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Glaucoma is a group of diseases characterized by an optic neuropathy with progressive retinal ganglion cell death. Elevated intraocular pressure (IOP) is a major risk factor for glaucoma. The balance between the secretion of aqueous humor and its drainage impacts the IOP. In primary open-angle glaucoma (POAG), there is resistance to aqueous outflow through the trabecular meshwork, whereas in primary angle closure glaucoma (PACG), the access to the trabecular meshwork is typically obstructed by apposition of the iris. The iris is also implicated in the pathophysiology of glaucoma through alterations in its biomechanical properties and fluid conductivity. Altered biomechanical function of the iris exhibits itself as increased mechanical stiffness in the PACG iris, which has been demonstrated in PACG irises both ex vivo and in vivo, in association with a reduction in iris volume/area changes in response to pupil dilation. This property of the PACG iris may be correlated with increase in extracellular matrix gene expression in the iris. Fluid conductivity is also central to iris function, enabling the iris to respond rapidly to light via rapid loss of water from the compressible and porous spongelike iris stroma to the anterior chamber. Fluid conductivity is also central to iris function, enabling the iris to respond rapidly to light via rapid loss of water from the compressible and porous spongelike iris stroma to the anterior chamber. In addition, being the only tissue in the anterior chamber with a vascular network, the iris can facilitate the direct exchange of extracellular fluid from the systemic circulation into the anterior chamber via osmotic gradients, with the potential to affect the IOP. This is clinically evidenced by hypotonicity of the blood osmotic pressure inducing a rise in IOP (e.g., in the water-drinking test) and hypertonicity inducing a fall in IOP (e.g., during administration of oral glycerol, intravenous mannitol, or intravenous hypertonic saline solution). Although alterations in iris fluid conductivity are suspected to play a role in glaucoma pathophysiology, its molecular basis has not been established.

Water transport through the iris may be facilitated by evolutionally conserved protein channels known as...
aquaporins (AQPs). They are integral membrane proteins that mainly facilitate the transport of water in response to osmotic gradients. In humans, the aquaporin family contains 13 isoforms and expression analyses of selected AQPs have been described in diverse areas of normal eyes, as well as in glaucoma and experimental glaucoma eyes, revealing in some cases differential expression levels, and therefore implying potential involvement of AQPs in glaucoma. However, the quantitative levels of AQPs in the iris of glaucoma eyes have not been reported.

Water transport across the iris may influence aqueous osmolality, which may in turn be intimately related to IOP. For instance, buphthalmic rabbits with higher IOP were found to have lower aqueous osmolality compared to those with normal eye pressures. On the other hand, it was reported that no significant difference in aqueous osmolality existed between chronic primary glaucoma and nonglaucoma eyes. Given the lack of consistency in observations and scarcity of studies, it remains unclear whether aqueous osmolality deviates from normal in glaucoma eyes.

In this study, we compared the expression of AQPs 1, 2, 3, 4, and 5 in PACG and POAG irises against nonglaucoma counterparts and further examined the levels of aqueous osmolality in a corresponding cohort of POAG, PACG and nonglaucoma subjects.

METHODS

Patient Recruitment

The study was reviewed and approved by the Institutional Review and Ethics Board at the Singapore Eye Research Institute (SERI) and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients. All patients were recruited from the Singapore National Eye Centre (SNEC).

The diagnosis of glaucoma was based on the presence of glaucomatous optic nerve head damage, defined as vertical cup/disc ratio >0.7, CD asymmetry >0.2, and focal notching with corresponding visual field loss on static perimetry (SITA Standard algorithm with a 24-2 test pattern, Humphrey Visual Field Analyzer II; Carl Zeiss Meditec, Dublin, CA, USA). PACG eyes additionally had occludable angles, defined as having the posterior trabecular meshwork not visible for at least 180° on nonindentation gonioscopy. Exclusion criteria included subjects with secondary glaucoma, previous selective laser trabeculoplasty or iridoplasty, previous intraocular surgery within five years, intraocular inflammation within six months of planned surgery, use of systemic diuretics, preoperative use of intravenous mannitol or intravenous fluids, and prior history of previous acute primary angle closure (APAC) or intermittent angle closure. Patients with previous laser peripheral iridotomy (LPI) were not excluded as the LPI was believed to have an insignificant effect on the iris samples collected for our study, which were sampled from a site separate to the LPI. Patients who were prescribed drugs that may cause uveal effusions (e.g., trimethoprim or sulphur drugs) and chronic topical miotics that may cause iris changes were also not excluded.

Iris Collection

The methodology for the collection and analyses of iris specimens has previously been reported. In short, iris tissues were obtained from surgical peripheral iridotomies performed as part of a standard trabeculectomy procedure from 34 PACG patients and 30 POAG patients between March 2011 and December 2012. Nonglaucoma iris specimens were harvested within eight days of death from 26 cadaver eyes with no known history of glaucoma, procured from the Lions Eye Institute for Transplant and Research (Tampa, FL, USA) with written consent from the next of kin. All iris specimens were collected in RNAlater solution (Thermo Fisher Scientific Inc, MA, USA) and frozen at −80°C on the same day of collection until analyses were performed.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Iris specimens were lysed by sonication, and total RNA was recovered with Trizol Reagent (Invitrogen Corp, CA, USA) as described previously. First-strand cDNA was synthesized using random hexamer primers (Invitrogen Corp) with Superscript III reverse transcriptase (Invitrogen Corp). All qPCR reactions, comprising the Power SYBR Green PCR Master Mix (Applied BioSystems, CA, USA), were performed in triplicates in volumes of 10 μL in 384-well microtiter plates and run using the Roche LightCycler 480 System (Roche Diagnostics Corp, Indianapolis, USA). All mRNA levels were measured as Ct threshold levels. The best house-
Iris Aquaporins and Aqueous Osmolality in Glaucoma

| Iris Specimens | Aqueous Specimens |
|----------------|-------------------|
| POAG           | PACG              | Nonglaucoma | P Value | POAG | PACG | Nonglaucoma | P Value |
| Age, years     | 70.4 (9.2)        | 67.3 (7.0)  | 25.8 (8.9) | <0.001 | 70.5 (9.8) | 71.2 (7.8) | 71.0 (6.8) | 0.132 |
| Gender, male   | 26 (89.7)         | 22 (64.7)   | 19 (67.9)   | 0.058  | 33 (66)    | 22 (45.8)  | 23 (46.9)  | 0.045 |
| Ethnicity      |                   |             |             | <0.001 |           |             |             | 0.298 |
| Chinese        | 26 (89.7)         | 26 (76.5)   | 0           |        | 38 (76.0)  | 43 (87.8)  | 45 (90)    |       |
| Malay          | 0 (0)             | 4 (11.8)    | 0           |        | 1 (2.0)    | 1 (2.0)    | 2 (4)      |       |
| Indian         | 2 (6.9)           | 3 (8.8)     | 23 (82.1)   |        | 8 (16.0)   | 4 (8.2)    | 3 (6)      |       |
| Caucasian      | 0                 | 0           | 23 (82.1)   |        | 0          | 0          | 0          |       |
| Black          | 0                 | 0           | 4 (14.3)    |        | 0          | 0          | 0          |       |
| Other          | 1 (3.4)           | 1 (2.9)     | 1 (3.6)     |        | 3 (6.0)    | 1 (2.0)    | 0          |       |

**TABLE 2.** Demographics of Subjects

**FIGURE 1.** Aquaporin gene expression in the irises of nonglaucoma, PACG and POAG patients. The irises of 26 nonglaucoma, 34 PACG and 30 POAG eyes were analyzed for mRNA expression of **AQP1**, **AQP2**, **AQP3**, **AQP4** and **AQP5**. Values shown indicate fold changes relative to nonglaucoma irises. Each symbol represents data from iris of each subject. Where * and ** are indicated, P < 0.05 (Bonferroni adjusted).

keeping gene, *ACTB* was determined using NormFinder. The average ΔCt value calculated from 26 cadaver irises for each gene was used for calculating the fold change in gene expression from each glaucoma subject by the 2−ΔΔCt method. Primers used for qPCR are shown in Table 1.

**Iris Histology**

Sections were deparaffinized through graded alcohol and rinsed in distilled water (Leica ST5010 Autostainer XL). Antigen retrieval was accomplished by submerging the sections in 10mM citrate buffer (pH6.0) supplemented with 0.05% Tween 20 and boiling in water bath at 95°C to 100°C for 25 minutes. Sections were then left to cool for 20 minutes in the buffer, rinsed in PBS (pH 7.4), and incubated overnight at 4°C with AQP-1 antibody (catalogue ab9566) obtained from Abcam Plc (Cambridge, UK). Labeling by the AQP1 antibody was detected using goat anti-mouse secondary antibodies conjugated to Alexa Fluor-488 (catalogue A11001) obtained from Life Technologies (Thermo Fisher Scientific, Waltham,
MA, USA). Labeled cells were visualized using the Nikon Eclipse Ti microscope (Nikon Instruments Inc., NY, USA).

**Aqueous Humor Osmolality**

Aqueous humour samples were separately collected from 50 patients with POAG and 49 patients with PACG requiring phacoemulsification surgery or combined phacoemulsification and trabeculectomy surgery between March 2016 and October 2018. Fifty patients with no evidence of glaucoma requiring cataract surgery were recruited as controls. Aqueous samples of 10 μL were obtained using a 30-gauge cannula under sterile conditions before commencement of phacoemulsification. The samples were then stored at minus 70°C within two hours of collection until further analysis.

Osmolality was measured using a Wescor 5520 Vapro Vapor Pressure Osmometer (Wescor, Logan, UT, USA), which measures the dew point, calculated as the difference between ambient temperature and the dew point temperature, and is a function of solution vapor pressure, which indirectly measures osmolality. The osmometer was calibrated using Wescor's Optimol ampule osmolality standards as recommended by the manufacturer. All specimens were applied onto filter papers measuring 6.0 mm in diameter provided by Wescor (SS-033). Each aqueous sample (10 μL) was measured three times, and an average of the three measurements was obtained.

**Statistical Analysis**

All statistical analyses were performed using the SPSS version 19 software (IBM SPSS Statistics for Windows, Version 19.0; IBM Corp, Armonk, NY, USA). Post hoc power analysis for the iris gene expression data has been previously described. Data are expressed as mean ± standard deviation (SD). Significance was determined by one-way ANOVA with Bonferroni post hoc adjustment. A p value of <0.05 was deemed to be significant.
RESULTS

Demographic Data for Gene Expression Study

The patient demographics are shown in Table 2. The mean ages of the PACG, POAG and nonglaucoma patients from which iris samples were taken were 70.23 ± 6.91, 70.47 ± 9.84, and 71.15 ± 7.77 years, respectively (P < 0.001). The subjects were predominantly male (64.7% in PACG, 89.7% in POAG, 67.9% in nonglaucoma eyes, P = 0.058) and mainly of Chinese ethnicity in glaucoma patients (76.5% in PACG and 89.7% in nonglaucoma eyes; P = 2.57), but mainly of Caucasian ethnicity in nonglaucoma cases (82.1%) (P < 0.001).

Aquaporin Gene Expression

The mRNA expression of AQP1, AQP2, AQP3, AQP4 and AQP5 of nonglaucoma, POAG and PACG subjects are described in Figure 1. Data from the nonglaucoma iris were used as baseline values for comparisons of fold changes in gene expression in POAG and PACG irises. AQP1 transcript was significantly higher in both POAG and PACG irises, compared to nonglaucoma irises, by 3.12-fold (P = 2.66 × 10^{-3}) and 3.48-fold (P = 5.57 × 10^{-3}), respectively. Similarly, AQP2 transcript was significantly higher in both POAG and PACG irises by 11.58-fold (P = 1.31 × 10^{-4}) and 8.07-fold (P = 4.17 × 10^{-4}), respectively, compared to nonglaucoma irises. AQP3, AQP4 & AQP5 were not significantly different between glaucoma and nonglaucoma irises. None of the aquaporin transcripts investigated were significantly different between POAG and PACG irises.

Demographic Data for Aqueous Osmolality Study

The mean ages of nonglaucoma, POAG and PACG patients from whom aqueous humor was collected were 70.23 ± 6.91, 70.47 ± 9.84, and 71.15 ± 7.77 years, respectively (P = 0.76) (Fig. 2A). There is no statistically significant relationship between the type of patients and gender (29.5%, 42.3%, and 28.2% males in nonglaucoma, POAG, and PACG patients, P = 0.069) (Fig. 2B) or race where they are mainly of Chinese ethnicity (90.0%, 76.0%, and 87.8% in nonglaucoma, POAG, and PACG patients, P = 0.015) (Fig. 2C). There were no significant differences in the topical and systemic glaucoma medications between the PACG and POAG subjects (Table 3). None of the subjects in the control group were on glaucoma medications. Clinically, none of our study subjects appeared to have any uveal effusions, and only one patient was on topical pilocarpine before surgery.

Aqueous Humor Osmolality

There was no statistical difference in aqueous osmolality between POAG and PACG patients. However, when compared against nonglaucoma subjects where the mean aqueous osmolality was 312.60 mmol/kg, the aqueous humor of glaucomatous eyes were significantly lower at 300.79 mmol/kg (P = 0.0012) in POAG eyes and 303.68 mmol/kg (P = 0.022) in PACG eyes (Fig. 2D).

Localization of AQP1 in the Iris

We further investigated the localization of AQP1 in the iris. Immunolabeling of a nonglaucoma and a glaucoma eye with an AQP1-specific antibody revealed that AQP1 was located predominantly in the iris dilator muscle of both irises, with relatively weaker expression in the posterior epithelium (Fig. 3). This is in contrast to studies on the adult rat eye, where AQP1 localized to the apical and basolateral plasma membranes of iris epithelial cell layers and of anterior ciliary nonpigmented epithelial (NPE) cells.

DISCUSSION

Our study provides novel findings that glaucoma eyes, irrespective of subtype, featured higher levels of iris AQP1 and AQP2 transcripts, providing a molecular basis for potential alterations in water conductance in glaucoma eyes. This molecular difference is associated with reduced aqueous osmolality in glaucoma eyes, which suggests enhanced water conductance through the iris into the anterior chamber, which potentially can increase the predisposition of diseased eyes toward IOP increase. Because PACG did not differ from POAG in both properties studied, it is likely that they are common to glaucoma disease in general. Although these properties may not be useful for differentiating PACG from POAG, they offer insights into the molecular basis for increased iris fluid conductivity and water transport in glaucoma eyes.

Because the selective upregulation of AQP1 and AQP2 in glaucoma irises was observed irrespective of disease subtype, we speculate that this molecular phenotype in the glaucoma iris may represent a specific response to disease. Inordinate stress in the glaucoma eye, especially hypoxic stress,12,13,32 which may be caused by ocular hypertension33–35 or reduced ocular blood flow,36,37 may provide the stimulus for specific AQP1/ AQP2 gene induction. Indeed, AQP1 has been shown to be inducible by hypoxia in human retinal vascular endothelial cells.88 Because inadequate oxygen supply in hypoxia is associated with a global repression of gene expression as a result of energy shortage, it follows that proteins that are increased are believed to be important for tissue survival under ischemic

Table 2. Demographic Data for Aqueous Osmolality Study

| Drug Class | Nonglaucoma Mean ± SD | POAG Mean ± SD | PACG Mean ± SD | P Value |
|------------|-----------------------|----------------|--------------|--------|
| Age        | 70.23 ± 6.91 years    | 70.47 ± 9.84 years | 71.15 ± 7.77 years | 0.76   |
| Gender     | 28.2% males           | 42.3% males      | 29.5% males   | 0.069  |
| Race       | 90.0% Chinese         | 76.0% Chinese    | 87.8% Chinese | 0.015  |

Data presented as n(%).
conditions. Indeed, AQP1 may have a protective role as seen by its capacity to promote viability of trabecular meshwork cells placed under mechanical strain. However, it remains possible that upregulation of AQP1 and AQP2 in the glaucoma iris may inadvertently contribute to pathophysiology of this disease.

AQP1 is the archetypal AQP and highly expressed in the eye. On the other hand, AQP2 expression appears to be low in the eye, and although its function in vasopressin-regulated osmotic water transport in the kidney is well established, little is known about its ocular role. Nonetheless, because water channels, AQP1 and AQP2 may be expected to facilitate fluid flow. In the iris, the stroma is highly permeable, and water could easily move in and out by bulk flow. If fluid movement in the iris occurs exclusively by a bulk flow mechanism, it is unlikely that AQP1 or AQP2 will be pivotal for aqueous fluid flow. This is indeed the case in the conventional aqueous outflow tract of human eyes, where overexpression of AQP1 in the trabecular meshwork was demonstrated to be not a significant contributor to bulk outflow.

The location of AQP1 in the iris may provide better clues to its potential role. Our study has highlighted the predominant location of AQP1 in the human iris dilator muscle, with lower expression found particularly in the basal lamina of the posterior pigment epithelium. The dilator muscle is a thin peripheral layer of myoepithelium with both muscular and epithelial functions. It was originally believed that the iris myoepithelium is impervious to fluid flow and forms a barrier preventing mixing of aqueous humor from the posterior and anterior chambers. Our data demonstrate the contrary and, in fact, reveal the iris myoepithelium as possibly actively participating in aqueous humor dynamics. Interestingly, AQP1 expression in myoepithelial cells have been reported in other tissues, including salivary and mammary glands. In the myoepithelial cells surrounding the acini of salivary glands, AQP1 is believed to ensure that water flow into the basal aspect of the acinar epithelial cells is not restricted and allow for the rapid control of myoepithelial cell volume, which may be required as structural support during salivary secretion. In myoepithelial cells underlying teat duct epithelia, the high abundance of AQP1 is believed to contribute to increased permeability of teat duct epithelia in the lactating bovine mammary gland. These two glands have in common roles in the production and secretion of specialized fluids. Although not a gland, the ciliary body, shown to be the dominant site of aqueous human production and secretion in the adult eye, also featured abundant AQP1 expression. We too have detected abundant AQP1 expression in the nonpigmented ciliary epithelium, which is continuous with the posterior pigmented epithelium of the iris (data not shown). Given the association between AQP1 expression in myoepithelia and fluid production in other organs and with aqueous humor production in the ciliary body, high AQP1 expression in the iris seems to support previous suggestion that the iris may contribute to aqueous humor production and, hence, IOP regulation.

The importance for AQP1 in IOP regulation has been established in several studies. First, mice lacking AQP1

![Image](iovsovs.com/ios/journal/figure/3.png)

**Figure 3.** Immunolocalization of AQP1 in the iris and ciliary body of a nonglaucoma and a glaucoma eye. (A) H&E image of the iris revealing the iris dilator muscle (arrowhead) and posterior epithelium (arrow). (B) Abundant AQP1 labelling in the iris dilator muscle (arrowhead) and weaker labeling in the posterior epithelium (arrow).
displayed a phenotype that included reduced aqueous fluid production and lower IOP. Second, disruption of AQP1 via CRISPR-Cas9 in the ciliary body resulted in reduced IOP in mouse models. Moreover, AQPs are known to modulate the gap junction proteins, connexins, and Cx43 in particular. This has been shown to be critical for the integrity of the posterior pigmented epithelium/myoepithelium junction, as well as aqueous humor production and IOP regulation. Although specific interaction between AQP1 (or AQP2) and Cx43 in the iris has not yet been reported, we predict that upregulation of AQP1 may influence Cx43 expression. Alternatively, it is possible for Cx43 to be upregulated in the glaucoma eye and in turn modulate AQP1 and/or AQP2 levels. Further work is required to determine these possibilities and provide further elucidation for the molecular network that connects aqueous humor production and IOP control. Overall, we speculate that elevated AQP1 in the glaucoma iris promotes aqueous humor production and in turn predisposes to higher IOP.

Given the sensitivity of AQPs to osmotic pressure, it is possible that elevated AQP1 or AQP2 in glaucoma eyes may cause an exacerbated response to altered serum osmolality. It has been reported that glaucoma patients were at a higher risk of acute IOP spike when undergoing hemodialysis. As serum osmolality was reduced during dialysis, it is thought that the osmotic difference causes water to migrate from the plasma into the aqueous humor, and when combined with an obstructed aqueous outflow, resulted in acute IOP increase. We speculate that the IOP rise in the event of a difference in aqueous and blood osmotic pressures in glaucoma patients may be further exacerbated by elevated AQP1/AQP2 causing accelerated water transport into the aqueous humor of glaucoma eyes.

Our finding that the aqueous humor osmolality was reduced in glaucoma eyes corroborated reports of the same phenomenon in buphthalmic rabbits. As with AQP1/AQP2, this aqueous humor characteristic was nondiscriminatory for PACG and POAG but was distinctly lower in glaucoma eyes compared to nonglaucoma counterparts. We speculate this is not a coincidence and likely reflects increase in water transport mediated by upregulated AQP1/AQP2. Taken together, we postulate that increased AQP1/AQP2 in the glaucoma iris, and possibly also in other tissues in the anterior chamber as a common stress response, results in aqueous humor of lower osmolality due to the propensity of more water channels to accelerate water transport.

There are several limitations to our study that may be further improved in future studies. First, the iris specimens and aqueous samples were not collected from the same patients. Second, iris samples from glaucomatous eyes were collected from patients, whereas those from nonglaucoma eyes were collected from non-Chinese cadaveric eyes, because of difficulty procuring them locally as a result of a small local population and the local organ donation act, which only allows for research tissues on an opt-in basis and the limited availability of iris tissue from living subjects. Because tissue-specific changes in gene expression are known to occur after death, this is a caveat in the gene expression analysis. The nonglaucoma subjects were also of significantly younger age and of different ethnicity to the glaucoma subjects, which is another limitation because it is not known what role age and ethnicity play in AQP expression. There was a preponderance of Chinese eyes among patients with glaucoma in our study, which are known to have the thickest irises. Given that the glaucoma irises in this study were derived from people of Asian ethnicity, the data may not be extrapolatable to Caucasian eyes. Our study did not exclude patients who were on carbonic anhydrase inhibitors, which can potentially cause a diuresis-related hyperosmolar state. However, the potential for carbonic anhydrase inhibitors to increase the serum osmolality should theoretically increase the aqueous osmolality, not reduce it. Finally, AQPs are also involved in aqueous production in the ciliary body and aqueous filtration in the trabecular meshwork, but our study is limited in that it has only examined AQPs in the iris in isolation.

In summary, we report that glaucoma eyes, regardless of subtype, may be distinguished from nonglaucoma eyes by the higher expression of iris AQP1 and AQP2 and by a significant reduction in aqueous osmolality. Our hypothesis is that elevated AQP1/AQP2 will promote aqueous humor production and water transport, and thus sustain high IOP. More studies are required to verify this hypothesis, as well as address the issue of whether AQP1 and AQP2 were similarly induced in other sections of the anterior chamber.

Acknowledgments

The authors thank Anita S. Chan for assistance with analyzing the histology samples and Li Zhen Toh and clinical research coordinators Lee Jia Yi and Stella Ng from Singapore Eye Research Institute for assistance with sample collections.

Supported by grants under the Health Research Endowment Fund (HREF) administered by the Singapore National Eye Centre to TTW, a New Investigator Grant (NMRC/NIG/1070/2012) administered by the National Medical Research Council to AN, as well as funding from the Singapore National Research Foundation under its Translational and Clinical Research (TCR) Programme (NMRC/TCR/008-SEBI/2013) administered by the Singapore Ministry of Health’s National Medical Research Council.

Disclosure: O.S. Huang, None; L.-F. Seet, None; H.W. Ho, None; S.W. Chu, None; A. Narayanaswamy, None; S.A. Perera, None; R. Husain, None; T. Aung, None; T.T. Wong, None.

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