Australian Journal of Basic and Applied Sciences, 4(8): 3553-3558, 2010
ISSN 1991-8178

The Role of Oxidative Stress Markers and Nitric Oxide Levels in the Pathogenesis of Glaucoma

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Abstract: This study tries to clarify the role of oxidant-antioxidant balance, oxidative stress and nitric oxide aqueous humor levels in the pathogenesis of primary open angle glaucoma (POAG) & Pseudoexfoliative glaucoma (PXG). This study was conducted on 25 patients with POAG, 25 patients with PXG and 15 patients with senile cataract serving as controls. Plasma glutathione levels were estimated & the aqueous humor levels of malondialdehyde, total antioxidant status, catalase, glutathione peroxidase activities and nitric oxide levels were estimated colorimetrically in all studied groups. Significant reduction of plasma glutathione and aqueous levels of total antioxidant status coupled to increase aqueous malondialdehyde and nitric oxide levels in aqueous in glaucoma's patient. Meanwhile, increased aqueous catalase and glutathione peroxidase activities in glaucoma patients compared to controls. There is sufficient evidence that oxidative and nitrative process play important role in the pathogenesis of glaucoma. However, it is desirable to undergo further studies and clinical trials for better understanding of the exact pathogenesis of glaucoma and help in design of more effective therapies.

Key words: Oxidative stress, nitric oxide, glaucoma

INTRODUCTION

Glaucoma is an optical neuropathy characterized by specific structure alteration of the head of the optic nerve accompanied by progressive damage to the visual field. Although increased intraocular pressure (IOP) is a major risk factor for primary open angle glaucoma (POAG), other concomitant factors affecting the eye play important roles including alterations in nitric oxide metabolism (Galassi *et al*, 2004), vascular alterations (Chung *et al*, 1999) and oxidative damage caused by reactive oxygen species (Moteno *et al,* 2004).

IOP elevation and visual field damage have been shown to be proportional to DNA oxidative damage in human trabecular meshwork. This finding provides a basis for the role of the oxidative stress in the pathogenesis of glaucoma (Sacca *et al*, 2007).The free radicals can be terminated by antioxidant defense systems including the enzyme glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and non-enzymatic compounds such as reduced glutathione and antioxidant vitamins. The net oxidative burden between prooxidant and antioxidant system is the oxidative stress that damage lipid, proteins and DNA culminating death (Kumar & Agawal, 2007).

Primary open angle glaucoma (POAG) is defined as an optic neuropathy which is characterized by the loss of optic nerve axons and the related retinal ganglion cells. One factor becoming more likely to be involved in pathogenesis of POAG is oxidative stress, (Welge- Lussen & Birke, 2010). Morphological and biochemical analyses of the trabecular meshwork TM of POAG patients revealed loss of cells, increased accumulation of extracellular matrix. Moreover, treatment of TM cells with oxidative stress induced POAG typical changes like extra cellular matrix accumulation (ECM) accumulation, cell death, disarrangement of cytoskeleton, advanced senescence and the release of inflammatory markers, (Welge-Lussen & Brike, 2010).

Pseudoexfoliation (PXG) glaucoma is the most common identifiable cause of open angle glaucoma worldwide. Increased evidence suggests that the oxidative-antioxidative balance is disturbed in patients with PXG, both in the anterior segment and through the body, and the resulting oxidative stress constitutes a major mechanism involved in pathophysiology of this fibrotic process, (Schlötzer-Schrehardt, 2010). Also, published evidence suggest that the biochemical changes related to PXG pathophysiology are linked with increased oxidative stress, which leads to exfoliation-induced tissue damage and pathological alteration of the extracellular matrix (Koliakos *et al*, 2008).

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The major mechanism of visual loss in glaucoma is retinal ganglion cell apoptosis, leading to thinning of the inner nuclear and nerve fiber layer of the retina and axonal loss in the optic nerve. (Fechtner & Weinreb, 1994). Nitric oxide-mediated cytoxicity and the capacity of NO to induce apoptosis have been documented in macrophage, astrocytes and neuronal cells. Reported studies also demonstrate the presence of nitric oxide synthase (NOS) in glaucomatous optic nerve head, indicating that reactive nitrogen species may contribute to retinal ganglion cell death associated with elevated intra-ocular pressure (Aslan *et al*, 2008).The biochemical function of nitric oxide (NO) in the eye might play an important role in the regulation of intraocular pressure (IOP), local control of ocular flow and loss of retinal ganglion cells by apoptosis (Neufeld, 1999). However, the exact mechanism of how NO is involved in the progression of glaucoma is still an open question for much speculation.

Using NADPH – diaphorase technique, the human anterior segment was found to be markedly enriched in NOS (Wiederholt *et al*, 1994). The vasculature of the ocular anterior segment is believed to play a role in mediating both aqueous inflow (Schuman et. al, 1994) and outflow (Nathanson, 1993). The possible role of NO in regulating aqueous humor outflow by relaxing contractile elements of the trabecular meshwork has been suggested by some animal experiments and clinical findings (Nathanson, 1993).

However, the pathologic mechanisms leading to trabecular meshwork and/or schlemm canal outflow pathway dysfunction in glaucoma are still unclear (Zanon- Moreno et. al, 2008).

This study clarifies the role of oxidative stress markers and nitric oxide levels in the pathogenesis of glaucoma.

MATERIALS AND METHODS

This study was conducted on 65 patients admitted from the outpatient clinic of the Research Institute of Ophthalmology. Twenty five with POAG, twenty five with PXG, and fifteen with senile cataract serving as controls. All patients and control groups are age matched 55-67 years.

Both patients and controls underwent a detailed ophthalmic examination including:

best corrected visual acuity, slit lamp biomicroscopy using 90 D lens, gonioscopy using Goldmann three mirror contact lens, intraocular pressure (IOP) measurement using Goldmann applanation tonometry and Humphrey visual field analyzer (24-2 program).

A detailed medical history was taken to detect patients with risk factors for vascular diseases such as, hypertension, diabetes mellitus, and hyperlipidemia.

Glaucoma patients had typical glaucomatous optic nerve head and visual field changes. Glaucoma patients continued their preoperative IOP lowering treatment.

Exclusion Criteria Included:

Patients with diabetes mellitus, evidence of renal or hepatic disease. Patients with ocular diseases other than glaucoma and senile cataract were excluded. Patients with history of previous laser procedures or ocular surgery were also excluded.

Sample Collections:

Fasting blood samples were obtained from patients before surgery on EDTA and were immediately centrifuged and plasma was separated.

- Quantitative determination of reduced glutathione (GSH) in plasma by colorimetric (412 nm) techniques provided by quantichrom glutathione kits Bio Assay systems, USA. (Blenn, C et. al, 2006).
- Routine laboratory investigations were carried out including fasting; 2 hour blood glucose levels, creatinine levels and liver function tests were also detected.
- Collection of aqueous humor was performed during cataract or glaucoma surgery. Aqueous humor 0.1-0.2 ml was aspirated at the beginning of surgery through a paracentesis using a 27 gauge needle on a tuberculin micro syringe. Blood contamination or iris touch was avoided. All aqueous humor samples were immediately stored at -70 °c till assays.
- In aqueous the following laboratory investigations were carried out:
- Quantitative determination of malondialdehyde (thiobarbituric acid reactive substances) by colorimetric method (535 nm) using quantichrom TBARS assay kits provided by Bio Assay system, USA, (Satoh, 19780).
- Quantitative determination of total antioxidant status by colorimetric (570 nm) methods by kits provided by Bio Assay System, USA. (Sies, 1997).

- Quantitative determination of catalase and glutathione peroxidase activities by colorimetric methods (570 nm) and (340 nm) respectively by kits provided by Bio Assay System, USA (GÖth, 1991), and (Jacobson et. al, 1988).
- Quantitative determination of nitric oxide by colorimetric (540 nm) method by kits provided by Bio Assay System, USA (Bulau et. al, 2007).

Statistical Analysis:

Data was expressed as mean ± SD. The three groups were compared using the ANOVA test; single factor test. The degree of association between the variables was assessed using Pearson's correlation coefficient (r); where value of P<0.05 were considered significant.

RESULTS AND DISCUSSION

Table 1: The mean levels of plasma glutathione levels and aqueous malondialdehyde, total antioxidant status, levels, catalase & glutathione peroxidase activities and nitric oxide levels in all studied groups.

	POAG Mean ± SD	PXG Mean ± SD	Controls Mean ± SD	P value
Plasma glutathione (n mol)	225.3± 72.9	227±69.1	362±89.5	P< 0.5
Aqueous Malonedialdhyde (µ mol/L)	0.48±0.1	0.49±0.1	0.1±0.07	P< 0.01
Total antioxidant status in aqueous (m mol)/L	49.1±7.1	47.9±7.5	124±5.2	P< 0.001
Glutathione peroxidase (µ /ml)	19.2±205	18.2±2.2	6.1±2.3	P< 0.5
Nitric Oxide (µ /M)	59.8±3.6	61.2±4.1	25.8±3.1	P<0.01
Catalase (µ /ml)	112±29.2	117±30.4	15.1±1.9	P< 0.001

P<0.5 significant

P< 0.01, P<0.001 high significant

Analysis of result:

Table (1) summarizes the laboratory investigations done in the studied groups.

- The plasma glutathione levels were significantly lower in POAG and PXG compared to controls P< 0.05.
- The mean aqueous malondialdehyde levels were significantly higher in POAG and PXG compared to controls P<0.01.
- The total antioxidant status levels in aqueous were significantly lower in POAG and PXG compared to controls P<0.001.
- The catalase and glutathione peroxidase activities in aqueous were significantly higher in POAG and PXG compared to control P<0.001 & P<0.05 respectively.
- The nitric oxide levels were of aqueous humor significantly higher in POAG and PXG compared to controls P<0.001.
- No significant differences of all studied parameters in both glaucoma groups.
- Significant positive correlation was detected between plasma glutathione and total antioxidant status in POAG r=0.65, P<0.001.
- Significant positive correlations were detected between aqueous malondialdehyde and nitric oxide levels in POAG r=0.63 p<0.001, PXG r=0.65, P<0.001.
- Significant positive correlations were detected between aqueous catalase & GPX activities in POAG r =0.54 and PXG r=0.56 r=<0.05.
- Significant negative correlations were found between aqueous malondialdehyde and total antioxidant status levels in POAG r=-0.54, PXG r= -0.55 P<0.05.
- A high significant negative correlation were detected between plasma GSH and aqueous NO levels in POAG r=-0.67 P<0.001 PXG r=0.62 P<0.001 respectively.

Discussion:

Oxidative stress has been claimed in POAG, as well as in neurodegeneration and death of TMC and retinal ganglion cells (Zanon-Moreno et. al, 2009).

Oxidative damage has been hypothesized to play a role in the pathogenesis of glaucoma. As there are high aqueous concentrations of hydrogen peroxide and photochemical reaction in the anterior segment arising from aerobic metabolism, the trabecular meshwork is exposed to high levels of oxidative stress. Aqueous humor is known to contain oxidative agents such as hydrogen peroxide and superoxide anion (Spector & Garner, 1981). Trabecular meshwork is exposed to course of the life time and therefore, it has a sophisticated defense mechanism against ROS, (Kolikos, *et al*, 2008).

In this study significant decrease in glutathione levels in plasma were detected in POAG and PXG. Clinical and laboratory studies performed have demonstrated a significant decrease in blood GSH in ocular diseases. Also, altered levels of GSH and GSH activity in trabecular meshwork and aqueous humor of patients with glaucoma has been demonstrated (Fekreira *et al*, 1994). Cherghel *et al* (2005), demonstrated a significantly lower GSH levels in glaucoma patients compared to controls, indicated that this mechanism played a role and implying the protection against ROS. Also, circulating GSH can be depleted either by subjecting cells to oxidative stress, or by inhibition of synthesis could be another possible reason for low GSH levels in glaucoma patients, (Cherghal *et al*, 2005).

In this study, significant decrease in total antioxidant status in aqueous humor of both POAG and PXG were found coupled to significant increase in malondialdehyde, CAT and GPX enzyme activities in aqueous humor of both POAG and PXG. Malondialdehyde is a lipid peroxidation end product that is generally accepted as a marker of oxidative stress. These results agreed with the result of De La Paz & Epstein (1996) thus supporting the view that oxidative stress may be etiologically involved in POAG. Also *Ylidlrim et al, 2005)* found elevated malondialdehyde levels in POAG compared to control.

Result's of Ferreira *et al*, 2004 as regard malondialdehyde and increased GPX coupled to decrease total antioxidant capacity in glaucoma patients agree with our results. Also, they illustrated that oxidative stress in the eye is significant enough to elicit compensatory activation of antioxidant defense mechanism.

On the contrary, they detected no significant change in catalase. The decrease in total antioxidant status levels reflects long lasing oxidative damage.

Also, Kolialos *et al*, (2008), found a prooxidant–antioxidant balance shifted in PXG. The antioxidants in PXG can not counterbalance the oxidative stress in the eye. The same results are also detected by Schrehardt, (2010). Significantly reduced levels of antioxidants such as glutathione and total antioxidant status in aqueous suggested a faulty antioxidative defense in PXG patients. To the contrary, they detect a decrease in antioxidant enzymes in aqueous which indicate an inadequate cytoprotection against oxidative insult, (Schrehardt, 2010). Also, *Zoric et al, (2006)* detected a lower catalase activity in PXG due to evidence of oxidative damage as well as decreased antioxidative protection. Indeed, decrease antioxidative potential, increase expression of oxidative stress markers and increased oxidative DNA damage, peroxide lipids, as well as the up regulation of inducible nitric oxide synthase has been described in the trabecular meshwork of glaucoma's patients (Liton *et al*, 2009).

The insignificant difference detected in all parameters studied in both POAG & PXG are quite logic, as PXG is one of POAG, however other parameters could aggravate EC protein damage in PXG.

In this study, we found also elevated nitric oxide levels in aqueous humor. This result agrees with the results of Schrehardt (2010), they suggest that chronic oxidative stress in combination with weakened cytoprotective and repair strategies affect abnormal matrix. The profibrotic growth factor TG F.β transformoring growth factor β oxidative stress, therefore, appears to represent a modifiable risk factor in PXG.

According to studies of Wang *et al*, (1996) NO production in the anterior segment of the eye affects the regulation of aqueous humor formation. It is possible that patients with glaucoma may stimulate an inflammatory reaction to induce a higher NO level in their eyes and that long term exposure to the higher NO level in the aqueous humor would lead to structural or biochemical impairment of outflow facility.

Studies on the action of NO in endotoxin -evoked ocular inflammation have suggested the importance of NO in ocular inflammation and toxicity (Wang *et al*, 1996). The inflammation can be induced by free radicals, e.g. NO or superoxide anion (O_2^-), but the actual damage is caused by peroxynitrite anion ($ONOO^-$) in the cells. (Kosthka, 1995) participated in the autocytotoxic responses to induce NO-mediated apoptosis by activating the endonuclease involved in the cleavage of DNA (Klem *et al*, 1997).

Neufeld *et al*, (1999), further demonstrated inducible NOS in the optic nerve heads from human glaucomatous eyes, and from glaucomatous rat experiments. They were convinced that the excessive NO could cause neurodegeneration of the axons of the retinal ganglion cells in glaucoma (Neufeld *et al*, 1997).

In accordance with the current study, the elevated levels of NO in the aqueous humor of patients with glaucoma reflected an increased NO production that causes neuroretinal damage. Although a high NO level in the anterior segment of the eye could increase the outflow of aqueous humor in POAG patients, it could still be diminished if the longitudinal ciliary muscle is structurally or biochemically impaired.It has been hypothesized that impaired nitric oxide products has a major effect on the equilibrium between the endothelial vasoconstrictor and vasodilator factor at the ocular levels that could result in a decrease ocular blood flow in susceptible patients with glaucoma. In addition to homodynamic role, nitric oxide has also been shown to induce relaxation of the trabecular meshwork & the ciliary muscle, result in decrease intraocular pressure. Any disturbance in nitric oxide balance could therefore act both at the ocular and systemic levels and have dramatic consequence on the progression of glaucoma. Cherghel *et al*, (2005).

The strong negative correlation between plasma glutathione and nitric oxide in aqueous detected in this study could be explained as the low levels of circulating glutathione may be due to depleted GSH aggravated by increase strong oxidative marker NO or impaired synthesis. The work of Izzotti *et al*, (2003) and Sacca *et al*, (2005), demonstrated that oxidative stress in anterior chamber of the eye may be overwhelming enough that the trabecular meshwork cells, the critical gateway to the draining of aqueous humor and regulation of IOP, can directly damage DNA of the cells and potentially propagate the pathophysiologic mechanism of glaucoma.

Also the capacity of nitric oxide to induce apoptosis has been documented, (Aslan *et al*, 2008). They claim nitric oxide to inhibit nuclear factor κß (which plays a protective role against apoptosis through the up regulation of gene encoding anti apoptotic protein) by inducing the expression of NF κß inhibitor. (Aslan *et al*, 2008).

Conclusion:

There is sufficient evidence that oxidative and nitrative processes play an important role in pathogenesis of POAG and PXG.

However, there is still a limited understanding to whether free radical generation is a primary or a secondary event in glaucoma. Oxidative damage in the cellular components of the trabecular meshwork could directly affect the regulation of extracellular matrix structure and lead to an alteration of flow of the aqueous humor. Perturbation of the eye's outflow will thus cause an elevation of intraocular pressure. In such set of circumstances, oxidative stress can be considered as secondary event in pathogenesis of glaucoma. It is desirable that future studies and clinical trials will further advance our understanding on the exact pathogenesis of glaucoma, and help in design of more effective therapies.

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