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Elevated histamine levels in aqueous humor of patients with glaucoma

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Purpose: Neurotransmitters (NTs) are the key mediators of essential ocular functions, such as processing the visual functions of the retina, maintaining homeostasis of aqueous humor, and regulating ocular blood flow. This study aims to determine variations in the levels of L-glutamate and γ-aminobutyric acid (GABA), histaminergic, adrenergic, cholinergic, and serotonergic NTs in patients with primary glaucoma versus patients with cataract.

Methods: This case–control study involved three age-matched groups of patients with primary open angle glaucoma (POAG, n = 14), primary angle closure glaucoma (PACG, n = 21), and cataract (control, n = 19). Patients' aqueous humor and plasma were collected, snap frozen at −80 °C, and subjected to ultrasensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis for quantification of NTs.

Results: Baseline intraocular pressure and the cup-to-disc ratio were found to be statistically significantly elevated in the POAG and PACG groups compared to the cataract control group. In aqueous humor, histamine was found to be statistically significantly elevated (5-fold, $p<0.0001$), whereas 1-methyl histamine was statistically significantly decreased ($p<0.05$) in POAG compared to the control group. A statistically significant increase in L-glutamate and GABA was observed among both patient groups with glaucoma compared to the cataract control group. Adrenaline was found to be elevated only in the PACG group (2.7-fold, $p<0.05$). No statistically significant difference was observed among the plasma NT levels between the groups.

Conclusions: This study demonstrated the prominent role of the histaminergic system apart from autonomic mechanisms in the progression of glaucoma. Elevated L-glutamate and GABA could be due to retinal ganglionic cell death. Further studies are required to evaluate the effects of histamine on Müller cell dysfunction.

Glaucoma is a multifactorial disease characterized by neurodegenerative optic neuropathy and retinal ganglionic cell death that leads to irreversible loss of vision without any symptoms [1-3]. Glaucoma is the leading cause of irreversible blindness worldwide, and its global prevalence is estimated to be up to 112 million in 2040 [4].

The eye is considered a highly complex and immune-privileged part of the nervous system, and neurotransmitters (NTs) are the key mediators for processing visual information and regulating the aqueous humor dynamics and ocular blood flow. The development of drugs for therapeutic management of glaucoma has been based on the involvement of the autonomic system and its related NTs in aqueous humor regulation. The localized autonomic nervous system controls the ciliary body and iris, thus influencing neuronal control of intraocular pressure (IOP) [5]. Blood perfusion control of individual ocular tissues is augmented by additional factors at postganglionic fibers which is controlled by autonomic inputs through sympathetic and parasympathetic nerve fibers. L-glutamate and γ-aminobutyric acid (GABA) are the principle excitatory and inhibitory NTs responsible for synaptic transmission of retinal signals for visual perception [6,7]. L-glutamate-induced excitotoxicity is one of the well-understood mechanisms responsible for retinal ganglionic cell death in glaucoma [8,9]. Although most of the NTs involved in the central nervous system are also reported in the visual signal transduction pathway of the retina, their influence on aqueous humor dynamics has yet to be elucidated in patients with glaucoma.

The advent of techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS) has made possible the precise quantification of a group of water-soluble analytes from a single sample to study the total variation in a sample compared to an age-matched control group (patients undergoing surgery for senile cataract). The purpose of the study was to evaluate the levels of NTs in the aqueous humor of patients with primary open angle (POAG) and primary angle

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closure glaucoma (PACG) undergoing trabeculotomy surgery compared with patients with normal IOP undergoing cataract surgery (the control group).

METHODS

Human ethics approval and patient recruitment: The experiments conducted according to the tenets of the Declaration of Helsinki and the ARVO guidelines for vision research in humans. The protocol was approved by the Institute Ethics Committee of All India Institute of Medical Sciences, New Delhi, India (Ref. No. IECPG-185/01.09.2017). This study involved 49 subjects, divided into three groups, POAG (n = 14), PACG (n = 21), and age-matched cataract (n = 19) as controls.

Sample collection: After written informed consent was obtained, patients of either sex between the age of 18 to 70 years diagnosed with either POAG or PACG were recruited from Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences (AIIMS), New Delhi, from December 2018 to March 2020. The cohort had 34.6% of females and 65.4% of males. Patients with any other ocular pathology and systemic illness were excluded from the study. Aqueous humor (about 100 µl) was collected by paracentesis using a 30-gauze needle during the trabeculectomy and cataract surgery for patients with glaucoma and cataract (control), respectively. Collected samples were stored at −80 °C and were analyzed within 6 months post-collection. Blood samples (approx. 2ml) were also collected before the procedure and stored in EDTA vials, and plasma was stored for analysis of NTs.

Chemicals: L-glutamic acid (G1251), GABA (A2129), acetylcholine (A6625), histamine (H7125), 1-methyl histamine dihydrochloride (M4910), adrenaline (E4250), noradrenaline (A7257), dopamine (PHR1090), and serotonin hydrochloride (H9523) were purchased from Sigma-Aldrich Pvt. Ltd. (St. Louis, MO). L-DOPA (D0600) and N-acetyl serotonin (A1277) were procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Mass spectrometry–grade acetonitrile and formic acid (FA) were procured from Merck (Darmstadt, Germany). Ammonium formate was obtained from Central Drug House (New Delhi, India). Ultrapure water of 18.2 mΩ resistance was used for the analysis which was purified through the Milli-Q system (Millipore Corp., Burlington, MA).

Liquid chromatographic conditions: Chromatographic separation was attained using the Agilent high performance liquid chromatography (HPLC) 1260 Infinity series system (Agilent, Santa Clara, CA) with a ZIC-cHILIC column (100 mm × 4.6 mm; Merck, Kenilworth, NJ). Liquid chromatographic (LC) conditions were adopted from Tufi et al. with minor modifications [10]. For analyzing L-glutamate, GABA, acetylcholine, histamine, and 1-methyl histamine, the mobile phase consisting of ultrapure water with 0.1% FA (A), acetonitrile with 0.1% FA (B), and 10 mM ammonium formate (pH 3.2, adjusted using FA; C) was used. The initial condition was started with the mobile phase comprising the solvents in the ratio of 10% (A):70% (B):20% (C) which was maintained for 8 min. Then, B and C were shifted to 40% and 60%, respectively, at 9 min and sustained up to 15 min; at 16 min, the mobile phase was reversed to the initial conditions with a total run time of 20 min. For analyzing adrenaline, noradrenaline, dopamine, levodopa, serotonin, and N-acetyl serotonin, use of ammonium formate as a buffer was avoided to improve sensitivity by ten times in terms of limit of detection, especially for catecholamine metabolites. Therefore, for this analysis, the mobile phase contained ultrapure water with 0.1% FA (A), acetonitrile with 0.1% FA (B), and pure acetonitrile (D). The initial condition started with 15%(A): 75%(B): 10%(D) which was maintained for 3 min and shifted to 40%(A):5%(B):55%(D) at 6 min. It was gradually shifted to 28%(A):40%(B):32%(D) by 11.20 min, 20%(A):70%(B):10%(C) at 13 min and reversed to the initial condition at 16 min with a total run time of 20 min. A constant flow rate of 500 µl/min was maintained throughout the run with ambient column compartment temperature and 20 µl injection volume. The standard calibration range, R^2 value, retention time, and limit of quantification (LOQ) of all the metabolites in both methods are listed in Table 1.

Mass spectrometry conditions: For optimizing all the source- and compound-related parameters, the constant infusion of individual analytes at 100 ng/ml was made into the positive electrospray ionizer $[M+H]^+$ of mass spectrometry (Q-trap 4000; AB Sciex, Foster City, CA) at a 5 µl/min flowrate using a Harvard infusion pump (Holliston, MA). Optimization and data analysis were performed using Analyst software ver 1.5.2 (AB Sciex). The details of all the parent-to-fragment ion (m/z, Q1→Q3) transitions of all the NTs used in the study are listed in Table 1, and the respective chromatograms are shown in Figure 1.

Calibration standards and sample preparation: Standard stock solutions of all the analytes (1 mg/ml) were separately prepared using ultrapure water and for catecholamines using 0.2% FA. For aqueous humor and plasma analysis, serial dilutions (of the calibration ranges mentioned in Table 1), were performed in ultrapure water and human plasma (obtained from the blood bank, AIIMS, New Delhi, India), respectively. For analyzing L-glutamate, GABA, acetylcholine, histamine,

Table 1. List of neurotransmitter metabolites studied, their respective precursor (Q1) to fragment ion (Q3) transitions, retention time, limit of detection (LOD), R2 values of standard calibration along with calibration range.

S.no	Neurotransmitter metabolites	Q1→Q3	Retention time (min)	LOD (ng/ml)	R^2	Calibration range (ng/ml)
1	Acetyl choline	146.2→87.2/60.2	3.5	0.2	0.998	0.313–20
2	GABA	104.1→87.1/69.1	7.65	2.5	0.998	5–320
3	Glutamate	148.1→130.1/ 84.1	8.25	90	0.999	125–8000
4	1-methyl Histamine	126.1→109.2	16.64	0.12	0.998	0.313–20
5	Histamine	112.2→95.1/83.1	15.4	0.12	0.996	0.313–20
6	N-Acetyl serotonin	219.1→160.1	2.91	0.09	0.994	0.19–12.5
7	Serotonin	177.1→160.1/132.1	9.12	0.04	0.995	0.19–12.5
8	Dopamine	154.2→137.2/119.1/91.1	10.2	0.03	0.995	0.19–12.5
9	Adrenaline	184.1→166.1	10.5	0.01	0.995	0.030–3.84
10	DOPA	198.1→181.1/152.1	12.6	0.25	0.992	0.39–12.5
11	Noradrenaline	152.1→135.1/107.1	12.7	0.06	0.999	0.19–6.25

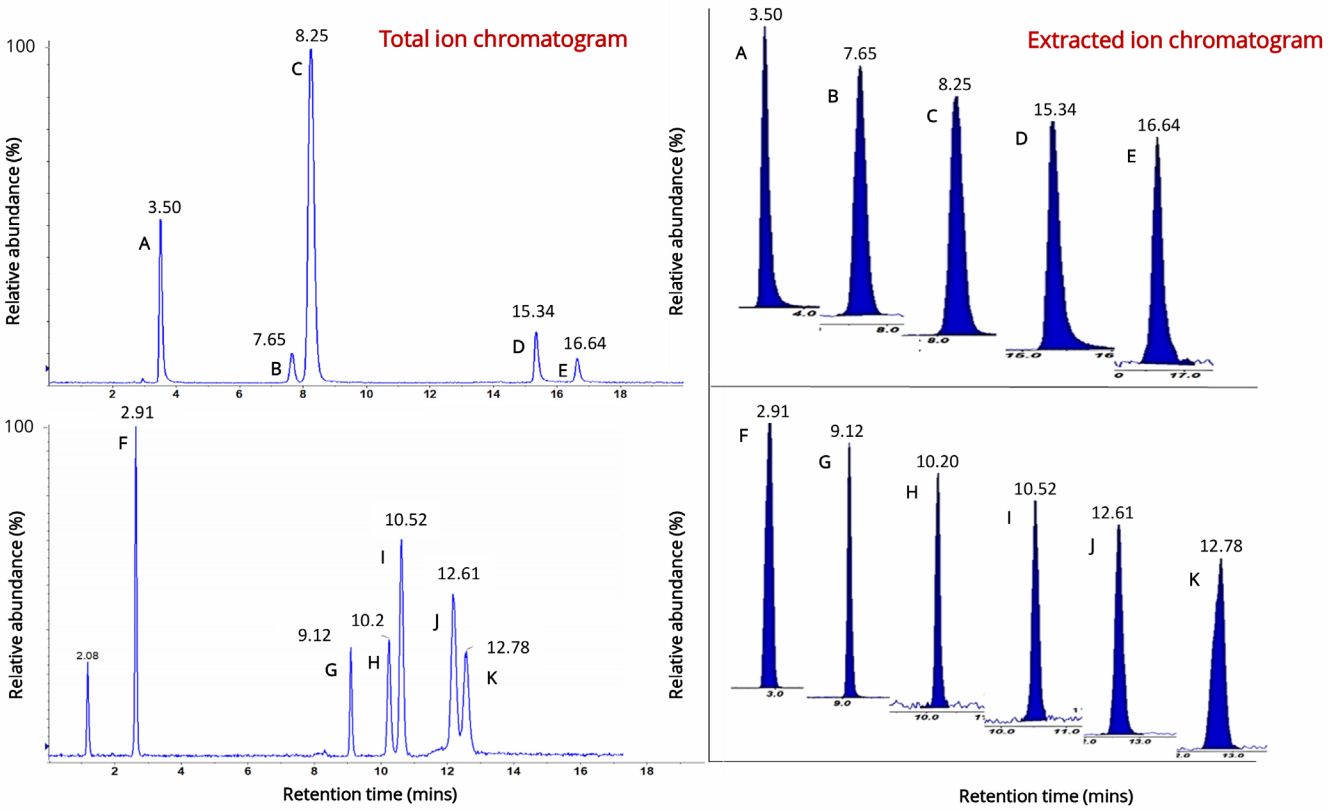

Figure 1. The figure depicts the total and extracted ion chromatograms of the analytes quantified in the adopted method: **A**) acetyl choline, **B**) γ-aminobutyric acid (GABA), **C**) L-glutamate, **D**) 1-methyl histamine, **E**) histamine, **F**) N-acetyl serotonin, **G**) serotonin, **H**) dopamine, **I**) adrenaline, **J**) L-DOPA, and **K**) noradrenaline.

and 1-methylhistamine, 10 µl aqueous humor/5 µl plasma was added to 90 µl of extraction solvent containing 75% of acetonitrile with 0.1% FA. This was vortexed for 1 min and centrifuged at 7,800 ×*g* for 10 min at 4 °C, following which 95 µl of the clear supernatant was loaded in 96 micro-well plates (Tarson, Kolkata, India), and 20 µl was injected in the analytical column. For analyzing adrenaline, noradrenaline, dopamine, L-DOPA, serotonin, and N-acetyl serotonin, 40 µl of aqueous humor was lyophilized and reconstituted with 40 µl of the extraction solvent containing 75% acetonitrile with 0.1% FA, then vortexed for 1 min, and centrifuged at 15,000 ×*g* at 4 °C. From this, 35 µl of the supernatant was loaded in a 384-well plate (MJ Research, Corning, NY), and 20 µl was injected in the analytical column. The analytical methods were individually validated per the recommendation of the U.S. Food and Drug Administration (FDA) for bioanalysis [11].

Statistical analysis: The data were presented in the form of number (%) for qualitative variables and mean (standard deviation [SD])/median (interquartile range [IQR]) for quantitative variables. The study groups were individually compared with the control (cataract) group using the unpaired Student *t* test, and the Mann–Whitney rank sum test was used for non-parametric data. To find the association of qualitative variables with a group, the chi-square test or the Fisher exact test was used as required. In addition, correlation analysis was performed to measure the linear correlation between aqueous humor and plasma collected from the same patients involved in this study. GraphPad Prism (version 7.0, GraphPad Software Inc., San Diego, CA) was used for the analysis. A p value of less than 0.05 was considered statistically significant.

RESULTS

Demographic data of subjects: There was no statistically significant difference observed between the mean age group of the patients with POAG (p=0.56) and PACG (p=0.61) versus the cataract control group. No association was observed between gender and study groups (POAG, p=0.17; PACG, p=0.22 versus control). Baseline IOP (POAG, p<0.0001; PACG, p<0.0001 versus control), drug-treated IOP (POAG, p<0.01; PACG, p<0.01 versus control), and cup-to-disc ratios (POAG, p<0.0001; PACG, p<0.0001 versus control) of the study eye were statistically significantly elevated in both groups with glaucoma compared to the control group. Mean arterial pressure was found to be statistically significantly elevated in the POAG group (p<0.05) compared to the control group. Other patient characteristics, such as weight, gender

Table 2. Demographic data of the POAG, PACG and control patients involved in this study.

Patient demography	POAG (n=14)	PACG (n=21)	Control (n=19)
Age in years, (mean ± SD)	53.21±11.32	56.42±7.88	58.6±5.42
Median (IQR)	52.5 (32,74)	58 (39, 66)	56 (36, 66)
Weight in Kg, (mean ± SD)	62.79±9.84	59.14±9.15	57.35±12.78
Median (IQR)	62 (49, 83)	60 (40, 75)	60 (40, 74)
Baseline IOP in mmHg, (mean ± SD)	33.5±10.76****	39.85±9.27####	14.03±3.70
Median (IQR)	30.5 (21, 56)	40 (29, 60)	14 (10, 18)
Treated IOP in mmHg, (mean ± SD)	20.5±10.28**	28.14±15.19##	NA
Median (IQR)	18 (14, 55)	22 (13, 60)	NA
Cup-to-disc ratio (X:1), (mean ± SD)	0.93±0.05****	0.86±0.12 ####	0.32±0.09
Median (IQR)	0.9 (0.85, 1)	0.9 (0.5, 1)	0.3 (0.3, 0.5)
Mean arterial pressure (mmHg), (mean ± SD)	105.05±10.64*	101.83±20.20	95.46±7.73
Median (IQR)	105.17 (84, 130.67)	101.33 (73.33, 128.35)	97.67 (72, 104)
Therapeutic classes of glaucoma medications used on therapy			
Prostaglandin analogs (%)	100	66.67	NA
Beta Blockers (%)	85.71	57.14	NA
Cholinergic agonist (%)	21.43	76.19	NA
Alpha agonist (%)	92.86	66.67	NA
Carbonic anhydrase inhibitors (%)	71.43	71.43	NA

POAG- Primary Open angle glaucoma PACG- Primary angle closure glaucoma. POAG VS Control group, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 PACG VS Control group- #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001

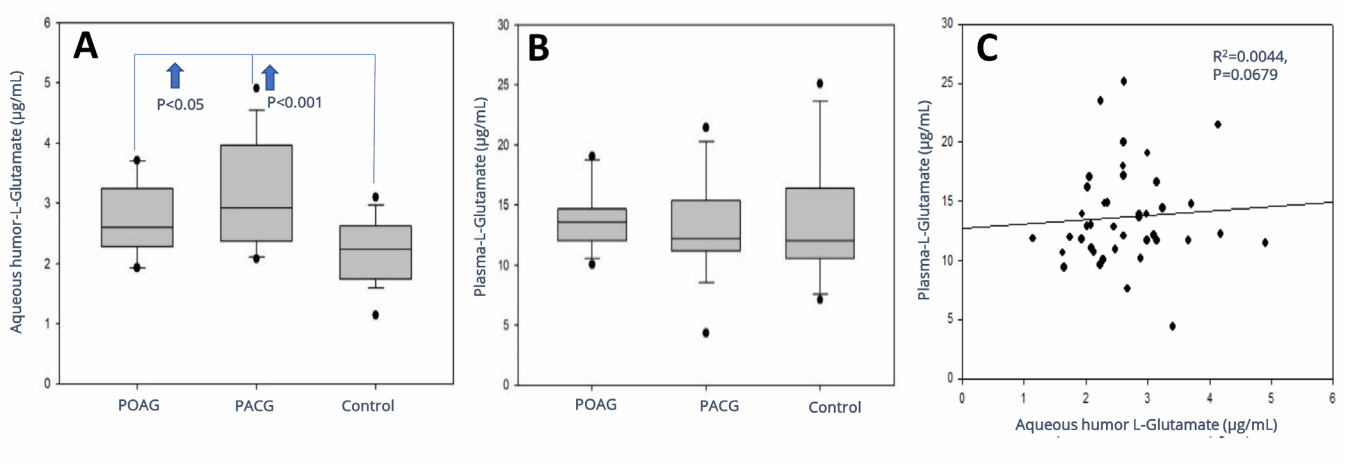

Figure 2. The figure shows the variation in the levels of L-glutamate in the **A**) aqueous humor, **B**) plasma of patients with primary open angle (POAG) and primary angle closure (PACG) glaucoma compared to the control (cataract) group, and **C**) linear correlation analysis of the aqueous humor and plasma L-glutamate levels collected among the same patients in this study.

distribution ratios, and the percentage class of drug used for glaucoma treatment for each group, are shown in Table 2.

L-glutamate and GABA: L-glutamate (POAG, 2.75±0.62 µg/ml, p<0.05; PACG, 3.12±0.86 µg/ml, p<0.001 versus control 2.23±0.53 µg /ml) and GABA (POAG, 44.75±19.01 ng/ml, p<0.05; PACG, 47.61±14.66 ng/ml, p<0.01 versus control 30.60±13.86 ng/ml) were found to be statistically significantly elevated in the aqueous humor of the POAG and PACG groups compared to the control group (Figure 2A and Figure 3A). However, the plasma concentration of L-glutamate (POAG, 13.86±2.530 µg/ml, p=0.76; PACG, 13.17±3.940 µg/ml, p=0.87 versus control 13.42±4.820 µg/ml) and GABA (POAG, 24.62±10.31 ng/ml, p=0.29; PACG, 27.13±6.770 µg/ml, p=0.64 versus control 28.26±8.34 µg/ml) in both glaucoma groups did not show any statistically significant difference compared to the control group (Figure 2B and Figure 3B). Poor correlation was observed for L-glutamate (p=0.0679) and GABA (p=0.177) among the aqueous humor and plasma levels, respectively (Figure 2C and Figure 3C).

Acetylcholine: Acetylcholine levels in the aqueous humor samples were below the lower limits of quantification (<0.3 ng/ml) of the developed method in 87.5%, 70.5%, and 40% of patients with PACG, cataract, and POAG, respectively. Plasma levels of acetylcholine were undetectable in all groups.

Histamine and 1-methyl histamine: It was observed that histamine was statistically significantly elevated only in the aqueous humor of the POAG group with respect to the cataract control group (2.94±1.79 ng/ml versus 0.59±0.29 ng/

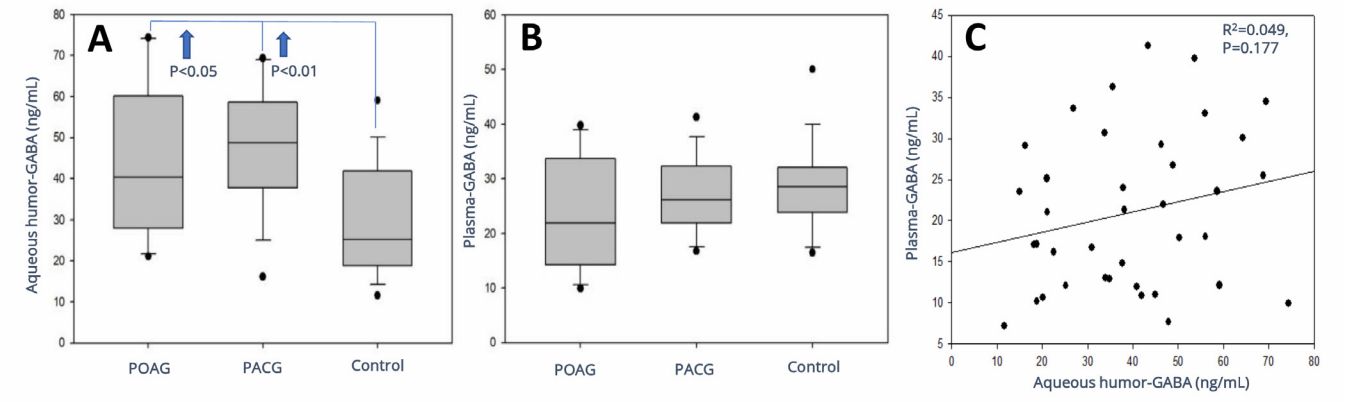

Figure 3. This figure shows the variation in the in the levels of γ-aminobutyric acid (GABA) in the **A**) aqueous humor, **B**) plasma of patients with primary open angle (POAG) and primary angle closure (PACG) glaucoma compared to the control (cataract) group, and **C**) linear correlation analysis of the aqueous humor and plasma GABA levels collected among the same patients in this study.

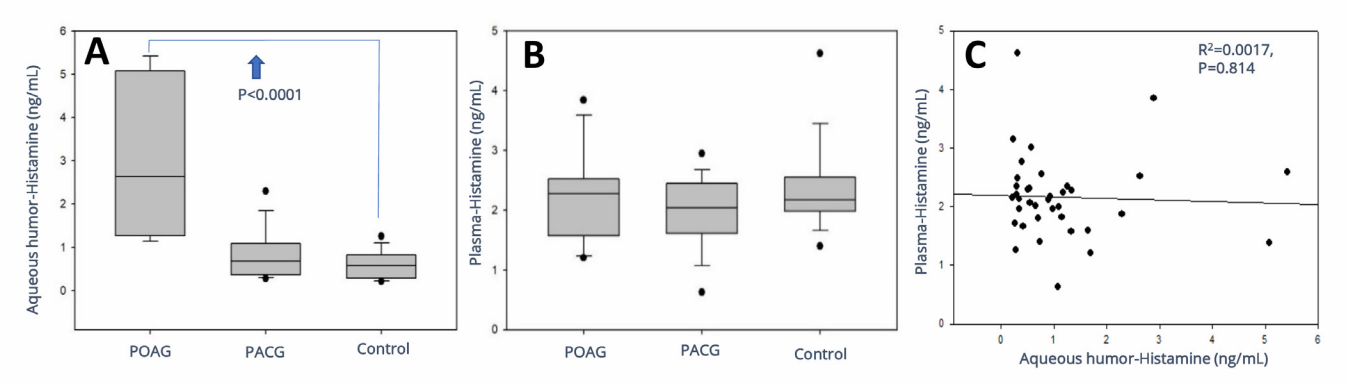

Figure 4. The figure shows the variation in the levels of histamine in the **A**) aqueous humor, **B**) plasma of patients with primary open angle (POAG) and primary angle closure (PACG) glaucoma compared to the control (cataract) group, and **C**) linear correlation analysis of the aqueous humor and plasma histamine levels collected among the same patients in this study.

ml; p<0.0001). However, no statistically significant alteration was observed in the PACG group (0.83±0.41 ng/ml, p=0.139; Figure 4A). 1-Methyl histamine (methylated metabolite of histamine) levels in aqueous humor were statistically significantly declined in the POAG group with respect to the control group (POAG, 1.71±0.88 ng/ml versus control 2.75±1.30 ng/ml; p<0.05; Figure 5A). No statistically significant difference was observed in the plasma levels of histamine (POAG, 2.19±0.72 ng/ml, p=0.55; PACG, 1.98±0.56 ng/ml, p=0.11 versus control 2.35±0.73 ng/ml) and 1-methyl histamine (POAG, 0.61±0.25 ng/ml, p=0.90; PACG, 0.60±0.26 ng/ml, p=0.98 versus control 0.60±0.17 ng/ml) in the glaucoma group compared to the control group (Figure 4B and Figure 4C). Poor correlation was observed between the aqueous humor and plasma levels of histamine (p=0.814) and 1-methyl histamine (p=0.728), respectively (Figure 4C and Figure 5C).

Adrenergic, dopaminergic, and serotonergic neurotransmitters: The adrenaline level was found to be statistically significantly elevated in the aqueous humor of the PACG group compared to the control group (p<0.05). Aqueous humor levels of other NTs, such as noradrenaline, dopamine, L-DOPA, 5-hydroxy tryptamine, and N-acetyl serotonin, did not differ statistically significantly across the different groups of patients (Table 3).

DISCUSSION

Although a plethora of literature is available that implies autonomic control and its modulation for the therapeutic benefit of IOP control in glaucoma [12-15], variation in the levels of various NTs has not been quantified to substantiate the same. Understanding the dominant NTs in patients with POAG and PACG is expected to rationalize other therapeutic strategies to

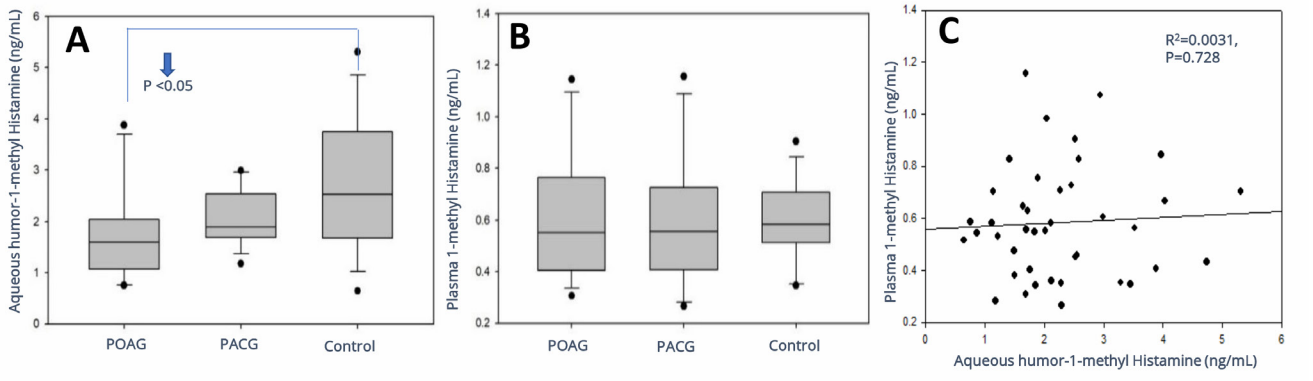

Figure 5. The figure shows the variation in the levels of 1-methyl histamine in the **A**) aqueous humor, **B**) plasma of patients with primary open angle (POAG) and primary angle closure (PACG) glaucoma compared to the control (cataract) group, and **C**) linear correlation analysis of the aqueous humor and plasma 1-methyl histamine levels collected among the same patients in this study.

Table 3. Variation in the Aqueous humor levels of adrenergic and serotonergic neurotransmitters of POAG and PACG patients versus control.

Neurotransmitter metabolites	Aqueous humor concentration (ng/ml)			Fold change versus control (p-value)	
	POAG	PACG	Cataract	POAG	PACG
Adrenaline (mean± SD)	0.07±0.05	0.12±0.08#	0.04±0.01	1.62 (0.11)	2.50 (0.015)
Median (IQR)	0.056 (0.03, 0.17)	0.10 (0.05, 0.27)	0.039 (0.03, 0.08)		
Noradrenaline (mean± SD)	0.34±0.14	0.36±0.11	0.41±0.19	0.81 (0.29)	0.87 (0.49)
Median (IQR)	0.37 (0.19, 0.60)	0.38 (0.19, 0.51)	0.38 (0.19, 0.65)		
Dopamine (mean± SD)	0.75±0.47	0.74±0.38	0.76±0.35	0.98 (0.95)	0.98 (0.94)
Median (IQR)	0.68 (0.2, 1.43)	0.86 (0.19, 1.18)	0.75 (0.19, 1.34)		
DOPA (mean± SD)	4.53±2.76	4.87±1.02	5.89±2.69	0.76 (0.35)	0.82 (0.43)
Median (IQR)	4.66 (1.14, 8.78)	4.37 (4.08, 6.60)	6.71 (2.17, 9.77)		
Serotonin (mean± SD)	4.69±1.64	5.06±1.17	4.55±1.79	1.02 (0.87)	1.11 (0.55)
Median (IQR)	4.53 (2.88, 7.32)	5.01 (3.46, 6.47)	3.84 (3.12, 8.58)		
N-acetyl serotonin (mean± SD)	1.87±1.27	1.54±1.05	1.17±0.76	1.59 (0.17)	1.31(0.43)
Median (IQR)	1.40 (0.69, 2.54)	1.07 (0.63, 3.38)	1.03 (0.40, 2.8)		

POAG- Primary Open angle glaucoma and PACG- Primary angle closure glaucoma. POAG VS Control group, *p<0.05 PACG VS Control group- #p<0.05

preserve deterioration of vision despite effective IOP control. India accounts for around 12 million glaucoma cases, which is one-fifth of the world's glaucoma burden [16]. POAG and PACG are highly prevalent and the two most common forms of all primary adult glaucoma (PAG) [17].

For this study, the group of major NTs comprising L-glutamate, GABA, histamine, noradrenaline, adrenaline, DOPA, dopamine, acetylcholine, and serotonin was selected to represent the excitatory amino acid, neuronal inhibitory, histaminergic, adrenergic, dopaminergic, cholinergic, and serotonergic pathways, respectively. N-acetyl serotonin and 1-methyl histamine were also selected to understand the metabolism of serotonin and histamine. Histamine is an important neurotransmitter, but its influence on ocular physiology and pathology has yet to be elucidated. Neurons of the histaminergic system innervate from the tuberomammillary nucleus of the posterior hypothalamus, and their projections are reported to be present in the retinas of rodents and primates. Histaminergic receptors (H_1R to H_4R) are distributed over the various retinal cell layers and ocular structures (H_1R, dopaminergic amacrine cells and horizontal cells; H_2R, choroidal blood vessels and retinal capillaries; H_3R, ON bipolar cells; H_4R, mast, leucocyte, and peripheral hemopoietic cells). Histamine plays a major role in maintaining retinal ganglion cellular activity, their light response, and regulation of circadian oscillations along with the circadian pacemaker, that is, the suprachiasmatic nucleus (SCN) of the hypothalamus. Resch et al. demonstrated that intravenous administration of histamine increased subfoveal choroidal blood flow and the diameter of retinal blood vessels via H_2 receptors, and its antagonist cimetidine did not alter its effects [18]. Histaminergic tone influences the circadian rhythm of IOP regulation and causes ciliary muscle contraction, and its dysfunction is expected to play a central role in glaucoma [19].

For the first time, this study observed a fivefold elevation of histamine levels in the aqueous humor of patients with POAG, compared to the levels found in the PACG and control groups. This elevation was independent of the concentration of histamine in plasma. This localized increase in the levels of histamine indicates the possibility of involvement of the localized histaminergic system apart from autonomic mechanisms. Histamine is an endogenous biogenic monoamine reported to be involved in mediating allergy, central nervous system neurotransmission, gastric acid secretions, and inflammatory response [20,21]. Histamine is metabolized by the intracellular enzyme N-methyltransferase to form 1-methylhistamine. In the present study, 1-methyl histamine levels were also found to be statistically significantly lowered in the aqueous humor of patients in the POAG group compared to the control group. However, the levels of 1-methyl histamine in plasma did not differ statistically significantly among the groups, indicating that plasma levels likely do not represent ocular levels of histamine and its metabolism.

Nowak and Nawrocki evaluated histamine levels in various ocular tissues (the iris, ciliary body, choroid, retina, sclera, and optic nerve) from enucleated human eyes from patients with endophthalmitis, perforated wounds of the cornea or sclera or both, or uncontrolled glaucoma. The study revealed that inflammation (endophthalmitis) increased tissue histamine levels up to five- to tenfold, followed by glaucoma compared to penetrating trauma [22]. Their observation indicated the involvement of an inflammatory process in glaucoma causing an increase in the tissue levels of histamine. In intravitreal hypertonic saline-induced transient and intracameral carbomer-induced stable models of ocular hypertension (OHT), Lanzi et al. reported that H_3R antagonists are capable of significantly reducing OHT and preventing retinal ganglion cell loss by improving vascular performance of the central ophthalmic artery, and reducing oxidative stress, thus indicating the possibility of using H_3R antagonists as a potential antiglaucoma medication. The authors reported the protein expression of H_1, H_3, and H_4 receptors in the retina, optic nerve, and trabecular meshwork (TM). However, mRNA expression of these receptors was reported only in the retina and the TM. Moreover, the immunofluorescence study showed the presence of histamine H_1, H_3, and H_4 receptors in retinal ganglion cells (RGCs) in the authors' model, and they reported significant choroidal mast cell degranulation [23]. Taking the observations of Lanzi et al. forward, the observations of the present study further substantiate the role of histamine in POAG and rationalize the development of H_3R antagonists as an adjunct to other agents.

Greferath et al. investigated retinal function and retinal blood flow dynamics using the histidine decarboxylase knockout mice model. The results of their study indicated that histamine plays a minor role in retinal structure and function [24]. As histamine is not expected to cross the blood–brain barrier, systemic histamine levels are unlikely to be responsible for the elevation of histamine levels in patients with POAG found in this study [24]. Moreover, the lack of difference in the plasma histamine levels across the groups shows that ocular levels of histamine may play an isolated and significant role in POAG. The major source of histamine found in the aqueous humor [23] and tissues [22] could be the choroidal mast cell degranulation in glaucoma which could be a source of RPE inflammation [25]. The H_3 receptor has been reported to promote cerebral ischemia reperfusion injury in experimental models which was explained by H_3R/chloride intracellular channel 4 (CLIC4) binding activated autophagy and showed that H_3R antagonists had a preventive role [26]. However, the role of this receptor in retinal ganglionic cell death has yet to be examined.

Ciproxifan is a H_3 receptor inverse agonist and antagonist reported to reduce the extracellular signal-regulated kinase (ERK)/synapsin I cascade and decrease L-glutamate release in the rat hippocampus [27]. In the present study, increased levels of histamine in aqueous levels were accompanied by an increase in aqueous humor L-glutamate levels in the POAG group. In the present study, statistically significant elevation of L-glutamate and GABA levels in the aqueous humor was observed in the POAG and PACG groups which could also be due to the loss of the function of Müller cells of removing extracellular GABA and L-glutamate [28,29]. This study recorded the lack of direct statistically significant influence of dopaminergic and serotonergic systems among the study groups. This could be due to the efficient reuptake of their NTs as well as their neuronal metabolism. However, increased levels of L-glutamate and GABA in aqueous humor of the PACG group were accompanied by an increase in adrenaline levels compared to the control group, indicating the predominant iris traction–related autonomic dysregulation.

In the mammalian central nervous system, GABA and L-glutamate are inhibitory and excitatory. GABA is one of the key determinants of neuronal conduction. Involvement of the excitatory amino acid pathway mediated by L-glutamate has been well emphasized in the mechanism of retinal ganglionic cell death in glaucoma [30]. L-Glutamate has also been implicated in IOP-related ischemia [31]. Interestingly, in the present study the increase in L-glutamate levels were statistically significant in the PACG group compared to the control group without any alteration in histamine levels. This indicates the fundamental difference in the occurrence of POAG and PACG with reference to the autonomic control. GABA-A/B receptor expression in the arcuate nucleus (ANC) was found to be statistically significantly elevated in rat models of high IOP and antagonization of the GABA receptors in ANC statistically significantly downregulated their expression. This indicated that the GABAergic system could be influenced by alterations in IOP [32].

Cooper et al. quantified the levels of catecholamines in aqueous humor in a small group of patients with glaucoma (n = 6) undergoing trabeculectomy and compared the levels with those of patients with cataract. In their study, adrenaline and noradrenaline were found to be elevated in patients with glaucoma. However, owing to the therapeutic use of adrenaline and its prodrug dipivefrine, the increased levels of adrenaline could not be isolated from the endogenous levels [33]. In the present study, we found a statistically significant increase (2.5-fold) in adrenaline in the PACG group compared to the control group. In contrast to Cooper et al.'s report [34], we did not observe any statistically significant

change in noradrenaline levels among the groups. Adrenaline is reported to have free access to the eye, but metabolism of adrenaline in eye is due to monoamine oxidase transporters and depends on catechol-O-methyl transferase (COMT) to inactivate adrenaline into metabolites in non-neuronal tissue sites. The involvement of a COMT polymorphism in the reduced metabolism of adrenaline, leading to PACG, has yet to be confirmed. However, involvement of a COMT polymorphism in various neurodegenerative disorders has been well documented [35,36].

To conclude, by analyzing the NT levels from the aqueous humor and plasma of patients with POAG, PACG, and cataract (control), this study revealed a fivefold elevation of histamine levels in patients with POAG, and a 2.5-fold increase of adrenaline levels in patients with PACG. Apart from this observation, GABA and L-glutamate were found to be elevated in the POAG and PACG groups compared to the control group. All these changes were observed to be independent of their respective plasma levels. This study did not show any statistically significant variation in the levels of 5HT, N-acetyl 5HT, DOPA, dopamine, and noradrenaline. This signifies that apart from autonomic mechanisms, there could be involvement of the histaminergic system in the progression of glaucoma. However, further studies are required to evaluate the effects of histamine on Müller cell dysfunction. Elevated L-glutamate and GABA showed that their release might be a reflection of retinal ganglionic cell death.

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