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

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Abstract

Purpose: To investigate correlations 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 presence of primary open angle glaucoma for statistical analyses. Intraocular pO_2 measurements with a fiberoptic probe were made in patients at the beginning of planned glaucoma and/or cataract surgery. Aqueous humor specimens were obtained for antioxidant analysis (AsA, TRAP).

Results: Following prior pars plana vitrectomy, pO_2 was significantly higher compared to the reference group (cataract; CAT) in the anterior chamber (AC) angle (16.2 ± 5.0 vs. 13.0 ± 3.9 mmHg, P=.0171) and in the posterior chamber (7.6 ± 3.1 vs. 3.9 ± 2.7 mmHg, P<.0001). AsA and TRAP levels were significantly lower (1.1 ± 0.4 vs. 1.4 ± 0.5 mM; 403.3 ±116.5 vs. 479.0 ± 146.7 Trolox unit; P=.004, P=.024, respectively) in patients following vitrectomy surgery. 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 alteration of the intraocular oxidantantioxidant balance. Our studies link physiologic factors, increased pO_2 in the AC angle and posterior chamber, to decreased antioxidant levels in aqueous humor following vitrectomy surgery. Oxidative stress/damage to the trabecular meshwork in such post-vitrectomy cases may contribute to intraocular pressure elevation and increased risk of glaucoma.

INTRODUCTION

The precise pathogenesis of primary open angle glaucoma (POAG) has not been fully elucidated. It likely represents a variety of different pathologies, genetic predispositions and contributing environmental factors. Alterations of the local environs of the trabecular

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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 via nitric oxide synthesis in the TM,[1] cyclic mechanical stress yields alterations in conventional outflow facility,[2] and TM cells undergo contractile changes, potentially via Rho-kinase mediated signaling.[3] In addition to these physiologic mechanisms, the concept of intraocular surgical procedures modifying this environment in a potentially deleterious manner has been previously noted.[4,5]

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 2001–2012.[6] This increase is likely related to improvements in the safety profile of the procedure, improved success of vision-saving surgical interventions, decreased utilization of alternative surgical therapies, and earlier intervention for non-vision threatening pathologies (e.g. “floaterectomy”). Evaluation of adverse events of this procedure between the years of 1994 to 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.[7]

The vitreous humor is an important ocular structure that plays a prominent role in maintaining biochemical homeostasis, consuming molecular oxygen and protecting the lens from oxidative damage.[8] Shui and colleagues found that gel vitreous, in comparison to liquefied vitreous due to myopia, aging, or surgical removal, has a higher concentration of ascorbate (AsA) and consumes oxygen at a faster rate. Previous studies indicated that antioxidant levels, specifically AsA and glutathione, are present in high concentrations in the vitreous humor.[9,10] The discovery of this role of gel vitreous 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 development within 1 to 3 years.[11] In patients undergoing PPV, it has been observed that a nuclear sclerotic cataract develops and progresses rapidly in the ensuing 12–18 months following vitrectomy, with 37–95% of patients requiring cataract extraction within 2 years.[12–18] Vitrector gauge size did not influence these results.[19] Increased vitreous liquefaction increases the risk of nuclear cataract development.[20] Identification of increased oxygen (pO_2) levels within the vitreous cavity and at the posterior surface of the lens following vitrectomy surgery led to the proposal that increased oxygen exposure leads to oxidative damage to the lens and nuclear cataract formation.[21]

Besides the lens, other ocular structures are continuously exposed to a broad spectrum of pO_2 levels, ranging from hyperoxic to markedly hypoxic. Cells exposed to high pO_2 levels as well as ultraviolet light (e.g. corneal epithelium) contain nuclear ferritin,[22,23] AsA,[24] glutathione,[25] superoxide dismutase, and catalase and otherantioxidants.[26,27] Ocular cells that function at low physiologic pO_2 are unlikely to adapt to altered (i.e. higher) levels of oxygen 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 anion, hydrogen peroxide, hydroxyl radical, peroxyl radical 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.[28] 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.[29,30]

Evidence supporting such oxidative damage to the TM was initially reported by Alvarado in 1981,[31] as he first suggested that aging and oxidative stress underlie the degeneration of TM cells in patients with glaucoma. Cell senescence has been shown to increase ROS generation leading to reduced number and function of mitochondria.[32] 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 hydrogen peroxide (H_2O_2) following depletion of glutathione in the TM increases outflow resistance.[33] TM tissue, as compared to corneal and iris tissue, was found to be most sensitive to oxidative damage induced by H_2O_2 exposure.[34] Subsequent studies provide evidence of oxidative damage and reduced resistance to oxidative stress in the outflow pathway.[35,36] Oxidative damage to DNA is greater in TM cells in glaucoma patients compared to controls.[37] and correlates with IOP level and visual field loss.[38] Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG), an established biomarker of oxidative DNA damage, are significantly higher in both aqueous humor and serum in glaucoma patients compared to controls.[39] Increased levels of 8-OHdG have also been found in TM specimens of POAG patients.[38]

Importantly, PPV has also been associated with increased oxygen exposure to the microenvironment of the TM and outflow pathways[40] and increased risk of developing open angle glaucoma in several retrospective clinical studies,[41–46] and a recent population based study.[47] The present study was undertaken to provide further understanding of the impact of exposure to increased pO_2 and/or its metabolites in the local environment of the aqueous outflow pathways following vitrectomy surgery. We hypothesize that increased pO_2 in these cases may contribute to alterations of oxidant-antioxidant balance leading to increased oxidative stress/damage of the TM. To assess these conditions in human subjects, we measured *in vivo* levels of pO_2, total reactive antioxidant potential (TRAP) activity, and AsA levels in aqueous humor of eyes 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 HIPAA guidelines. Informed consent was obtained from subjects after explanation of the nature and possible consequences of the study. This study was designed to measure oxygen 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_2 MEASUREMENTS

A complete general medical and ophthalmic history and comprehensive ophthalmic examination, 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 one 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 (Hodapp Parrish Anderson criteria).[48] Central corneal thickness was measured by ultrasound technique (DGH 55 Pachmate, DGH Technology Inc., Exton, Pennsylvania, USA), and axial length measurements were recorded (IOLMaster 500, Carl Zeiss Meditec, Inc., Germany) for patients undergoing cataract extraction. Racial background was based on self-report as indicated on a standardized registration questionnaire.

As per routine surgical protocol, the patient was placed in supine position, intravenous sedation was administered, the eye was prepped and draped and a lid speculum was placed. Supplemental oxygen (21–30%) was provided via nasal cannula and separated from the ocular region by adhesive sterile drape to avoid any additional oxygen exposure. This technique did not impact intraocular oxygen measurements as previously reported.[21] 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 1–3 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 utilized for entry through peripheral clear cornea into the anterior chamber (AC) and the Oxylab™ pO_2 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 three 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.[49,50] In pseudophakic patients or those scheduled to undergo cataract extraction, two additional measurements were obtained (4) at the central anterior lens surface and (5) in the posterior chamber just behind the iris. These latter two measurements were not performed in patients remaining phakic 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_2 level, duplicate testing in the same locations were performed for verification.

AQUEOUS HUMOR SPECIMEN COLLECTION

Following pO_2 measurements, the needle entry site in the cornea was slightly enlarged with a 15-degree blade or side port instrument and an aqueous humor sample (50–100 μl) was drawn into a 1-ml tuberculin syringe via 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

Ascorbate—AsA concentration was quantified in triplicate, based on its ability to reduce Fe^{3+} to Fe^{2+} and the resulting change in the A_{525} of complexes of Fe^{2+} with 2,2”-dipyridyl.[51] Assay modifications enabled the analysis of 10-μl samples and a standard curve was used for all measurements as in our previous report.[8] 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 ($^{13}C_6$-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, CA) using a 30-m, 0.25-mm-internal GC column with a 0.25-μm film (DB-5ms column; P.J. Cobert Associates Inc., St. Louis, MO), maintained at 80°C for 1 minute, and then eluted with a temperature gradient of 80°C to 300°C at 15°C/min. 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, MA) operated in the electron ionization mode at 70 eV. The concentration of AsA was calculated from the ratio of carbon 13- to carbon 12-labeled ascorbate. In order to confirm the specificity of the AsA assay, 2 units of ascorbate oxidase (AO; A 0157, Sigma Chemical, St. Louis, MO) were added to each of the 10-μl samples, 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, MO, USA) and 40 μM luminol (3-aminophthalhydrazide, Sigma Aldrich, St. Louis, MO, USA). ABAP combines with oxygen to produce alkyl peroxyl 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 peroxyl radicals. Since 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 vitamin E analog and reported as “Trolox units,” with one unit equal to amount of time required to quench luminescence by a sample containing 1 μM Trolox. In addition to measuring TRAP in the aqueous humor samples, samples were treated with ascorbate oxidase to remove AsA as described above. Repeat measurements were then performed in order to differentiate AsA and 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 using SPSS software (Version 24.0, Chicago, Illinois, USA). T-test, one-way ANOVA with multiple comparison analysis (Bonferroni correction), and Spearman correlation analyses were performed with GraphPad Prism (Version 8.0, La Jolla, CA, USA). Results are expressed as mean values ± standard deviation (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 to July 2010) of 112 eyes of 112 patients were included from a previously published study evaluating intraocular pO_2 measurements.[49] We extended this work to study total 288 eyes for pO_2, AsA, and TRAP measurements, after exclusion of 24 eyes due to inadequate specimen collection. Patients with secondary open angle glaucoma (i.e. 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 prior history of ocular surgery, glaucoma, or exposure to ocular glaucoma medications. This group served as the reference/control group for select statistical analyses. Consistent with our previously published data,[49] patients with a diagnosis of POAG (GL) were subdivided into: a) patients undergoing glaucoma surgery or combined cataract and glaucoma surgery (GL/CAT), and b) pseudophakic patients undergoing glaucoma surgery (GL/IOL). Patients with a history of vitrectomy who had undergone previous pars plana vitrectomy for vitreoretinal conditions including rhegmatogenous retinal detachment, epiretinal membrane and macular hole and excluding proliferative retinopathy comprised the VIT group. All of 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 compared to VIT group (P =.0005 and P=.0001, respectively). The GL/IOL patients were also older than the CAT reference group (P=.005). Subgroup analyses were performed to identify correlations of race, age, sex, lens and vitreous status, and ocular medications with pO_2 levels and antioxidant status. We randomly selected one eye for the final data analysis in patients who had measurements/ specimens from both eyes.

INTRAOCULAR pO_2 MEASUREMENTS

Oxygen measurements at five intraocular locations were analyzed by multiple comparison analysis with Bonferroni correction (Figure 1). Intraocular pO_2 measurements were significantly higher following vitrectomy (VIT) compared to the reference (CAT) group in the AC angle (16.2 ± 5.0 vs. 13.0 ± 3.9 mmHg; P=.0171) and posterior chamber (7.6 ± 3.1 vs. 3.9 ± 2.7 mmHg; P<.0001). In the GL/IOL (pseudophakic) group, there were significantly higher levels of pO_2 at the anterior lens surface compared to compared to the reference group (8.0 ± 4.1 vs. 2.5 ± 2.4 mmHg; P<.0001), in the mid-AC (11.1 ± 3.8 vs. 8.4

± 3.9 mmHg; *P*=.007), and in the posterior chamber (5.7 ± 3.4 vs. 3.9 ± 2.7 mmHg; *P*=.0384).

ASCORBATE MEASUREMENTS

AsA levels were significantly lower in VIT group (1.1 ± 0.4 mM; *P*=.004) compared to the CAT reference group (1.4 ± 0.5 mM). We further confirmed that AsA is correlated with prior vitrectomy surgery in a multivariate regression model (Beta = −.198, P=.004). AsA levels were increased in phakic patients with POAG diagnosis (GL/CAT; 1.8 ± 0.7 mM; *P*=.002) as shown in Figures 2A and 2D. Multivariate regression analyses did not identify any correlations of pO_2 with 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 have designated the calculated remainder TRAP value as non-AsA dependent TRAP (non-AsA TRAP) in our subsequent analyses. As shown in Figures 2B, 2C and 2D, there were significantly lower TRAP levels following vitrectomy (VIT; 403.3 ± 116.5 Trolox units; *P* =.024) in comparison to reference CAT group (479.0 ± 146.7 Trolox unit). 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 ± 51.9 Trolox unit, 41.4%) compared to CAT (114.5 ± 61.9 Trolox unit, 24.3%; *P*=.014). Multivariate regression also showed correlation between non-AsA TRAP and vitreous status (Beta = .135, *P*=.05). There were no differences between the CAT group and both GL/CAT and GL/IOL TRAP activity. Figure 4 illustrates the comparative contributions of the components of TRAP in each group. AsA contributed 76% of TRAP in CAT group while AsA in the VIT group only contributed 58%. Multivariate regression analyses did not indicate any correlations of pO_2 with 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 analogues (bimatoprost, latanoprost, travoprost). Fixed combination agents (Combigan,® Cosopt,® Simbrinza®) were categorized by their individual medication components. As most patients were on 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 (47.2%) of this group of patients were utilizing topical CAIs as a component of their medical regimen. Four of 35 (11.4%) of the VIT group were taking CAI agents. Following adjustment 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 oxygen levels and aqueous humor antioxidant status were obtained. Precise *in vivo* measurement techniques of pO_2 by our colleagues in rabbits and in human vitreous led to these studies of the anterior segment of the human eye revealing consistent oxygen gradients.[21,52,53] Our studies of how oxygen homeostasis is altered by surgical intervention, aging, and disease may reveal important insights of physiology and pathology. As the ocular anterior segment represents a "protected" environment from direct blood flow, it provides an ideal scenario 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 aimed to separate 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 Freddo and colleagues' description of this pathway facilitating movement of plasma proteins through the TM[54] and our previously published hypothesis regarding the correlation of pO_2 levels in the AC and posterior chamber.[49] Other body tissues exposed to excess levels of molecular oxygen have been shown to accumulate ROS. For example, pulmonary epithelial cells are adapted to much higher oxygen levels than other cells in the body (21% O_2 or 160 mmHg), but during prolonged exposure to levels as high at 40% O_2 or greater, increased intracellular ROS leads to pulmonary oxygen toxicity.[55,56] Physiologic conditions for TM cells are relatively hypoxic as we discovered in the rabbit, monkey and human.[40,49,53] 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/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.

Adaptation of ocular structures to specific levels of oxygen is revealed in studies of oxidative damage and defense. For example, the basal layer of corneal epithelium is accustomed to high levels of oxygen, essentially equivalent to air with pO_2 of 160 mmHg (21% oxygen). In contrast, pO_2 in inner retinal tissue and the vitreous adjacent to retinal blood vessels is approximately 20 mmHg, consistent with other body tissues.[21,57,58] The environment surrounding the lens is notably hypoxic under normal conditions, measuring approximately 7 mmHg at the posterior surface[21] and 3 mmHg at the anterior and lateral surfaces of the lens,[49] with oxygen consumption by lens cells further decreasing pO_2 within the lens nucleus.[59,60] 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 oxygen 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 the reference group (Figure 2). Vitrectomy surgery, 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 the reference group (Figure 4). Interestingly, we found that patients with POAG diagnosis (GL/CAT, GL/IOL) had higher levels of AsA and no difference in TRAP when compared to the reference group (Figure 2). Lee and coworkers also noted increased aqueous humor AsA in glaucoma patients compared to cataract surgery controls,[61] while Ferreira and colleagues found that AsA levels were decreased in patients with both POAG and exfoliation syndrome,[62] and TRAP levels from glaucoma patients were significantly lower compared to the cataract group.[63]

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

A recently published systematic review and meta-analysis of oxidant-antioxidant stress markers in glaucoma demonstrated decreased total antioxidant status in serum and aqueous humor in glaucoma patients with the exception of two enzymatic antioxidants, superoxide dismutase and glutathione peroxidase.[66] 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.[67] However, in males, levels increased only up to the age of 51–74 years when they were noted to decline. Increases of antioxidant potential, especially in response to oxidative stress, were due to unidentified antioxidants which comprise 35% of TRAP in both sexes.

Huang and coworkers described “extreme” exposures to increased pO_2 (42.7 ± 12.4 mmHg at the corneal surface) in patients with Fuchs’ dystrophy,[68] another ocular condition associated with oxidative stress.[69] Aqueous humor levels of AsA and TRAP were significantly lower compared to a cataract reference group (P=.012 and P=.032, respectively; unpublished data). In addition, we previously reported[50] increased pO_2 in the anterior segment of patients with African American background compared to 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,[70] supporting the role of oxidative damage and accumulation of free radicals in cataract development. We did not identify correlations of AsA with 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. As the TM represents the target tissue of glaucoma in the anterior segment, understanding the role of antioxidants in the trabecular tissue,[71,72] 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,[73,74] including AsA, L-tyrosine, L-cysteine, uric acid and glutathione. Antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase,[75] and catalase have also been identified in aqueous humor. In contrast to nocturnal animals, the corneal epithelium,[76,77] lens,[78] vitreous[9] and aqueous humor[79,80] of diurnal animals contain extremely high levels of AsA as compared to plasma.[81] Shui and colleagues 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.[8]

AsA is actively concentrated from the plasma via the sodium-dependent vitamin C (SVC2) transporter located in the pigmented epithelial cell layer of the ciliary body with uptake of dehydroascorbic acid (dAsA) via glucose transporter (GLUT1) receptors in the non-pigmented epithelial layers.[82] 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 elucidated to provide specific information about antioxidant protection of the aqueous humor and TM.[83]

Review of the literature has led us to propose that oxygen is consumed by AsA in aqueous humor via a two-step reaction.[84–88] These reactions gradually decrease oxygen levels as AsA is converted to dAsA:

$$2O_2 + 2\text{AsA} \xrightarrow{\text{Fe / Cu}} \underset{\text{(dehydroascorbic acid)}}{2\text{dAsA}} + 2H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Direct *in vivo* measurements of ROS are problematic due to their reactivity and transient nature.[89] As a result, quantification of antioxidants is frequently performed as a surrogate marker of oxidant-antioxidant balance. Using gas chromatography–mass spectrometry, we have measured dAsA byproducts in ocular fluids and identified 2,3-diketogulonate and L-threonate (data not shown). 2,3-diketogulonate reacts with H_2O_2, producing L-threonate. This provides indirect evidence that H_2O_2, an important ROS,2 exists in aqueous humor.[90]

Some have suggested that elevated oxygen may produce enough H_2O_2 to exceed the ability of catalase to remove it,[41,91] 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.[31,92–94] If increased oxygen oxidizes AsA and other antioxidants, one would expect antioxidant molecules to be depleted in the aqueous humor of patients with elevated oxygen. Our findings of decreased AsA and TRAP levels in eyes following vitrectomy and IOL implantation support this theory.

VITRECTOMY AND RISK OF OPEN ANGLE GLAUCOMA

Increased pO_2 in the AC angle and altered antioxidant status may be clinically significant. Alternate 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 surgery and subsequent lens extraction is inconsistent,[42–45] with contrary conclusions in some studies.[46,95,96] The mechanisms of post-vitrectomy glaucoma likely represent a multifactorial process with various yet unidentified genetic and/or environmental risk factors.

Lelazary and colleagues recently completed the Prospective Retinal and Optic Nerve Vitrectomy Evaluation (PROVE) study.[97] The three- year data[98] (Patel S, et al. AAO, 2016; AAO abstract P0562) revealed a significant increase of 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.[41] A recently published retrospective, population-based cohort study confirmed these findings of increased 10-year risk of POAG in post-vitrectomy eyes (10%; 95% CI) and following vitrectomy combined with scleral buckle (17.5%; 95% CI) compared to the nonoperative group (1%; 95% CI).[47]

Of the patients in the VIT group who underwent glaucoma surgery, 5/10 (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.[42,43] Our findings of further increases of pO_2 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.[40] 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 carbonic anhydrase inhibitors (CAIs) with AsA levels in aqueous humor from 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.[99] These findings confirmed Becker's prior studies and were noted to be a reflection of decreased aqueous production and flow in this region.[100] However, these measurements were based on 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 on 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.[101] Timolol, a beta blocker, has also been shown to exert direct antioxidant protection of human endothelial cells in culture.[102] Metipranolol, in addition to its active metabolite, desacetylmetipranolol, also exhibits antioxidant properties *in vitro*.[103] 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,[104] but no changes in antioxidant levels of the anterior segment. Pre-incubation of cultured human TM cells with prostaglandin analogues followed by exposure to H_2O_2 has been shown to reduce glaucomatous TM changes in these cells.[105] 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 literature review limits our understanding of how responses to oxidative stress occur over time in a given patient. Future longitudinal analyses may aid 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 patient's historical information regarding medication (e.g. antioxidant supplements) and social history (e.g. 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, in spite of similar protocol techniques. Although we designated patients undergoing cataract surgery as reference/controls for our comparisons, it is important to note that these are not "normal controls" as they do have 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-vitrectomy subjects (with and without glaucoma) could be informative since vitreoretinal pathology may independently influence antioxidant levels. Since both patients and specimen quantities are limited, assays of multiple antioxidants cannot 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 measure of antioxidant potential, but other molecules may play a significant role in antioxidant defense (i.e. non-AsA TRAP).

CONCLUSIONS

Our observation of increased pO_2 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_2 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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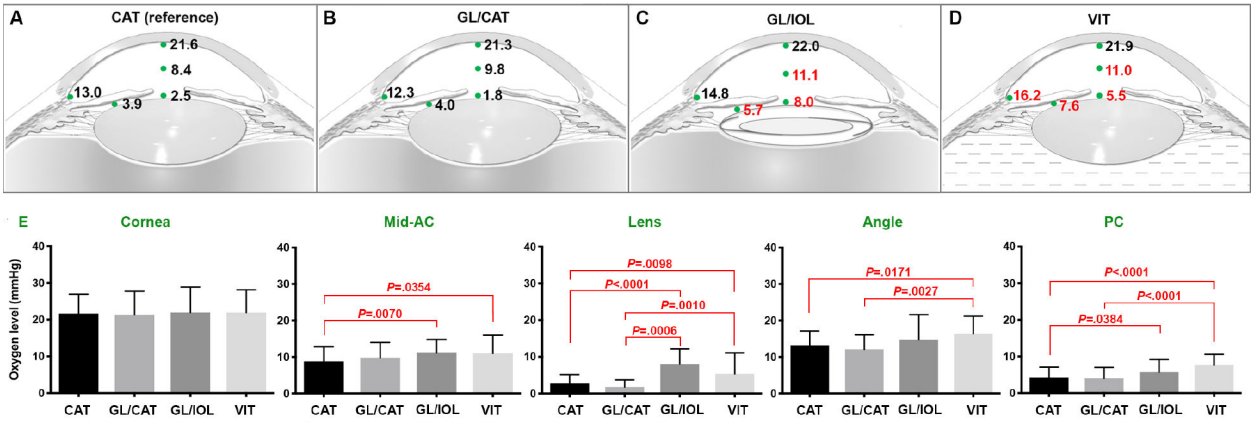
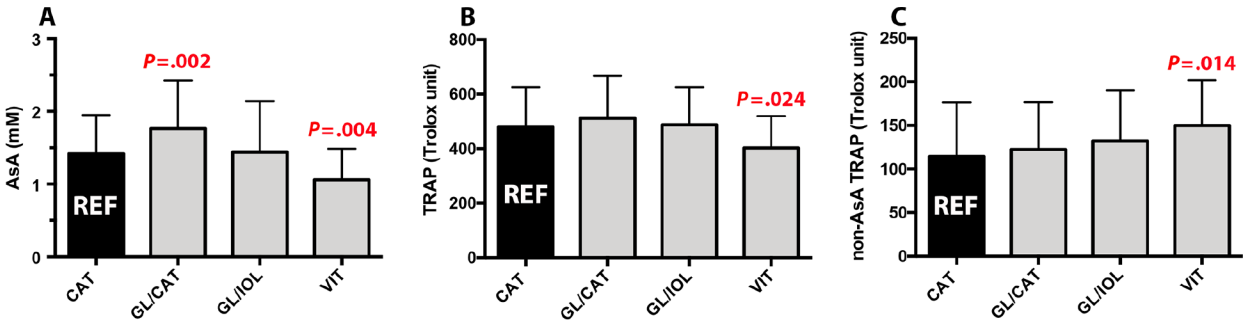


Figure 1.

Intraocular pO_2 measurements (mmHg) at indicated locations (green dots). A. Cataract (CAT) group used as reference for comparison with other groups. Red numbers indicate *P* values <.05. B. GL/CAT = glaucoma diagnosis with cataract C. GL/IOL = glaucoma diagnosis with history of prior cataract surgery D. VIT = history of prior pars plana vitrectomy. E. Comparison of pO_2 at intraocular locations. (ANOVA with multiple comparison analysis and Bonferroni correction; *P* values <.05). Error bar: mean mmHg ± standard deviation.

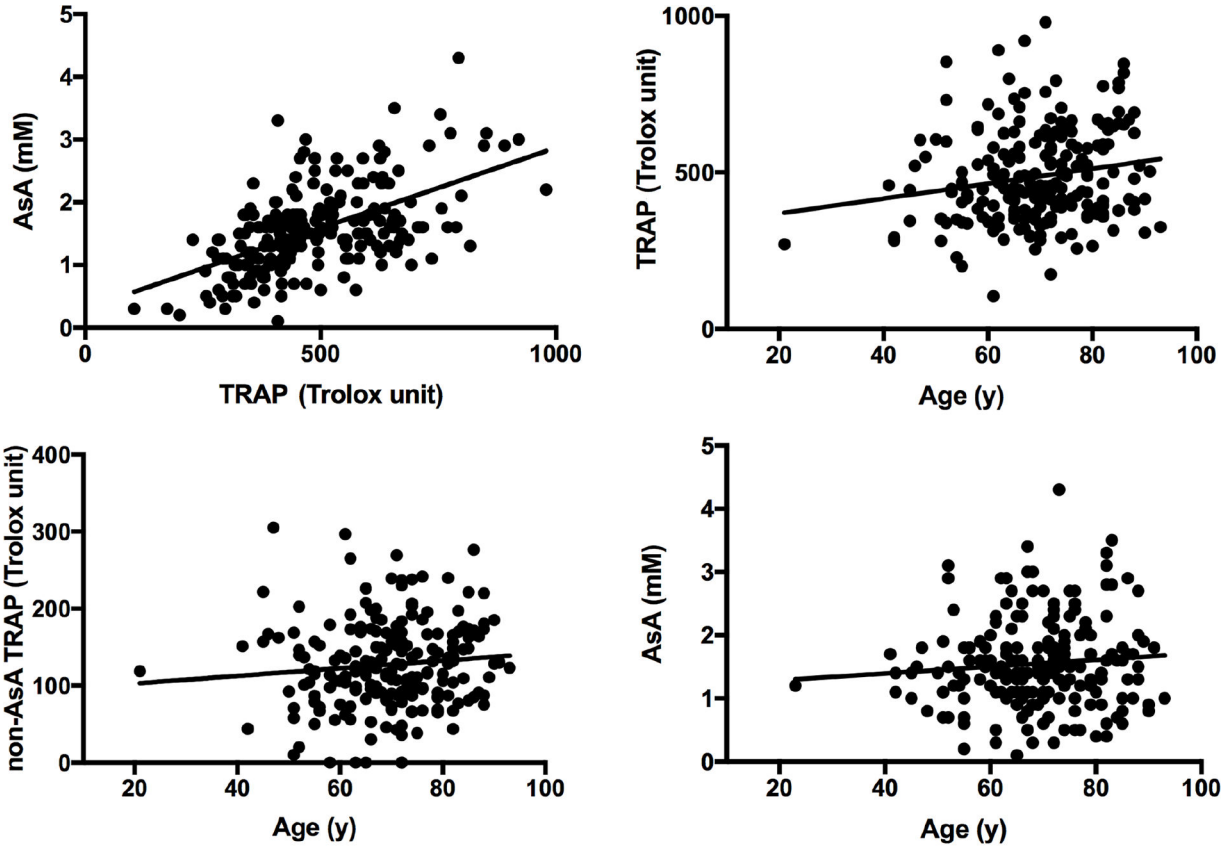


D

	CAT(Reference)	GL/CAT	GL/IOL	VIT
AsA	1.4 ± 0.5	1.8 ± 0.7	1.4 ± 0.7	1.1 ± 0.4
TRAP	479.0 ± 146.7	511.4 ± 154.6	487.5 ± 137.8	403.3 ± 116.5
non-AsA TRAP	114.5 ± 61.9	122.4 ± 53.9	132.2 ± 58.4	149.9 ± 51.9

Figure 2.

Comparison of aqueous humor antioxidant levels to reference group (CAT; black bar). A. Ascorbate (AsA) B. Total Reactive Antioxidant Potential (TRAP) C. Non-AsA TRAP D. Table shows mean value ± standard deviation (SD). *P* values are calculated from unpaired *t*-test. Bar: SD.



Variables	Spearman R	95% Confidence interval	*p* value
AsA and TRAP	0.546	0.4425 to 0.6359	**0.0001**
TRAP and Age	0.175	0.0388 to 0.3047	**0.0100**
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 showing relationships between A. Ascorbate (AsA) and Total Reactive Antioxidant Potential (TRAP), B. TRAP and age, C. non-AsA TRAP and age, D. AsA and age in all cases demonstrating the best linear fit to the data. Table shows Spearman's rank-order correlations.

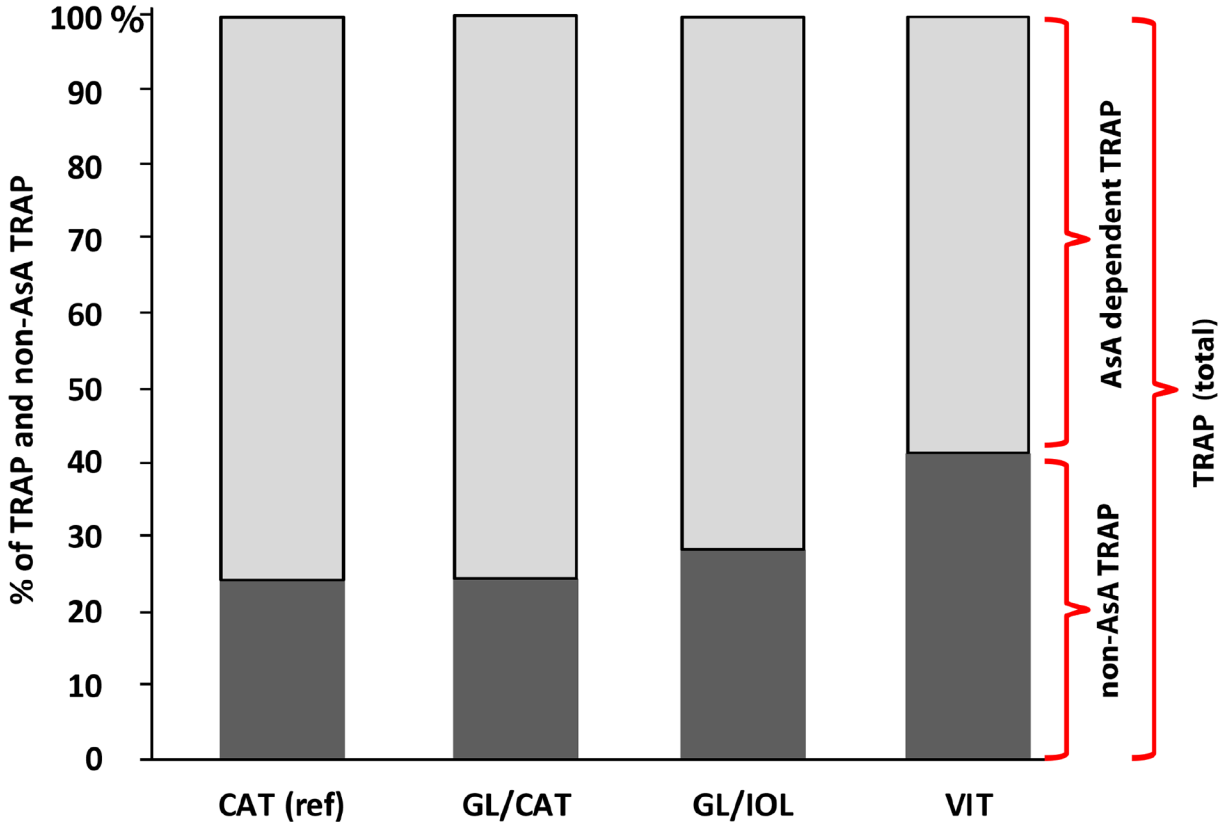


Figure 4.

Comparison of ascorbate (AsA) and non-AsA components of Total Reactive Antioxidant Potential (TRAP). TRAP was designated as 100%, and AsA component (light gray) and non-AsA dependent component (dark gray) are shown as percentages of TRAP values in each patient group.

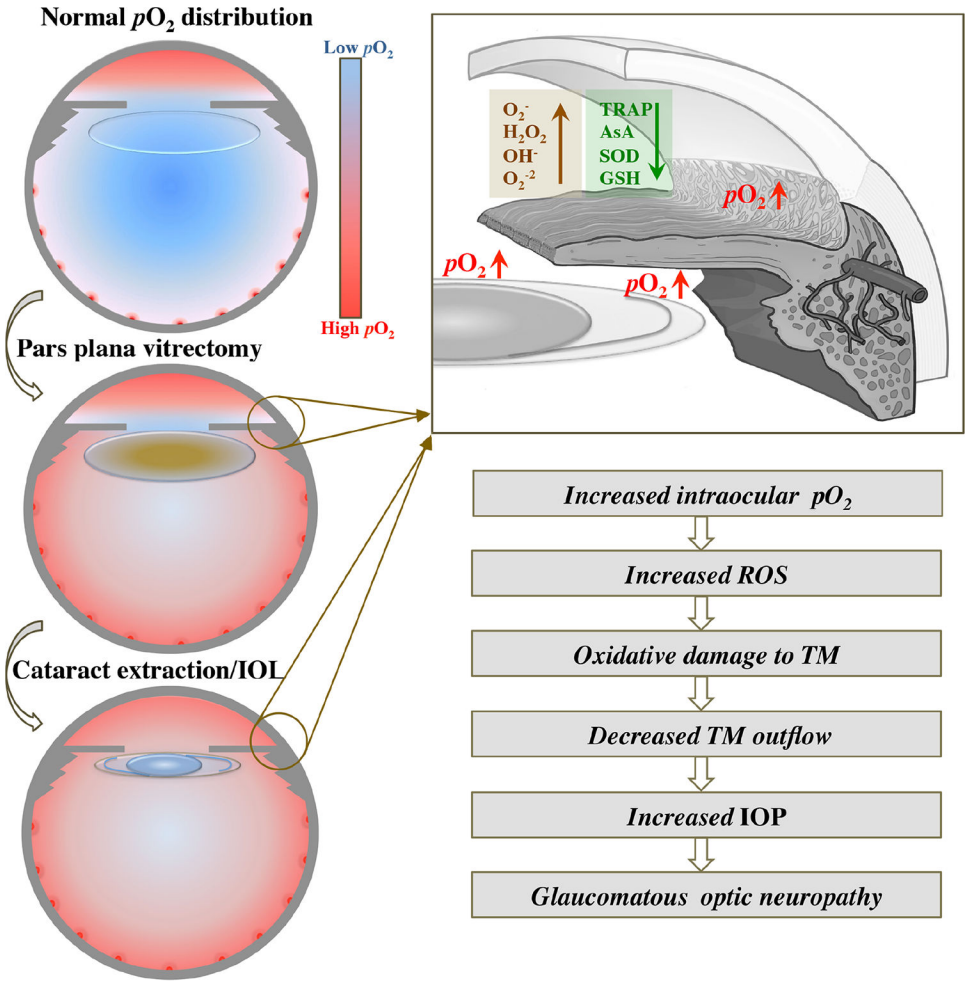


Figure 5.

Proposed mechanism for development of post-vitrectomy glaucoma. Upper left image: 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. Center left image: Following pars plana vitrectomy surgery, pO_2 increases in the vitreous cavity, around lens, in AC angle and posterior chamber with development of nuclear sclerotic cataract. Lower left image: Following cataract extraction and intraocular lens implantation (IOL), pO_2 increases in the AC angle, posterior chamber and mid-AC. Upper right image: 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). Lower right image: Proposed cascade of events following vitrectomy and IOL implantation ultimately leading to increased intraocular pressure (IOP) and risk of glaucoma.

O_2^- = superoxide anion, H_2O_2 = hydrogen peroxide, OH^- = hydroxyl ion, O_2^{-2} = peroxide, TRAP = total reactive antioxidant potential, AsA = ascorbate, SOD= superoxide dismutase, GSH= glutathione, TM = trabecular meshwork

Table 1.

Patient demographic information and group descriptions.

Groups	Group description	Eyes N=	Age (yrs)	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

Key: POAG = primary open angle glaucoma, GL = glaucoma, CAT = cataract, VIT = prior vitrectomy, IOL = intraocular lens, F= female, M=male, AA=African American, CC=Caucasian.

Table 2.

Results of multivariate regression analyses evaluating effect of topical glaucoma medications on ascorbate (AsA) in aqueous humor.

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

Key: Alpha = Alpha-2 agonists, Beta = Beta blockers, CAI = Carbonic anhydrase inhibitors, PG = prostaglandin analogues.