF = reference; TRAP = total reactive antioxidant potential; VIT = vitrectomy.

**TABLE 2.** Results of Multivariate Regression Analyses Evaluating Effect of Topical Glaucoma Medications on Ascorbate (Asa) in Aqueous Humor

Dependent Variable	Independent Variable	$\beta$	P value
AsA	Alpha	0.009	.927
	Beta	0.111	.306
	CAI	0.274	.004
	PG	0.041	.696

Alpha = alpha-2 agonists; AsA = ascorbate; Beta = beta-blockers; CAI = carbonic anhydrase inhibitors; PG = prostaglandin analog.

Our present study did not support these findings of decreased AsA in patients with glaucoma compared to those in cataract controls. Leite and coworkers<sup>64</sup> found that AsA levels were significantly lower in secondary aqueous obtained from patients with a history of previous intraocular surgery than in primary aqueous in patients with glaucoma and cataract. Our separate analysis of phakic glaucoma patients (GL/CAT) with a history of intraocular surgery confirmed this finding of decreased AsA compared to patients without history of surgery ( $P = .04$ ; data not shown). Confounding variables such as frequent use of CAIs in the glaucoma subgroups significantly correlated with increased AsA and contributed to these contradictory findings. In addition, systemic ascorbate supplementation was not specifically documented in our medication review and might have also affected our results, especially in patients taking high doses of vitamin C (2 g/day).<sup>65</sup>

A recently published systematic review and meta-analysis of oxidant-antioxidant stress markers in glaucoma demonstrated decreased total antioxidant status in sera and aqueous humor in glaucoma patients, with the exception of 2 enzymatic antioxidants, superoxide dismutase and glutathione peroxidase.<sup>66</sup> These entities may represent a compensatory protective response to oxidative stress reflected in this study as non-AsA TRAP. A study of age-related changes in TRAP plasma levels showed that TRAP increased with age in both females and males.<sup>67</sup> However, in males, levels increased only from 51 to 74 years of age, at which point they were noted to decline. Increases of antioxidant potential, especially in response to oxidative stress, were due to unidentified antioxidants which made up 35% of TRAP in both sexes.

Huang and colleagues<sup>68</sup> described “extreme” exposures to increased  $pO_2$  ( $42.7 \pm 12.4$  mm Hg at the corneal surface) in patients with Fuchs’ dystrophy,<sup>68</sup> another ocular condition associated with oxidative stress.<sup>69</sup> Aqueous humor levels of AsA and TRAP were significantly lower than in the cataract reference group ( $P = .012$  and  $P = .032$ , respectively [unpublished data]). In addition, we previously reported<sup>50</sup> increased  $pO_2$  in the anterior segment of patients with African American background compared to that in caucasians and confirmed in this expanded study cohort (data not shown). These increased levels of  $pO_2$  did not correlate with differences in antioxidant status, potentially suggesting alternative mechanisms for this racial group’s increased risk and severity of POAG.

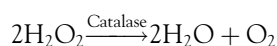
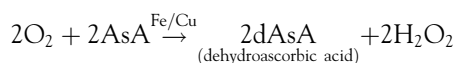
Finally, AsA levels in aqueous humor of patients with cataract have been shown to decrease with age,<sup>70</sup> supporting the role of oxidative damage and accumulation of free radicals in cataract development. We did not identify correlations between AsA and age in this study (Figure 3). We acknowledge that the oxidant-antioxidant balance in the eye as reflected in the aqueous humor is undoubtedly highly complex and requires further study.

• **ASCORBATE METABOLISM:** Ocular exposure to ultraviolet and visible light irradiation is greater than any other organ except skin. Consequently, this organ requires protective mechanisms against ROS generation. Because the TM represents the target tissue of glaucoma in the anterior segment, understanding the role of antioxidants in the trabecular tissue,<sup>71,72</sup> as well as in the aqueous humor in which it bathes, is critical to our comprehension of oxidant-antioxidant balance and its role in glaucoma development. The “pecking order” of aqueous humor antioxidants is affected by both the concentration and electrochemical activity of several low molecular weight water soluble species,<sup>73,74</sup> including AsA, L-tyrosine, L-cysteine, uric acid, and glutathione. Antioxidant enzymes such as superoxide dismutase, glutathione peroxidase,<sup>75</sup> and catalase have also been identified in aqueous humor. In contrast to nocturnal animals, the corneal epithelium,<sup>76,77</sup> lens,<sup>78</sup> vitreous,<sup>9</sup> and aqueous humor<sup>79,80</sup> of diurnal animals

contain extremely high levels of AsA compared to that in plasma.<sup>81</sup> Shui and colleagues<sup>8</sup> noted that metabolism of molecular oxygen in the vitreous gel occurs in an AsA-dependent manner, without an exogenous catalyst and independent of light, revealing the significance of AsA as a primary regulator of intraocular molecular oxygen.

AsA is actively concentrated from the plasma through the sodium-dependent vitamin C (SVC2) transporter located in the pigmented epithelial cell layer of the ciliary body with uptake of dehydroascorbic acid (dAsA) through glucose transporter (GLUT1) receptors in the non-pigmented epithelial layers.<sup>82</sup> AsA secretion into aqueous humor has been described in animals and humans with subsequent dAsA recycling back to AsA. However, neither the transporters implicated in the uptake of AsA and its metabolites nor other transporters of key antioxidants such as glutathione have been observed to provide specific information about antioxidant protection of the aqueous humor and TM.<sup>83</sup>

A review of published studies led us to propose that oxygen is consumed by AsA in aqueous humor through a 2-step reaction.<sup>84–88</sup> These reactions gradually decrease oxygen levels as AsA is converted to dAsA:



Direct in vivo measurements of ROS are problematic due to their reactivity and transient nature.<sup>89</sup> As a result, quantification of antioxidants is frequently performed as a surrogate marker of oxidant-antioxidant balance. Using gas chromatography-mass spectrometry, we measured dAsA byproducts in ocular fluids and identified 2,3-diketogulonate and L-threonate (data not shown). The 2,3-diketogulonate reacts with H<sub>2</sub>O<sub>2</sub>, producing L-threonate. This provides indirect evidence that H<sub>2</sub>O<sub>2</sub>, an important ROS, exists in aqueous humor.<sup>90</sup>

Some investigators have suggested that elevated oxygen may produce enough H<sub>2</sub>O<sub>2</sub> to exceed the ability of catalase to remove it,<sup>41,91</sup> potentially increasing exposure of the aqueous outflow pathway to this toxic metabolite. Oxidative damage or death of TM endothelial cells could result as a consequence of this exposure, as observed in glaucoma patients with decreased cellularity of the TM.<sup>31,92–94</sup> If increased oxygen oxidizes AsA and other antioxidants, one would expect antioxidant molecules to be depleted in the aqueous humor of patients with elevated pO<sub>2</sub>. Our findings of decreased AsA and TRAP levels in eyes after vitrectomy and IOL implantation support this theory.

• **VITRECTOMY AND RISK OF OPEN-ANGLE GLAUCOMA:** Increased pO<sub>2</sub> in the AC angle and altered antioxidant status may be clinically significant. Alternative mechanisms of TM damage and physiologic responses may be

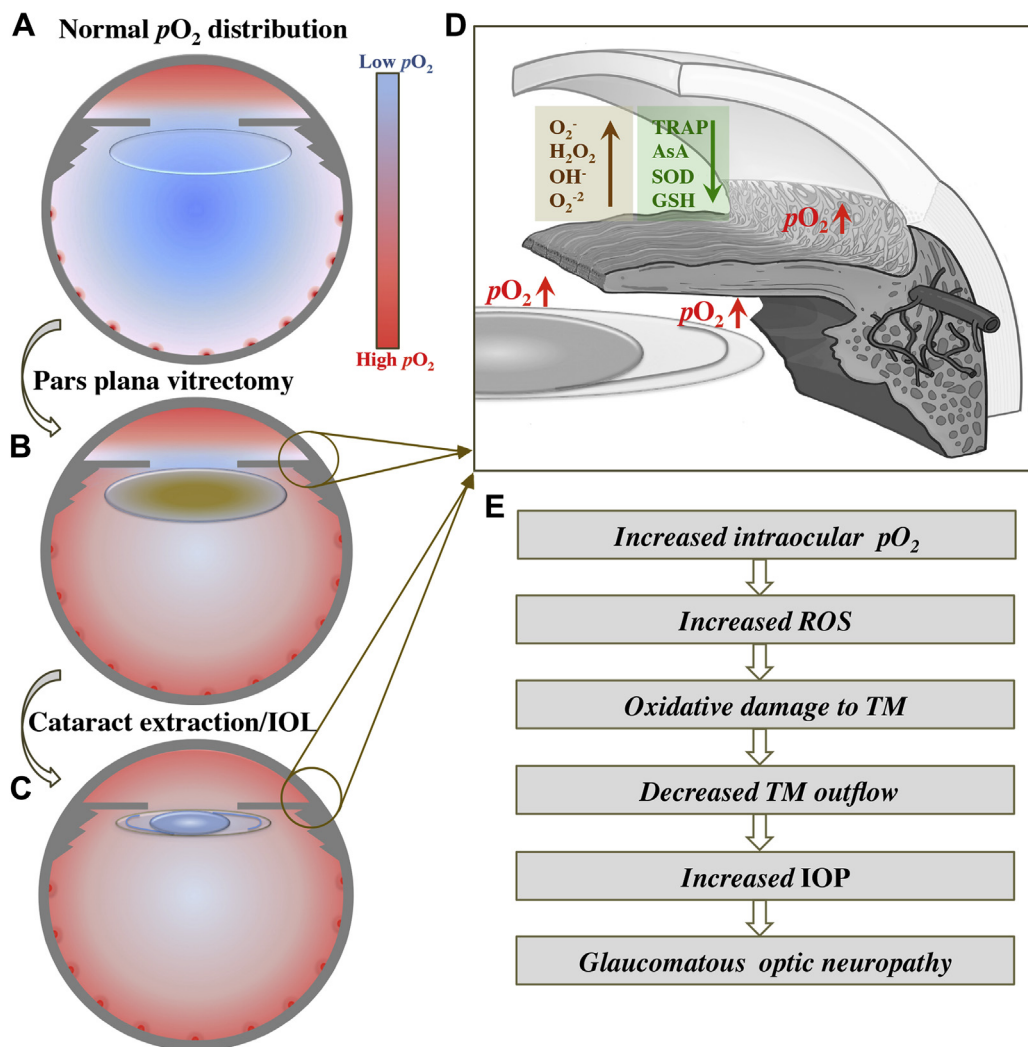
represented in these vitrectomized patients compared to other forms of POAG. As indicated in several retrospective studies with varying inclusion/exclusion criteria and follow-up periods, the prevalence (2%–19.2%) of ocular hypertension and glaucoma following vitrectomy and subsequent lens extraction is inconsistent,<sup>42–45</sup> with contrary conclusions in some studies.<sup>46,95,96</sup> The mechanisms of post-vitrectomy glaucoma likely represent a multifactorial process with various yet unidentified genetic and/or environmental risk factors.

Lelazary and colleagues<sup>97</sup> recently completed the Prospective Retinal and Optic Nerve Vitrectomy Evaluation (PROVE) study. The 3-year data from Patel and colleagues<sup>98</sup> revealed a significant increase in mean IOP in eyes having undergone both vitrectomy and cataract surgery with IOL implantation compared to baseline (*P* < .05) and compared to the fellow eye (*P* < .05), consistent with original reports by Chang.<sup>41</sup> A recently published retrospective, population-based cohort study confirmed these findings of increased 10-year risk of POAG in post-vitrectomy eyes at 10.0% (95% confidence interval [CI]: 3.0–17.0%) and following vitrectomy combined with scleral buckle at 17.5% (95% CI: 0–34.9%) compared to the nonoperative group at 1% (95% CI: 1%).<sup>47</sup>

Of the patients in the VIT group who underwent glaucoma surgery, 5 of 10 (50%) had a history of controlled glaucoma prior to PPV surgery and subsequent lens extraction. The mean time from PPV to glaucoma surgery was 51.3 ± 39.4 months (range = 12–118 months). Delayed onset of elevated IOP and protective effects of the crystalline lens have been reported, consistent with our data.<sup>42,43</sup> Our findings of further increases of pO<sub>2</sub> in the AC angle and posterior chamber in these cases following cataract extraction provides additional support for the theory of prolonged oxidative stress causing TM damage.

Future recruitment of a subgroup of patients who have undergone PPV and lens extraction without a diagnosis of ocular hypertension or POAG may provide additional information. We performed longitudinal assessments of aqueous and vitreous humor oxidant-antioxidant balance in an older monkey model of PPV with subsequent lensectomy.<sup>40</sup> Our results indicated progressive decrease of both TRAP and AsA as well as increased 8-OHdG, a marker of oxidative damage, in both aqueous and vitreous specimens following each surgical procedure.

• **ANTIOXIDANT PROPERTIES OF TOPICAL GLAUCOMA MEDICATIONS:** An interesting finding in this study was the correlation of topical CAIs with AsA levels in aqueous humor collected from human patients in vivo (Table 2). CAIs administered topically or systemically to rabbits resulted in increased concentrations of AsA in the aqueous of the posterior chamber but not the AC.<sup>99</sup> These findings, confirming prior studies by Becker,<sup>100</sup> were noted to be a reflection of decreased aqueous production and flow in this region. However, these measurements were based on



**FIGURE 5.** Proposed mechanism for development of post-vitrectomy glaucoma. **A:** Low levels of  $pO_2$  in vitreous cavity, surrounding clear lens and in anterior chamber (AC) angle. Oxygen diffuses across cornea into the AC and from retinal blood vessels into the vitreous cavity. **B:** Following pars plana vitrectomy,  $pO_2$  increases in the vitreous cavity, around lens, in AC angle and posterior chamber with development of nuclear sclerotic cataract. **C:** Following cataract extraction and intraocular lens implantation (IOL),  $pO_2$  increases in the AC angle, posterior chamber and mid-A(C). **D:** Expanded view of AC angle illustrating alterations in aqueous humor including increased  $pO_2$  surrounding lens implant and in AC angle leading to increased reactive oxygen species formation (brown; ROS) and decreased antioxidants (green). **E:** Proposed cascade of events following vitrectomy and IOL implantation ultimately leading to increased intraocular pressure (IOP) and risk of glaucoma. AsA = ascorbate; GSH = glutathione;  $H_2O_2$  = hydrogen peroxide;  $O_2^-$  = superoxide anion;  $O_2^{-2}$  = peroxide;  $OH^-$  = hydroxyl ion; SOD = superoxide dismutase; TM = trabecular meshwork; TRAP = total reactive antioxidant potential.

acute therapy with systemic carbonic anhydrase inhibitors, and may not be reproduced with chronic topical use, a common component of glaucoma therapy.

Our findings of significantly higher AsA concentrations in patients taking topical CAIs may represent a potential secondary mechanism of action, as revealed in the reduction of free radical formation in glaucoma patients taking topical dorzolamide.<sup>101</sup> Timolol, a beta-blocker, also has been shown to exert direct antioxidant protection of human endothelial cells in culture.<sup>102</sup> Metipranolol, in addition to its active metabolite

desacetylmepipranolol, also exhibits antioxidant properties in vitro.<sup>103</sup> Brimonidine, an alpha-2 adrenergic agonist, has been shown to exert a neuroprotective effect on rat retinal ganglion cells in the presence of glutamate, oxidative and hypoxic stress,<sup>104</sup> but no changes in antioxidant levels of the anterior segment. Pre-incubation of cultured human TM cells with prostaglandin analogs followed by exposure to  $H_2O_2$  has been shown to reduce glaucomatous TM changes in these cells.<sup>105</sup> Further studies of potential antioxidant effects of glaucoma therapies may be warranted.

• **STUDY LIMITATIONS:** The cross-sectional design of this study and others identified in our review of published studies limits our understanding of how responses to oxidative stress occur over time in a given patient. Future longitudinal analyses may help to understand questions surrounding progressive TM damage and glaucoma development. As in any human study, individual patient variation may affect group mean data analysis. Dependence on a patient's historical information regarding medication (eg, antioxidant supplements) and social history (eg, tobacco use) may significantly alter results, especially with limited sample size within each of the study groups. Our results did not confirm previously published findings of decreased antioxidant protection in glaucoma versus cataract patients, despite similar protocol techniques. Although we designated patients undergoing cataract surgery as references/controls for our comparisons, it is important to note that these were not "normal controls," as they did have a condition(s) associated with oxidative damage (cataract and aging). Differences in cataract type and glaucoma severity may have had an impact on the results as well as our observation of the effect of specific glaucoma medications on AsA in aqueous humor. Given our limited number of VIT subjects, recruitment of additional post-vitreotomy subjects (with and without glaucoma) could be informative as vitreoretinal pathology may independently influence antioxidant levels. Because both patients and specimen quantities were limited, assays of multiple antioxidants

could not be performed for all patients, depending on volumes required. We identified AsA and TRAP as the most promising agents, given their biochemical reactions with oxygen as the dominant measurements of antioxidant potential, but other molecules may play a significant role in antioxidant defense (eg, non-AsA TRAP).

## CONCLUSIONS

OUR OBSERVATION OF INCREASED PO<sub>2</sub> LEVELS IN THE ANTERIOR segment and decreased levels of AsA and TRAP in the group of patients who had undergone PPV compared to the reference group may provide important insights into how this surgery may increase oxidative stress and glaucoma risk in select patients. We propose increased intraocular pO<sub>2</sub> levels in these patients could be a potential source of pro-oxidants for generation of ROS, decreasing antioxidant defenses in the ocular anterior segment (Figure 5). Further understanding of this surgical intervention's impact on oxygen homeostatic mechanisms, antioxidant balance, and oxidative stress is vital, and these investigations may lead to future therapies targeted to this specific population as well as to other individuals afflicted with this leading cause of irreversible blindness.

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