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# Oxidative stress and primary open-angle glaucoma: A correlative study of serum bilirubin, albumin, and thiobarbituric acid reactive substance

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## ABSTRACT

**Background:** Primary open-angle glaucoma (POAG) is a major health burden throughout the world. The imbalance between antioxidant defence and pro-oxidant plays an important role in this multifactorial disorder. Few recent studies have shown that serum total bilirubin and albumin play an important role in POAG. **Aims and Objectives:** The aim of the study was to estimate serum albumin, total bilirubin, thiobarbituric acid reactive substances (TBARS), and advanced oxidative protein products (AOPP) in POAG cases and controls and find out the association between serum total bilirubin, albumin with TBARSs. **Materials and Methods:** The study was conducted in the department of Biochemistry, Calcutta National Medical College, Kolkata, after getting ethical clearance. Fifty-five cases and 55 comparisons were taken. Samples were processed, and tests were performed. **Results:** It showed that the mean intraocular pressure in the case is 22.51 ± 1.77 mm of Hg much higher than the comparison group 13.78 ± 1.49 mm of Hg. Serum total bilirubin and serum albumin were significantly decreased in POAG cases. Serum TBARS and serum AOPP were significantly increased in POAG cases. Spearman's rho correlation showed that there was a significantly negative correlation between TBARS and total bilirubin ($r = -0.370$). Spearman's rho correlation showed that there was a significant positive correlation between serum AOPP and TBARS ($r = 0.556$). Spearman's rho correlation showed that there was a negative correlation between serum albumin and TBARS ($r = -0.076$, which was statistically not significant $P = 0.428$). **Conclusion:** Oxidative stress plays an important role in POAG. Bilirubin and albumin, through their antioxidant and anti-inflammatory action, try to prevent the progression of POAG.

**Key words:** Bilirubin; Albumin; Antioxidant; Thiobarbituric acid reactive substance; Advanced oxidative protein product; Oxidative stress; Primary open angle glaucoma

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## INTRODUCTION

Glaucoma is a neurodegenerative disorder[1] with complex etiology. It is the second leading cause of visual impairment and irreversible blindness globally. As estimated, there would be a 45% increase in glaucoma cases by 2040.[2] Primary open-angle glaucoma (POAG) is the most common type of glaucoma among all. In POAG, there is a slowly progressive optic nerve atrophy along with optic disc changes and retinal ganglion cell (RGC) damage. Although high intra-ocular pressure (IOP) is the major risk factor,[3,4] other factors, including vascular factors,

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neuroinflammation, and oxidative stress (OS) are also associated with glaucoma pathogenesis. Among all OS is considered as a critical etiological factor in the pathogenesis of glaucoma.[5,6] OS is an imbalance between anti-oxidative defense and pro-oxidant state in glaucoma patients[7-9] that causes damage to the lipid, protein, and DNA molecules.[10] However, the current lack of success in early diagnosis and prevention of glaucoma creates a dire need to find simple biomarkers such as serum albumin and bilirubin, which may help to assess the disease as early as possible and also aid in the assessment of disease progression and its severity. Serum albumin and serum bilirubin are conventionally two markers of hepato-biliary function in clinical practice. However, both of them are reported to have powerful antioxidant properties and could serve as serum biomarkers of OS.[11] Various activities of albumin as a major extracellular antioxidant have been observed in many studies. Albumin has the ability to bind with molecules such as metal-ions, fatty acids, drugs, and also hormones. The putative antioxidant bilirubin is transported in an albumin-bound form. Three-domain design of albumin structure provides a variety of binding sites. Among the cationic ligands, iron and copper, as transition metals mostly bind with albumin as they are potential reactive oxygen species (ROS) generators after reaction with oxygen. As free $Cu^{2+}$ and $Fe^{2+}$ can react with hydrogen peroxide and produce hydroxyl radical via Fenton reaction, so after binding with albumin they become less susceptible to participate in Fenton reaction. Hence, albumin acts as a sponge to absorb the potential ROS generators with its ligand binding activity. Hence, among all the plasma antioxidants, albumin is considered the most important molecule for the maintenance of plasma redox state.[12,13] Advanced oxidative protein products (AOPP) are formed when plasma proteins, including albumin, react with chlorinated compounds during OS. They are involved in many diseases including atherosclerosis, diabetes, and chronic kidney disease. AOPPs in the context of POAG refers to specific type of damaged protein molecules formed due to OS, which is considered a potential contributing factor to the development and progression of the disease by causing cellular damage and inflammation, particularly in the optic nerve head, where the damage is characteristic of POAG.

Bilirubin is derived mainly from two sources. The majority of bilirubin is made from the breakdown of hemoglobin in senescent red blood cells. The reminder originates from the turnover of various heme-containing proteins found in other tissues. These proteins include myoglobin, cytochromes, catalase, peroxidase, and tryptophan pyrrolase. Bilirubin is generally considered a useless metabolite with little physiological function. Like Roman god Janus Bifrons, bilirubin has two faces in human. When elevated excessively, it causes neurotoxicity; low level of bilirubin is also dangerous. As an antioxidant, it scavenges the ROS and reduces the nicotinamide adenine dinucleotide phosphate hydrogen oxidase activity and protects cells from damage caused by free radicals. According to the studies conducted in a clinical setting, serum bilirubin levels are also related to cardiovascular disorders. Existing evidence has shown reduced levels of serum bilirubin in patients with neurodegenerative or neuroinflammatory disorders such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis. Bilirubin is a potent antioxidant, anti-inflammatory and cytoprotective activity. Bilirubin is a powerful scavenger of peroxyl radicals and singlet oxygen.[14] Recent studies have shown that bilirubin is a potent endogenous compound that activates the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway under conditions of OS.[15] Keum et al., demonstrated that bilirubin nanoparticles can cause significant anti-inflammatory activity against various OS-related disorders.[16]

ROS-mediated oxidation of lipid molecules in the cell membrane produces lipid peroxidation end products. Malondialdehyde (MDA) is a widely accepted marker of lipid peroxidation and indicator of cellular OS and also known as thiobarbituric acid reactive substances (TBARS) as it can be assessed using thiobarbituric acid assay to assess the OS.

### Aims and objectives

The aim of the study is to find out the role of OS in POAG.

#### *Objective*

- To measure serum bilirubin and albumin levels in POAG patients and healthy individuals.
- To measure TBARSs and AOPP in POAG patients and healthy individuals.
- To assess the correlation between serum bilirubin, albumin with TBARSs in POAG patients.

## MATERIALS AND METHODS

A hospital based cross-sectional observational study is carried out in the department of biochemistry, Calcutta national medical college, Kolkata, in collaboration with department of ophthalmology after getting ethical clearance from institutional ethical committee (EC-CNMC/199/dated May 30th, 2023). 5 mL of blood is collected from cases and controls. Blood samples are centrifuged at 2500 rpm for 5 min. Serum is separated, and bilirubin, albumin are measured using a standard protocol by Konelab Prime 60i autoanalyser. Serum TBARS is measured by the method of Buege and Aust (1978)[17] by measuring TBARS. MDA is the end product of lipid peroxidation. MDA reacts with thiobarbituric acid

to form a pink-colored complex which can be measured spectrophotometrically at 546 nm. Concentration of serum TBARS is then determined using molar extinction coefficient of MDA. AOPP is a measure of oxidative damage to protein. They are formed when proteins are oxidized by chlorinated oxidants such as hypochlorous acid and chloramines. The serum level of AOPP is measured by monitoring the oxidation of iodide radical ($I^-$) at 340 nm (Witko-Sarsat et al., 1996)[18] and expressed as micromole chloramine T equivalent.

### Inclusion criteria

Persons suffering from POAG within the age group 45–64 years, irrespective of male and female, were included in the study.

### Exclusion criteria

Angle closure glaucoma, normal tension glaucoma, intraocular surgery within 6 months, opacity obscuring the visual field, neurodegenerative disorders, uncontrolled hypertension >150/90 mm of Hg, diabetes mellitus, any hepatobiliary disease with deranged serum bilirubin and albumin, malnutrition.

## RESULTS

This cross-sectional observational study was conducted with 55 cases and 55 healthy persons in comparison. Cases of POAG were selected on the basis of diagnostic criteria with IOP >20 mm of Hg and vertical cup disc ratio (VCDR) >0.5. Statistical analysis was carried out using the Statistical Package for Social Sciences 27 software. Normal or abnormal data distribution is assessed by using Shapiro–Wilk test and the Kolmogorov–Smirnov test, which have shown that all the data are nonparametric in nature. For comparison of different parameters between cases and controls, we have performed Mann–Whitney U-test.

Table 1 shows that the serum total bilirubin is significantly reduced in POAG cases (median value 0.55 mg/dL) compared to controls (median value 0.86 mg/dL). Serum albumin is also significantly reduced in cases (median value 4.2 g/dL) compared to controls (median value 4.5 g/dL). Serum TBARS level is significantly increased (median value 9.87 micromole equivalents MDA) compared to controls (median value 4.63 micromole equivalents MDA. Serum AOPP level is significantly increased (median value 317 μmol chloramine T equivalent) compared to controls (median value 205.01 μmol chloramine T equivalent).

Table 2 shows Spearman correlation analysis of different blood parameters, IOP, and VCDR. Spearman's rho correlation shows that there is a significantly negative correlation between TBARS and Total Bilirubin (r=−0.370). Spearman's rho correlation shows that there is a significant positive correlation between serum AOPP and TBARS (r=0.556). Spearman's rho correlation shows that there is a negative correlation between serum Albumin and TBARS (r=−0.076, P=0.428, which is not statistically significant). Spearman's rho correlation shows that there is a significantly positive correlation between TBARS and IOP (r=0.323). Spearman's rho correlation also shows that there is significantly negative correlation between serum bilirubin and VCDR (r=−0.595). Figure 1 shows violin plot comparison between POAG cases and controls.

**Table 1: Comparison of serum analytes between cases and controls by Mann-Whitney U-test**

| Serum analytes | Median (Cases) | Median (Controls) | Mann-Whitney U | P-value |
|---|---|---|---|---|
| Total bilirubin | 0.55 | 0.86 | 489.5 | <0.001* |
| Albumin | 4.2 | 4.5 | 786.5 | <0.001* |
| AOPP | 317 | 205.01 | 872.0 | <0.001* |
| TBARS | 9.87 | 4.63 | 911.5 | <0.001* |

*P<0.05 regarded as statistically significant. AOPP: Advanced oxidative protein products, TBARS: Thiobarbituric acid reactive substances

**Table 2: Spearman correlation analysis of different blood parameters, IOP, VCDR**

| Blood parameters | rho | P-value |
|---|---|---|
| Serum TBARS: Serum total bilirubin | −0.370 | <0.001* |
| Serum AOPP: Serum TBARS | +0.556 | <0.001* |
| Serum TBARS: Serum albumin | −0.076 | 0.428 |
| Serum TBARS: IOP | 0.323 | 0.001* |
| Serum TBARS: VCDR | 0.385 | <0.001* |
| Serum total bilirubin: IOP | −0.588 | <0.001* |
| Serum total bilirubin: VCDR | −0.595 | <0.001* |

*P<0.05 as significance level. IOP: Intra-ocular pressure, VCDR: Vertical cup disc ratio, TBARS: Thiobarbituric acid reactive substance, AOPP: Advanced oxidative protein products

## DISCUSSION

POAG is a multifactorial disorder involving a variety of pathogenic mechanisms, including OS. This stress is the consequence of the imbalance between excessive formation and insufficient protection against ROS. It is the leading cause of visual impairment and irreversible blindness worldwide. The disease is characterized by progressive damage and death of the RGCs and optic fibers. Neurodegeneration process extends beyond the retina and optic nerve into the visual pathway among the pathogenic mechanisms of glaucoma OS,[19] inflammation,[20] excitotoxicity, vascular impairment, hypoxia, glial dysfunction, and altered axonal transport are the most recognized processes.

In this case–control study, we have estimated serum total bilirubin and albumin (antioxidant) and TBARS, an

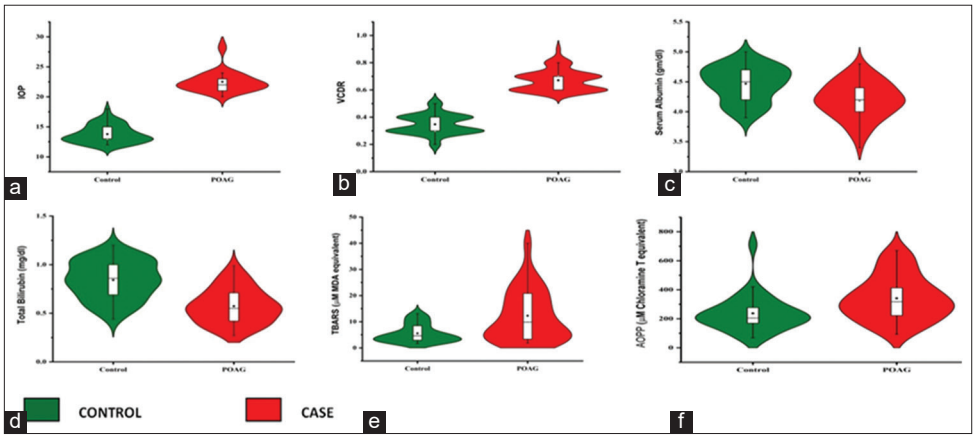


**Figure 1:** (a-f) Violin plot comparison between primary open angle glaucoma cases and controls

end product of lipid peroxidation reaction, AOPPs, OS related modified protein. We have also tried to find out the association of total bilirubin and albumin with TBARS in POAG cases.

There is a significant difference in serum total bilirubin between cases and controls (P<0.001). He et al.,[21] in their study, have also shown that serum total bilirubin is significantly reduced in glaucoma cases than controls. Rubia et al.,[22] have also shown in their study that serum total bilirubin is reduced significantly in cases compared to controls. Bilirubin is the end product of hem catabolism. It has both antioxidant and anti-inflammatory properties. Bilirubin is a powerful scavenger of peroxyl radical and singlet oxygen. Bilirubin alsoupregulates antioxidant enzymes through Nrf2 pathway. Activation of the Nrf2/HO-1 system is a critical defense mechanism against OS. Under normal conditions, Nrf2 is bound to Keap1 in the cytoplasm and is degraded through ubiquitination. However, when cells are exposed to OS, Keap1 is oxidized and dissociates from Nrf2, allowing Nrf2 to translocate into the nucleus where it can bind to the antioxidant response element and activate the transcription of various antioxidant genes, including HO-1.[23] This activation of the Nrf2/HO-1 system is an important step in counteracting OS and preventing cell damage. There is a significant reduction of serum albumin levels in POAG cases compared to controls in our study. He et al.,[21] and Rubia et al.,[22] have also shown similar results. Albumin is major extracellular antioxidant in our body. It binds to hem, copper, and putative antioxidant bilirubin. Albumin is a multifunctional protein and due to its free Cys34 thiol group, represents a main source of free thiols in the circulation. This property of albumin, combined with its ability to sequester redox-active Cu (II) ions, makes albumin a dominant circulatory antioxidant. There is also a significant increase of AOPPs in POAG cases compare to controls. AOPP are derived from oxidation-modified albumin, fibrinogen, and lipoproteins. AOPP are markers of oxidative damage to proteins through reactions with oxidants particularly chlorinated oxidants such as chloramines, hypochlorous acid, which are generated by myeloperoxidase in activated neutrophils. Rusmayani et al.,[24] have shown increase in ischemia-modified albumin in both aqueous humor and serum of POAG as well as primary angle-closure glaucoma (PACG) cases. Another study conducted by Dong Chang shows that there is increased serum AOPP levels in PACG cases.[25] Serum albumin is oxidatively modified under OS, which is seen in POAG. Oxidative modifications of albumin impair its quantification by the standard laboratory albumin assay, bromo-cresol green,and the gold standard nephelometry, rendering part of the serum albumin undetected, causing apparent hypoalbuminemia in POAG patients.[26] AOPP further interact receptor for advanced glycation endproducts receptors and activates inflammation via nuclear factor kappa B activation and further OS to cause further complications in POAG. There is a statistically significant increase in TBARS in POAG cases compared to controls. High TBARS can damage retinal ganglion cells, which are crucial for vision, contributing to visual field loss in POAG. Measuring of TBARS levels may be a useful to assess the severity of OS in POAG cases and potentially monitor the effectiveness of antioxidant therapy. Rokicki et al., have shown a significant increase in serum TBARS level.[27] Ateş et al., have also demonstrated a significant increase in serum TBARS level in POAG cases.[28] Spearman's rho correlation to check the association between serum total bilirubin and TBARS shows a significant negative correlation between serum total bilirubin and TBARS. As the OS increases in POAG more TBARS are generated. Spearman's rho correlation is performed to check the association between AOPP and TBARS. As the OS is increasing AOPP, OS marker, and TBARS, lipid peroxidation end products, are both increasing. Thus, both these parameters show a statistically significant positive correlation. Spearman's

rho correlation to check the association between serum albumin and TBARS shows a negative correlation that is not statistically significant.

## CONCLUSION

This study identifies the role of OS in POAG. Oxidative damage is an important pathogenic factor in glaucoma, which triggers trabecular meshwork degeneration and high IOP. Serum bilirubin and albumin levels are significantly decreased, whereas TBARS and AOPP are significantly increased in POAG cases. The study also suggests that modulation of pro-oxidant/antioxidant status might be a relevant target for glaucoma prevention and therapy.

### Limitation of the study

We have taken 55 cases of POAG and 55 controls. If the sample size increases, the result can provide more clear understanding about the role of serum total bilirubin, albumin, TBARS in pathogenesis of POAG.

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**Authors' Contribution:**
**SS-** Conceptualization, design of the study, and drafting of manuscript; **SB-** Supervision, and final approval; **JD-** Test performance and review of manuscript; **RM-** Test performance and review of manuscript; **PM-** Standardization of methods; **SG**- Supervision.

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