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To evaluate the role of oxidative stress in developmentof primary open angle glaucoma

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ABSTRACT

Introduction and Aims: Primary open angle glaucoma is a chronic optic neuropathy with possible involvement of oxidative stress in its pathogenesis. The present study was conducted to evaluate the role of oxidative stress in terms of lipid peroxide (LPO) and the protective role of antioxidant enzymes catalase (CAT), superoxide-dismutase (SOD) and glutathione peroxidase (GPX) in patients of primary open angle glaucoma.

Materials and Methods: A total of 120 patients were enrolled. After taking informed consent, 5 ml of venous blood was taken under aseptic conditions in EDTA tube. These samples were transported from Eye OPD to Laboratory in icebox maintaining temperature for enzymatic activity of enzymes in blood samples. The blood was centrifuged, plasma was separated and was used for estimation of lipid peroxide (LPO). RBC lysates were prepared for estimation of antioxidant enzymes namely; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx).

Results: A total of 120 patients were enrolled. There was no significant difference in LPO levels of POAG patients and controls. No statistically significant difference was observed in the levels of SOD and catalase. There was a significantly raised value of GPx in POAG patients but since SOD activity was normal, we can conclude that the SOD level was sufficient to meet the above exigency.

Conclusions: Oxidative stress is not the only cause for development of primary open angle glaucoma.

1. Introduction

Primary open angle glaucoma (POAG) is defined as a progressive, chronic optic neuropathy in adults where intraocular pressure (IOP) contribute to damage and there is characteristic acquired atrophy of optic nerve and loss of retinal ganglion cells and their axons. The prevalence of POAG varies in different populations. In general it affects about 1 in 200 of populations (of either sex) above the age of 40 years worldwide. In India, glaucoma is the leading cause of irreversible blindness with at least 12 million people affected and nearly 1.2 million people blind from the disease. Clinical and experimental studies have shown the possible involvement of oxidative stress in the pathogenesis of open angle glaucoma. The major types of free radicals and their nonradical reactive species are reactive oxygen species (ROS) and reactive nitrogen species (RNS). The present study was conducted to evaluate the role of oxidative stress by measuring the level of lipid peroxide (LPO) and antioxidant enzymes catalase (CAT), superoxide-dismutase (SOD) and glutathione peroxidase (GPX) in patients of primary open angle glaucoma and age matched controls.

2. Materials and Methods

This prospective, randomized study was conducted at a tertiary referral hospital for a duration of one year after obtaining approval from the hospital ethical committee and informed consent from patients was obtained .

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Sample size: Standard deviation (SD) of catalase levels among POAG and control population was as 1.126 & 0.993 mU/l respectively.[1] Pooled SD was calculated to be 1.075 mU/l. Assuming margin of error as 0.4 mU/l, alpha error of 5%, power of 80% and non-response rate of 90%, sample size for each group was calculated as 60.

Inclusion criteria included patient of age >40 years of either sex, patient with IOP greater than 21 mm Hg (measured with Applanation tonometry), patient with possible glaucomatous visual field defects (nasal step – 5dB in three adjacent points or 10 dB in two adjacent points, any bundle -type defect or any enlarged blind spot)and patient with Cup disc ratio (CDR) higher than 0.5 or difference of CDR in both eyes of > 0.2.

2.1. Sample collection

After taking informed consent, 5 ml of venous blood was taken under aseptic conditions in EDTA tube. These samples were transported from Eye OPD to Laboratory in icebox maintaining temperature for enzymatic activity of enzymes in blood samples.

The blood was centrifuged, plasma was separated and was used for estimation of lipid peroxide(LPO). RBC lysates were prepared for estimation of antioxidant enzymes namely; catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx).

2.2. Preparation of haemolysate

Plasma and RBC were separated by centrifugation (at 1500 g for 15 min). Packed cells remaining after removal of plasma were washed three times with normal saline to remove buffy coat. Haemolysis was performed by pipetting out 1ml of washed cell suspension into propylene centrifuge tubes which contained 1ml ice cold distilled water. Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at 20,000 g for 40 minutes. The cell contents in supernatant (or haemolysate) was separated out carefully and stored in at -20°C until analyzed. Protein estimation was performed on lysate by method of Lowry et al.[2,3]

2.3. Procedure

0.1 ml of sample (lysate) was made up to 1ml with 0.9 ml of normal saline and added with 1.5 ml of 10% Trichloroacetic acid (TCA). The content was kept in cold for 4 hours and protein precipitate was recovered by centrifugation. Protein was dissolved in 1ml of 0.1N NaOH. An aliquot of 0.2 ml of protein solution was taken in which 5 ml of alkaline copper sulphate solution was added and incubated at 37° C. After 30 minute, 0.5 ml of Folin's reagent was added at the same temperature. After incubation for 20 minutes the optical density of developed blue colour was read at 625 nm. Standard solution (BSA, 20-100μg) and a blank reagent were also run simultaneously.

Protein values were expressed as mg /ml of RBC lysate.

2.4. Lipid Peroxide(LPO) Estimation

2.5. Method

The Lipid Peroxide,Thiobarbituric Acid Reactive Substances (TBARS) was estimated in plasma according to modified method by Ohkawa et al.[4]

2.6. Principle

Acetic acid segregates the lipid and protein of tissue. The protein in the reaction mixture is dissolved by addition of Sodium Dodecyl Sulphate. 2-Thiobarbituric acid (TBA) reacts with lipid peroxide, hydrogen peroxide and oxygen labile double bond to form the coloured products with absorption maximum at 532 nm.

2.7. Reagents

1. Sodium Dodecyl Sulphate (SDS) 8%: 8.0 gm of SDS in 100 ml double distilled water.
2. Acetic Acid (20%): Diluting glacial acetic acid according to make 20 % solution.
3. Thiobarbituric acid (0.8%): 800 mg of TBA suspended in 80 ml distilled water, the pH was adjusted to 7 by 0.1 N NaOH, it was dissolved and volume was adjusted to 100 ml.
4. n-Butanol

2.8. Procedure

0.2 ml of plasma was mixed with 1 ml of 20% acetic acid. Subsequently 0.5 ml of 8% aqueous SDS was mixed in above reaction mixture, the pH of the mixture was adjusted to 4.0 using concentrated NaOH solution if needed.1.4 ml of 0.8 %TBA solution and sufficient amount of distilled water was added to final volume of 4 ml. The reaction mixture was kept in boiling water bath for one hour. After cooling to room temperature, 3.0 ml of n-butanol was mixed, the reaction mixture was then centrifuged at 10,000 rpm for 15 minutes. A clear butanol fraction obtained after centrifugation was used for measuring the absorbance at 532 nm in spectrophotometer. An appropriate standard 0.2 ml made up of malondialdehyde (MDA) 2.5 nmol MDA was run simultaneously.

2.9. Calculation

Standard absorbance of MDA (2.5 nmol) which was 0.400 used to calculate the amount of lipid peroxide in the samples and results were expressed as nmol of MDA /ml of plasma.

2.10. Catalase (CAT) Activity

Activity in lysate was estimated by method of Aebi et al.[5]

2.11. Principle

In ultraviolet (UV) range hydrogen peroxide (H_2O_2) shows continuous increase in absorption with decreasing wavelength which is maximum at 240 nm. The decomposition of H_2O_2 can be followed directly by relative decrease in optical density at 240 nm. The difference in optical density at 240 nm per unit time is the measure of CAT activity.

2.12. Reagents used

1. 50mM Sodium potassium phosphate buffer (pH 7.0).
2. 30mM Hydrogen peroxide: 0.34ml of 30% H_2O_2 was diluted to 100 ml with phosphate buffer.

2.13. Procedure

1.5 ml of phosphate buffer and 1 ml diluted H_2O_2 was added into the cuvette, along with 20μl RBC lysate, and contents were mixed thoroughly. Immediately the decrease in absorbance at 240 nm was recorded after every 30 seconds for three minutes. One unit of enzyme decomposes H_2O_2 that cause to decrease in optical density by 0.1.

2.14. Calculation

Δ OD/Minute = Change in OD $\times$ 60/180

Enzyme activity was expressed as units/mg protein.

2.15. Superoxide Dismutase (SOD) Activity

2.16. Method

The SOD activity in RBC lysate was estimated by method of McCord and Fridovich.[6]

2.17. Principle

Superoxide anion were generated in system comprised of NADH and PMS. These free radicals reduce the nitrobluetetrazolium (NBT), forming a blue compound named formazan which was measured at 560 nm on spectrophotometer. SOD inhibited the reduction of NBT and thus the enzyme activity was measured by monitoring the rate of decrease in optical density that corresponds to amount of formazan formed at 560 nm.

2.18. Reagents used

1. Tetra- sodium pyrophosphate: 454.9 mg of Tetra-sodium pyrophosphate was dissolved in distilled water. The volume was made upto 50 ml and pH was adjusted to 8.2.
2. NADH (2.34mM): 16.59 mg of NADH was dissolved in 10 ml of pyrophosphate buffer.
3. Phenazine methosulphate (93 μM): 2.9 mg of phenazine methosulphate was dissolved in 100 ml pyrophosphate buffer (pH maintained at 9.2).
4. Nitro-bluetetrazolium (1.5mM): 32.13 mg of Nitro–bluetetrazolium was dissolved in pyrophosphate buffer (pH 9.2) and volume was made up to 25 ml with same buffer.
5. Glacial acetic acid.

2.19. Glutathione Peroxidase (GPx) Activity

2.20. Method

GPx activity in RBC lysate was measured by method of Paglia and Valentine.[7]

2.21. Principle

GPx catalyses the reduction of hydro-peroxides (ROOH)in presence of glutathione (GSH).

2.22. Reagents

1. Sodium Phosphate Buffer:pH 7.0 (0.50 mM)
2. Sodium azide :10 mM
3. Glutathione (Reduced): 2 mM.
4. Hydrogen peroxide :1.0 mM
5. TCA:10%
6. Disodium hydrogen phosphate :0.3 M
7. DTNB :40 mg of (5,5'-dithiobis2-nitrobenzoic acid) in 1% sodium citrate.
8. Standard glutathione (reduced) : 1.0 mM solution of GSH in phosphate buffer was prepared.

2.23. Procedure

The incubation mixture containing 0.1 ml sodium azide, 0.2 ml reduced glutathione and 0.1 ml H_2O_2 to a final volume of 2.4 ml in phosphate buffer (1.9 ml) and preincubated at 37° C for 10 minutes. After this 0.1 ml RBC lysate as enzyme source was added to reaction mixture and further incubated for 20 minutes at 37ºC. The reaction was stopped by adding 0.5 ml TCA. The standard tubes containing 0.1 ml GSH added with 2.4 ml of phosphate buffer were also run simultaneously. After completion of reaction in both sets, 1ml of DTNB solution was added, mixed well and kept at 37ºC for 10 minutes. Then yellow colour developed, and optical density against reagent blank was read at 412 nm wavelength in UV-spectrophotometer.

2.24. Calculation

Enzyme activity was expressed as μg GSH utilized /min/mg protein.

2.25. Statistical Analysis

Statistical analysis was done by student unpaired t- test using SPSS 17/MS-EXCEL, used for the analysis purpose. P value < 0.05 was considered as statistically significant.

3. Results

Out of the 120 patients enrolled for the study, 60 were POAG patients and 60 were age matched controls of either sex. The parameters measured were as follows:

1. Lipid peroxide (in plasma)
2. Antioxidant enzymes: Superoxidedismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) (All in haemolysate).

Age of patients enrolled in the study ranged between 40 and 80 years in both the groups.

There was no significant difference in LPO levels of POAG patients and controls although the mean level of TBARS in POAG patients was slightly higher (4.4±0.824nmol/ml) than controls (4.2±0.794nmol/ml).

In the present study no difference was observed in the level of SOD among patients (1.26±0.23 units) and control (1.31±0.30 units).

There was a significantly raised value of GPx in POAG patients. GP_x value in controls was 30.88±2.4μgm GSH utilized per min. per mg protein and it was 33.19±3.8 μgm GSH utilized per min. per mg protein in POAG group.

The level of catalase in the control group was 50.66± 4.039 units per mg protein and 51.36± 6.05 units per mg protein in patients. There was no significant difference among the two groups.

The values of the enzymes in the control and POAG group are shown in the Table 1, and statistical analysis is depicted in Table 2.

4. Discussion

Glaucoma is a heterogeneous optic neuropathy with a varied environmental and genetic factor. It progressively diminishes vision without any major symptoms.[8] POAG is an age-related neurodegenerative disease, triggered by mechanical stress due to intraocular pressure, diminished blood flow to retina, reperfusion injury, glutamate excitotoxicity, abnormal immune response and raised oxidative stress. POAG is a distinct neurodegenerative entity in which oxidative stress is a palpable risk factor.[9] Multiple risk factors leads to retinal ganglion cell loss including apoptosis. Apoptosis involves Oxidative species (OS), reactive oxygen species (ROS) and reactive nitrogen species (RNS) for its function and modulation. Redox sensitive metabolites like thiols play a major role in several aspects of apoptosis signalling.[10] In the recent years oxidative stress has received ample attention as it is proposed to be an independent risk factor or more importantly as intervening risk factors for POAG. Oxidative stress (OS) is widely reported in many diseases and it has been hypothesized to play an important role in the pathogenesis and progression of POAG.[11,12]

Oxidative processes may initiate and mediate apoptotic death of retinal ganglion cell (RGC) during reoxygenation.[13] Further nitric oxide, a free radical, after reacting with O_2^- may damage RGC.[14] Several harmful substances such as excess glutamate induces fatal apoptotic cascade mediated by free radical damage.[15,16][15,16] It has also been found that Glutathione a tripeptide thiol group containing antioxidant, protects against glutamate induced toxicity Sucher.[17] There is ample evidence that age has direct relationship with oxidative stress(OS).[18–20] Since POAG is an age related disease, we were encouraged to evaluate the role of oxidant and antioxidant in POAG patients.

In our study the patients were in the range of 40-80 years with a mean age of 57.1 years. They were diagnosed as cases of POAG. IOP in these patients was more than 21 mmHg except in four patients. This decrease in IOP may be due to given treatment or may be due to diurnal variation but other findings of POAG were present. Nearly 43% patients were above 60 years, 20% patients were between 50-60 years and 36.67% patients were between 40-50 years. These observations along with general observations of clinicians strongly suggest that POAG is an age related disease. We have not found any correlation between age and oxidative stress, which suggests that other risk factors are also involved in POAG and they must be contributing their additional influence.

The net observation suggests that involvement of OS is an extremely complex one and may or may not show any relationship with health and disease and that OS may because or consequence. We have not observed raised OS in POAG patients but we cannot rule out the possibility of raised oxidant activity in the body which is able to manage the OS within normal range. From the data obtained in our study we can conclude that OS is not a cause in the etiopathogenesis of POAG and OS may be raised at some later period of the disease. Therefore, the theory of OS in ageing[21,22] have experienced a profound change though basics have remained the same. Ageing or age related disease may not necessarily be inversely related to OS, and that raised OS, even a mild one, may exacerbate ‘ ageing process or age related diseases. My observations suggested slightly raised oxidant activity but normal OS in POAG patients. The results in this study indicated that reactive oxygen species(ROS) is produced in extra amount in POAG patients but normal level of SOD is able to dismutate it to hydrogen peroxide hence did not require induction. However, extra hydrogen peroxide produced in cytosol required glutathione peroxidase (GPx) induction,

Table 1:

Parameters	Range (n=60 in each group)		Mean		± SD	
	Controls	POAG	Controls	POAG	Controls	POAG
Age (years)	40-80	40-80	57.3	57.1	7.85	11.33
IOP-Right eye(mm Hg)	14.4 -17.5	17.3 -26.4	15.54	22.05	0.99	2.56
IOP – Left eye (mm Hg)	14.2 -17.3	17.3 -26.4	15.55	21.7	0.95	2.72
LPO (nmol MDA/ml plasma)	2.63 -6.38	3.18 -6.36	4.222	4.439	0.794	0.824
SOD (unit/mg protein)	0.97 - 1.95	0.90 -2.02	1.306	1.257	0.304	0.227
CAT (units/mg protein)	40.95 - 62.82	35.13 -66.76	50.655	51.356	4.039	6.050
GPx (μgm GSH utilized per min. per mg protein)	26.53 -36.57	26.73 -41.38	30.876	33.193	2.399	3.832

Table 2: 2 : Unpaired t-test (case vs control)

Parameters	t- test	p- Value	Significance
Age	-0.079	0.937	NS
IOP (Right Eye)	12.98	0.001	S
IOP (Lefy Eye)	11.671	0.001	S
LPO	− 1.038	0.304	NS
SOD	0.707	0.482	NS
CAT	− 0.527	0.600	NS
GPx	− 2.806	0.007	S

NS=Non-Significant, S=Significant

that is why GPx activity was raised. There are six types of GPx[17] but quantitatively it is GPx1 which deals with cytosolic H_2 O_2 . RBC also contains GPx1 and its activity is obviously proportional to tissue activity.

There is evidences of raised OS in neurodegenerative diseases such as Alzheimer's disease, Amyotrophic Lateral Sclerosis, Parkinson's disease, Huntington's disease etc. because of abnormal activity of Cyclin Dependent Kinase (CdKS) helps in central nervous system development by phosphorylating the specific serine or threonine (amino acids) of several proteins that are responsible for neuronal migration, synaptogenesis, synaptic transmission and synaptic plasticity. OS through biochemical mechanisms leads to neuronal degeneration or neuronal death.[18] Similar type of studies are not available for POAG. This study opens two platforms for future research. First, longitudinal studies in POAG patients with regard to OS, free radicals and antioxidants along with concomitant anatomical or pathological changes and second to workout molecular mechanism to explicate interconnectivity with neurodegeneration.

In our study for evaluation of systemic changes in OS (LPO), enzymic endogenous antioxidants SOD, GPx, and CAT were evaluated in the blood of 60 POAG patients and 60 matched controls.

OS has been measured as Lipid peroxidase (representative of MDA). MDA is an end product of polyunsaturated fatty acid especially arachidonic acid in human body. Since cellular membranes are rich in lipids, they are preferential targets for attack by reactive oxygen species (ROS) and reactive nitrogen species (RNS) causing lipid peroxidation. MDA is one of the final end product and is representative of degree of damage of lipids. Lipid peroxidation in turn is an index of damage of other cellular biomolecules such as DNA, RNA, proteins, sugars etc.

Overproduction of ROS or deficiency of antioxidants can exacerbate OS. While normal OS is presently overwhelmingly recognised as an essential process for maintaining "cellular redox homeostasis", the raised oxidative stress is reported to disturb redox box thereby leading to numerous pathologies including neurodegenerative diseases.[23] Lipid peroxidase(LPO) level in serum or plasma has been found to be a good biomarker of neurodegenerative diseases.[24] In our study the mean level of LPO was 4.439±0.824 nmol MDA/ml plasma whereas it was found to be 4.222±0.794 nmol MDA/ml plasma in the control group and the difference was not found to be statistically significant.

Antioxidants are of two types: exogenous and endogenous. Exogenous antioxidants such as nutrient antioxidants (vitamin C, vitamin E and beta-carotenes) have various other metabolic responsibilities besides antioxidants. They needs to be supplemented on daily basis whereas endogenous antioxidants are more powerful and their synthesis is inducible, i.e. they can sense increased requirement and gene regulated enzymes are produced in increased amount quickly. Quantitatively the most important enzyme antioxidants are SOD, GPx, CAT and non-enzymatic antioxidant GSH. Keeping in view their importance in human body, we have also analysed the level of CAT, SOD, GP $_X$ in RBCs as RBC level are better index of in vivo status in patients of POAG so as to determine their protective role in their pathogenesis.

In the present study no difference was observed in the level of SOD among POAG (1.26±0.23 units) and control (1.31±0.30 units). The study does not show any influence on SOD activity. This enzyme is also called as "Primary Endogenous Antioxidant enzyme" as it controls superoxide radicals which is first oxygen radicals produced in body. Our observation with regard to GPx conveys very important information. They clearly point out "Raised antioxidant activity". GPx is major enzyme in mitochondria and cytosol as is quantitatively involved in conversion of H_2O_2 to $H_2O + O_2$ with help of GSH, because it has high sensitivity for H_2O_2 and acts at very low concentration of H_2O_2. On the contrary, catalase is confined to peroxisomes and requires higher concentration of H_2O_2 for its action. We have observed significantly raised values of GPx in haemolysate of POAG patients. It was significant but mild, only suggesting consistent higher concentration of H_2O_2 in patients (controls=30.88±2.4 and patients 33.19±3.8). It can be safely assumed that normal level of SOD was able to scavenge this slightly enhanced level of H_2O_2, hence the activity of SOD was not raised. The level of catalase was found to be 51.36±6.1 unit/mg protein in POAG groups and 50.66±4.0 unit/mg protein in control group and the difference was not found to be statistically significant.

Our study points out many pertinent suggestions which may be pondered upon. Firstly we found that POAG is an age related disease. Secondly though various literature repeatedly stresses that oxidative stress is a strong risk factor in the genesis of POAG, the actual studies in humans and animals in this regard are few and our observations do not suggest a strong role of OS and protective effect of antioxidant in the pathogenesis of POAG. Third, mildly raised oxidant activity was visible in POAG patients in our study which was indicated by raised glutathione peroxidase level. Hence our observation suggests that most of the accusations for OS or ROS or RNSinvolvement in aetiopathogenesis of POAG are conjectures. OS could be a cause or just a consequence i.e. a secondary effect set in during the course of the disease. To set these queries straight two types of studies are necessary firstly longitudinal studies in human and animals to assess OS at regular intervals and secondly, studies in animals to seek molecular explanation.

5. Conclusion

Based on the outcome of the present study we conclude that Oxidative stress is not the only cause in the pathogenesis of primary open angle glaucoma.

6. Source of funding

None.

7. Conflict of interest

None.

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