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# Evidence of Oxidative Stress Damage in Glaucoma

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## 1. Introduction

Oxidative stress has been implicated as a risk factor at several levels in the pathophysiology of glaucoma (Ferreira et al., 2004, 2009, 2010) as well as neurodegenerative diseases (Famulari et al., 1996); growing evidence supports the role of oxidative stress in glaucomatous neurodegeneration (Tezel, 2006). Reactive oxygen species are involved in signalling pathways during retinal ganglion cells death by acting as second messengers and/or modulating protein functions (Neufeld et al., 1999). Evidence of oxidative and nitrative processes was found in glaucoma in terms of activity of antioxidant enzymes, levels of low-molecular weight antioxidants and markers of lipid peroxidation (Aslan et al., 2008). Moreover it has been reported that nitric oxide may be an important mediator in retinal ganglion cells death in glaucoma (Neufeld et al., 1997). In the glaucoma eye, an altered oxidant/antioxidant balance may result in a number of molecular changes that contribute to the development of this ocular disease.

Glaucoma is a disease characterized by a specific pattern of optic nerve head and visual field damage in which if it is not controlled may lead to blindness. Although it has been traditionally associated with high intraocular pressure (IOP), glaucoma is now considered as a multifactorial disease. In this context, IOP is the most important known risk factor for the development of glaucomatous optic nerve damage. Even in normal-pressure glaucoma, reducing IOP can be beneficial in terms of halting visual field damage progression. However, lowering IOP may not be enough in every case, since different mechanisms that may or may not depend on the IOP level could contribute to this damage. Proposed mechanisms include ischemia (Lander, 1982), obstruction of axoplasmic flow (Anderson & Hendrickson 1974) and deprivation of one or more trophic factors (Quigley et al., 1995), excitotoxicity (Vorwerk et al., 1997) and oxidative stress damage (Ferreira et al., 2004, 2009, 2010).

IOP is not elevated in all the eyes that exhibit characteristics of glaucomatous neurodegeneration but experimental elevation of IOP induces oxidative stress in the retina.

Aqueous humor is known to contain several active oxidative agents such as hydrogen peroxide and superoxide anion. Low molecular weight antioxidants, such as glutathione (GSH), and ascorbate, together with molecules with free radicals scavenging properties like cysteine and tyrosine, have been identified in the aqueous. Ascorbate is present at high concentrations in it (1-2 mM) (Richer & Rose, 1998) and antioxidant enzymes such as

superoxide dismutase (SOD), catalase and glutathione peroxidase have been reported in aqueous humor (Garland, 1991; Varma, 1987).

It has been suggested that a chronic oxidative stress insult induced by these agents can compromise the trabecular meshwork function, the major route for aqueous outflow from the anterior chamber. The trabecular meshwork (TM) is exposed to chronic oxidative stress over the course of lifetime and therefore it has a sophisticated defense mechanism against ROS. Previous studies estimated that the rate of loss of cells of the TM is linear and approximately 0.58 % per year from birth through 81 years old (De la Paz & Epstein, 1996).

Exfoliation syndrome (XFS) is a clinically significant systemic disorder, involving abnormal production or turnover of extracellular matrix material or a combination of both processes (Ritch & Schlötzer-Schrehardt, 2001). The exact etiology of this syndrome remains unknown; the most accepted theory postulates that it is an age-related process of buildup of an abnormal elastotic material (Schlötzer-Schrehardt & Naumann, 2006). XFS is the most common cause of secondary open-angle glaucoma (Ritch, 1994). It is a generalized age-related disorder of the extracellular matrix with abnormalities in the basal membranes.

Exfoliation syndrome: The clinical diagnosis is made by the presence of exfoliative material on the surface of the anterior capsule of the lens. Exfoliative material may also be present in the corneal endothelium and the trabecular meshwork. Other clinical features include atrophy of the pupillary border and iris transillumination defects (Naumann et al., 1998).

XFS may be associated with ocular problems such as high intraocular pressure (IOP), and glaucomatous optic neuropathy. It may be also associated with poor mydriasis, zonular instability, corneal endotheliopathy, central retinal vein occlusion and cataract (Ritch & Schlötzer-Schrehardt, 2001). Systemic associations found in XFS include angina pectoris, hypertension, myocardial infarction, and stroke. An active involvement of the trabecular meshwork in this abnormal matrix process that leads to a progressive accumulation of XFS material in the juxtacanalicular tissue is considered as the possible cause of chronic high pressure in XFS eyes (Schlötzer-Schrehardt et al., 1992; Koliakos et al., 2001). The principal ocular cells implicated in the production of XFS material are those closely associated with the aqueous humor circulation and are influenced by the substances that are present in it. Increasing evidence suggests that ascorbic acid plays an important role in the defense mechanisms of the ocular tissues against free radical damage (Varma, 1987, 2001). A decrease in ascorbic acid and an increase of 8-isoprostaglandin F<sub>2a</sub> have been reported in the aqueous humor of patients with XFS (Koliakos et al., 2003). The principal ocular cells implicated in the production of exfoliative material are those closely associated with the aqueous humor circulation and are influenced by the substances present in it. Investigation of qualitative and quantitative alterations of the aqueous humor composition might, therefore, provide an important insight into the factors involved in this disorder. Recent studies reported differences in the concentration of matrix metalloproteinases (Schlötzer-Schrehardt et al., 2003) and growth factors (Koliakos et al., 2000, 2001) in the aqueous humor of XFS patients. It is well known that growth factors and proteases can be activated by free radicals, so the occurrence of oxidative stress and therefore the antioxidant status of the aqueous humor may play a role in the oxidative metabolism of the cells implicated in the production of exfoliative material.

The antioxidant status was evaluated in order to assess the occurrence of oxidative stress in the aqueous humor of glaucoma patients. It was measured through the determination of Total Reactive Antioxidant Potential (TRAP) levels and antioxidant enzymes activities. Antioxidant status of biological samples is regarded as an indicator of oxidative stress (Evelson et al., 2001). A decrease in the antioxidant capacity of tissues and body fluids may

be the consequence of increased oxidative processes. The activities of superoxide dismutase, glutathione peroxidase and catalase were determined in the aqueous humor of both types of glaucoma patients and compared with those measured in control group.

An experimental glaucoma model in rats was performed by our group in order to evaluate the time course changes in oxidative stress markers. This model induced high intraocular pressure and optic nerve head damage. It appears to mimic features of primary open angle glaucoma; therefore, it may be useful to understand the time-course of this ocular disease (Ferreira et al., 2010).

The relationship between the development of glaucoma and oxidative stress was evaluated. The occurrence of oxidative stress was evaluated by the following markers: *in vivo* chemiluminescence of the eye surface, the total antioxidant capacity in the aqueous and vitreous humor, nitrite concentration and markers of lipid peroxidation in the optic nerve head.

Among oxidative stress markers, organ chemiluminescence seems to afford a non-invasive assay that integratively measures the rate of formation of excited species, mostly singlet oxygen, through the measurement of light emission (Boveris et al., 1985).

Chemiluminescence from *in situ* organs is related to the *in vivo* steady state concentration of reactive oxygen species. An increased chemiluminescence level reflects an increased intracellular concentration of excited states, singlet oxygen, excited carbonyls and peroxy radicals. Increased levels of chemiluminescence indicated the occurrence of oxidative stress. Therefore chemiluminescence can be considered a non-invasive, nondestructive assay that can be useful in monitoring cellular damage in glaucomatous eyes (Ferreira et al., 2010).

## 2. Materials and methods

### 2.1 Aqueous humor sampling

Aqueous humor (0.1 mL to 0.2 mL) was rapidly and carefully collected at the beginning of the surgery through a paracentesis, using a 27 gauge needle connected to a tuberculin syringe under an operating microscope. Aqueous humor was immediately cooled at  $-70^{\circ}\text{C}$  and transported to the laboratory to run all the assays. All the samples were protected from light. Samples were evaluated as soon as possible during the first 24 hours after the surgery.

### 2.2 Patients

Glaucoma patients included in the study had a diagnosis of Primary open angle glaucoma (POAG) or XFG. Structural definition: Vertical C/D of 0.7 or more, asymmetry in the C/D of 0.2 or more and/or thinning of the neuroretinal rim to disc ratio of less than 0.1 with corresponding perimetric damage. The Disc Damage Likelihood Scale system was used to evaluate the rim to disc ratio. Functional definition: Glaucoma hemifield test outside normal limits, and 3 adjacent points in the 5% level on the pattern deviation plot, using the 24-2 strategy of the Humphrey perimeter. Visual fields were considered reliable if false negative and false positive responses were below 33%. Unreliable visual fields were repeated on the same day. If the second visual field was also unreliable, inclusion was made only on the basis of structural damage (Foster et al., 2002; Quigley et al., 2001).

All individuals had advanced glaucoma and elevated IOP despite the use of maximum tolerated medical therapy, and were scheduled for trabeculectomy. Patients with glaucoma (XFG and POAG) were using a variety of topical anti-glaucoma medications. Maximal tolerated medical therapy usually included a combination of timolol-dorzolamide (or timolol and brimonidine), although we have eliminated patients on prostaglandin analogues. Anti-glaucoma medications were not stopped before the procedures. Patients

enrolled in the cataract group had senile cataract. Cataract patients received topical phenylephrine and tropicamide as dilating drops before surgery. Non-steroidal anti-inflammatory agents were not administered before the procedure. All patients in this group, did not have glaucoma. In all cases, this was the first intraocular surgical procedure.

All subjects underwent a complete ophthalmic examination. This included an anamnesis, best corrected visual acuity, slit-lamp examination, Goldmann appplanation tonometry, and fundus examination with a dilated pupil. Gonioscopy was performed in all cases with a 4-mirror gonioscope. All subjects (POAG and XFG) had an open-angle (grade 3 or 4 of the Shaffer classification). The optic nerve was evaluated with a 78 diopter lens, and the vertical and horizontal cup to disc ratio (C/D) was recorded, as well as the presence of any notch or hemorrhage, and the appearance of the neuroretinal rim. Computerized perimetry was performed with the Humphrey 750, threshold strategy 24-2, or similar program with the Octopus. In all glaucoma patients, no other explanation for the optic nerve damage and the visual field loss should be found apart from the glaucoma.

XFS was defined by the presence of exfoliation material on the anterior surface of the lens. Exfoliation material was also investigated in the pupillary border, corneal endothelium, anterior hyaloid, and angle. However, for the diagnosis of XFS only the presence of material in the anterior surface of the lens was considered. This surface, with the pupil dilated, was carefully examined for the presence of the exfoliative material, using the high magnification of the slit-lamp and adequate illumination. Patients with previous intraocular surgeries, laser treatment, uveitis, any posterior segment pathologies, diabetes mellitus or any other systemic disease that may have influenced the measurements were excluded. Patients with other ophthalmic conditions such as angle-closure, low-tension, congenital glaucoma, trauma, or pigment dispersion syndrome were excluded. None of the subjects smoked, had special diets, or were taking antioxidant vitamins, such as  $\alpha$ -tocopherol, ascorbic acid, nonsteroidal anti-inflammatory agents, or on prostaglandin analogues treatment. The only systemic medications allowed were those for blood hypertension.

The patients were divided into three groups: patients with XFG, POAG and cataract. Each group consisted of 25 patients. All subjects were Caucasians and matched for age and sex. There were no statistically significant differences between the groups in terms of age and sex. Age was  $73 \pm 2$  years old for the XFG group,  $70 \pm 10$  years old for the POAG group, and  $73 \pm 2$  years old for the cataract group.

Preoperative intraocular pressure (IOP) was  $25 \pm 5$  mmHg for the XFG patients,  $26 \pm 4$  mmHg for the POAG group and  $14 \pm 6$  mmHg for the cataract group.

Vertical cup/disc ratio was  $0.89 \pm 0.01$  for XFG patients and  $0.89 \pm 0.01$  for the POAG group. A total of 75 aqueous humor samples were analysed, 25 for each of the following groups: patients with XFG, POAG and cataract. Aqueous humor samples were compared regarding the alterations in the levels of non-enzymatic antioxidants with two parameters: ascorbic acid concentration and the total reactive antioxidant potential. The activities of the antioxidant enzymes: glutathione peroxidase, catalase and superoxide dismutase were also measured. This study was approved by the Human Subjects Committee of the University of Buenos Aires, and adhered to the Declaration of Helsinki. A written informed consent was obtained from all participants.

### 2.3 Experimental glaucoma model

The chronic ocular hypertension model following episcleral venous occlusion in rats was used (Shareef et al., 1995). Female Wistar rats ( $n= 18$ ) weighting 250-300 g were operated

under a microscope with a coaxial light. Animals were anesthetized with ketamine hydrochloride (50 mg/ kg) and xylazine hydrochloride (0.5 mg/ kg) administered intraperitoneally. A specially designed small lid speculum was used to retract the eyelids. One drop of 0.5% proparacaine hydrochloride (Alcon Laboratorios Argentina) was instilled. Vannas scissors and a conjunctiva forceps were used to open the conjunctiva and expose the limbal veins. A cyclodyalisis spatula was used to gently lift the vein from the underlying sclera, and an ophthalmic cautery was used to cauterize the vein. Care was taken in order not to damage the sclera. Two of the large veins of the left eye were cauterized using this method for the glaucoma group (n=9). Retraction without bleeding was noted after cauterization. Only one eye per animal was used for the experiment.

A sham operation without cauterizing the vessels was performed in the left eye of the control group (n= 9). The right eye was only controlled in both groups.

Rats were housed in standard animal room in a 12 hours light/ dark cycle and were fed with food and water ad libitum under controlled conditions of temperature ( $21 \pm 2^\circ \text{C}$ ) and humidity. After different periods of time (0, 7, 15, 30, 45, 60 days), eyes were enucleated under dim light immediately after anesthesia, and aqueous humor, vitreous humor and retinas were carefully removed. Vitreous and aqueous humor were collected in a syringe under a surgical microscope and the retinas were detached by blunt dissection. Immediately after dissecting, the optic nerves heads were homogenized.

To assess the occurrence of oxidative stress the following markers were evaluated: in vivo chemiluminescence of the eye surface, the total antioxidant capacity in the aqueous and vitreous humor, nitrite concentration and markers of lipid peroxidation (TBARS) in the optic nerve head.

All animals used procedures were in accordance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### 3. Results

#### 3.1 Glaucoma patients

The ascorbic acid concentration in the aqueous humor was  $230 \pm 20 \mu\text{M}$  for the XFG group, which represents a 45 % decrease when compared to the POAG group. The mean value of the POAG group was  $415 \pm 17 \mu\text{M}$  ( $p < 0.001$ ). Ascorbic acid levels of both types of glaucoma were lower than in the cataract group ( $720 \pm 30 \mu\text{M}$ ;  $p < 0.001$ )

Ascorbic acid was significantly lower (67 %) in the exfoliation group in accordance with previously published values. Significantly reduced levels of ascorbic acid, an important free radical scavenger in the eye, have been reported in the aqueous humor of exfoliation patients, suggesting a faulty antioxidant defense system (Packer et al., 1979). Ascorbic acid is secreted into the aqueous by the ciliary epithelium and is essential in cells for its antioxidant capacity and its role in regenerating vitamin E and glutathione (Koliakos et al., 2003; Zhou et al., 1998). Beside its protective role against free radical damage, ascorbic acid modulates the synthesis of various extracellular matrix molecules such as collagen, elastin, laminin and glycosaminoglycans (Yimaz et al., 2005). The ascorbic acid levels in XFG patients were decreased 45 % compared to POAG patients. These findings seem to be significant because ascorbic acid is essential in cells for its antioxidant capacity and its role in regenerating vitamin E and glutathione (Parker et al., 1979). In addition to the protective role of ascorbic acid against free radical damage, it also modulates the synthesis of various extracellular matrix molecules such as collagen, elastin, laminin and glycosaminoglycans (Zhou et al.,

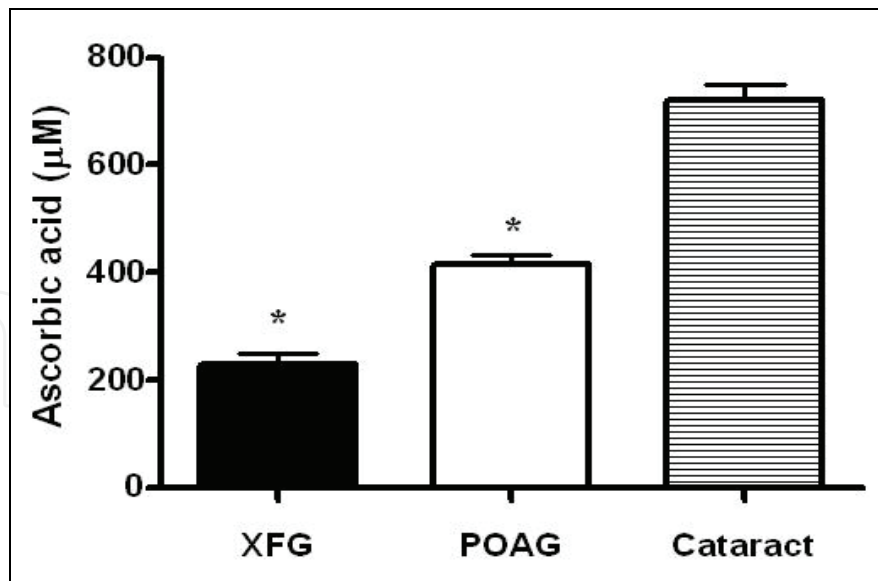


Fig. 1. Ascorbic acid concentration from the aqueous humor in patients with glaucoma associated with exfoliation syndrome (XFG), compared to primary open angle glaucoma (POAG) and cataract patients. The values are represented as mean  $\pm$  SEM for 25 XFG patients, 25 POAG patients and 25 cataract patients. \*  $p < 0.001$ .

1998). Thus, alterations in ascorbic acid concentrations may produce an increase in the fibrillar material deposits that affect the normal aqueous flow through the trabecular meshwork. Significantly reduced levels of ascorbic acid have been reported in the aqueous humor of exfoliation patients with cataract, suggesting a faulty antioxidant defense system (Koliakos et al., 2002).

The induction time for the aqueous humor from a glaucoma patient was 9.6 minutes, while the same volume of aqueous humor from a control patient rendered an induction time of 39.4 minutes. These results indicate that the concentration of antioxidants in the aqueous humor of glaucoma patients was lower than in control patients. The average TRAP values from glaucoma patients was significantly lower, by 64 %, than that obtained from control patients ( $124 \pm 5 \mu\text{M}$ ;  $p < 0.001$ ). On the other hand, the mean values of the total reactive antioxidant potential were found to be  $55 \pm 8 \mu\text{M}$  Trolox in the aqueous humor of POAG group and  $28 \pm 2 \mu\text{M}$  Trolox in aqueous humor of the XFG group. In other words, the levels of TRAP were significantly decreased (49 %) in the XFG aqueous humor when compared to the POAG group ( $p < 0.001$ ). TRAP values of both types of glaucoma were lower than the cataract value ( $124 \pm 5 \mu\text{M}$  Trolox;  $p < 0.001$ ) (Figure 2).

In addition to the decrease in ascorbic acid levels, a significant decay in the aqueous humor total reactive antioxidant potential level of XFG group was found when compared to the POAG group. These results indicate a significant reduction in the concentrations of water-soluble antioxidants in the aqueous humor, such as glutathione, ascorbic acid, tyrosine and cysteine. Homocysteine is a non-essential sulphur aminoacid produced as an intermediate in the metabolism of cysteine. In recent studies, the role of homocysteine in the development of exfoliation glaucoma was investigated (Bleich et al., 2004). The toxicity mechanism of homocysteine may be due to its oxidation in the presence of transition metals, generating superoxide anion, hydrogen peroxide, hydroxyl radical, and sulphurated radicals (Halliwell & Gutteridge, 1989). Homocysteine was found to be elevated in the aqueous humor and

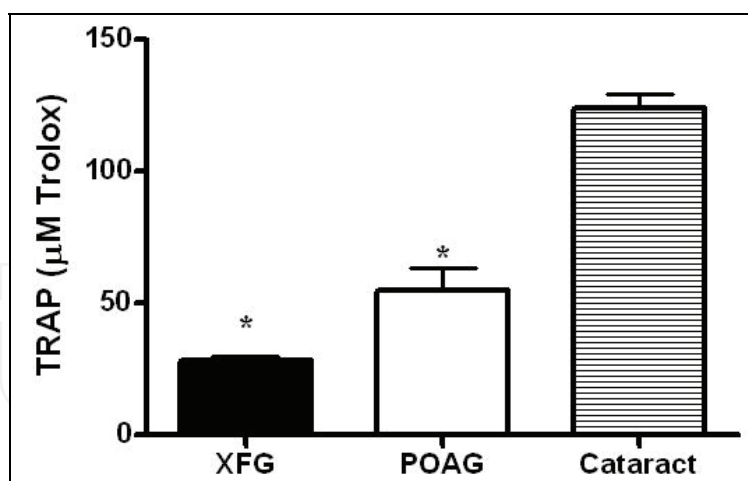


Fig. 2. Total reactive antioxidant potential from the aqueous humor in patients with glaucoma associated with exfoliation syndrome (XFG), compared to primary open angle glaucoma (POAG) and cataract patients. The values are represented as mean  $\pm$  SEM for 25 XFG patients and 25 POAG patients and 25 cataract patients. \*  $p < 0.001$ .

the plasma of patients with exfoliation syndrome coexistent with cataract and normal IOP (Bleich et al., 2002; Vessani et al., 2003). Moreover, low levels of glutathione were found in the aqueous humor of patients with exfoliation syndrome coexisting with cataract and normal IOP (Gartaganis et al., 2005).

These results indicate a significant reduction in the level of water-soluble antioxidants in the aqueous humor (that includes glutathione, ascorbic acid and tyrosine). These results indicate a significant reduction in the level of water-soluble antioxidants in the aqueous humor, mainly represented by glutathione and ascorbate. This decrease may be due to the occurrence of oxidative stress in a glaucomatous eye that make the organ more susceptible to damage associated with ROS production.

The activity of glutathione peroxidase (GPx), catalase (CAT) levels and superoxide dismutase (SOD) activity, were determined in the aqueous humor of XFG group and compared to those measured in the POAG and cataract groups. A significant increase in GPx activity was found in the aqueous humor of the XFG group, when compared to the POAG group, whereas no significant changes were found in CAT levels and SOD activity. Glutathione peroxidase activity in the aqueous humor of XFG patients showed a 87 % of increase when compared to the POAG group. The mean value of GPx in aqueous humor from the XFG group was  $30 \pm 2$  U/ mL, and for the POAG group was  $16 \pm 3$  U/ mL ( $p < 0.001$ ). GPx of both glaucomas showed an increase when compared to the cataract group ( $6 \pm 2$  U/ mL,  $p < 0.001$ ). On the other hand, no significant changes were found in catalase activity. Catalase levels in the aqueous humor from the XFG and POAG groups showed a mean value of  $40 \pm 5$  fmol/ mL, and  $42 \pm 4$  fmol/ mL respectively, while in the cataract patients it was  $38 \pm 7$  fmol/mL. The mean value of SOD in aqueous humor of the XFG group was  $44 \pm 7$  U SOD/ mL versus  $42 \pm 5$  U SOD/ mL in the POAG group. A significant increase of 67% in the superoxide dismutase activity was observed in both glaucoma groups versus the cataract group ( $27 \pm 3$  U/ mL,  $p < 0.001$ ), but no changes were found between both glaucomas. A significant increase in SOD and glutathione peroxidase activities was found in the aqueous humor of glaucoma patients, whereas catalase activity was not affected. These results imply a 57 % increase in SOD activity. Glutathione peroxidase activity in the aqueous



humor of glaucoma patients showed a threefold increase when compared with value obtained in the control group. The changes observed in the antioxidant enzymes in glaucoma patients are consistent with the presence of oxidative stress.

SOD is an antioxidant key enzyme in the metabolism of oxygen free radicals, as it removes superoxide radical and prevents formation of other reactive radical species, such as peroxynitrite (Aslan et al., 2008). Superoxide anion is the first species in the cascade of univalent reductions of molecular oxygen and therefore the first indicator of an increased production of ROS. Steady state concentrations of superoxide anion are directly proportional to its rate of production and inversely proportional to the concentration of scavenging enzymes, such as SOD. Thus alterations in SOD activity will have important consequences on the steady state concentrations of superoxide in the aqueous humor.

Nitric oxide reacts extremely rapidly with superoxide to form peroxynitrite. The deleterious effects of peroxynitrite may be prevented by limiting its formation, lowering the concentration of superoxide radicals by increasing SOD levels (Enghild et al., 1999). When peroxynitrite is added to a biological fluid, rapid losses of important antioxidant defenses, like ascorbic acid, uric acid, and -SH groups present in small molecules and proteins are observed (Giuseppe et al., 1998). If there is an increase of SOD activity, there will be a decrease in superoxide anion and an increase in hydrogen peroxide production.

H<sub>2</sub>O<sub>2</sub> is removed by two enzymes: catalase and glutathione peroxidase. Catalase directly catalyzes the decomposition of H<sub>2</sub>O<sub>2</sub> to ground state oxygen and water. Glutathione peroxidase removes H<sub>2</sub>O<sub>2</sub> using glutathione as a cofactor. Hydrogen peroxide is an essential component of several signal transduction pathways (Evelson et al., 2000).

In this pathological condition in the eye, there is an increase rate of superoxide production, which leads to a depletion of low weight molecules antioxidants and an increase in H<sub>2</sub>O<sub>2</sub> levels. According to this, glutathione peroxidase activity in the aqueous humor of glaucoma patients was found three fold higher than control group. Glutathione peroxidase activity was found increased and acts as a compensatory mechanism to ameliorate the oxidative stress in the aqueous humor of XFG and POAG patients (Ferreira et al., 2009). No significant changes were found in catalase levels, and this may be due to the nitric oxide (NO) inhibition of the catalase because of the union of NO to the heme group of the enzyme. NO competes with hydrogen peroxide for the union to the complex I of the catalase (Brown, 1995); if catalase is inhibited, hydrogen peroxide has to be metabolized by glutathione peroxidase. Nevertheless, in the present study we did not find any statistically significant differences in superoxide dismutase activity between XFG and POAG groups. Our results indicate that in both glaucoma groups, the activity of superoxide dismutase is increased compared to cataract group. Ischemia produced by changes in IOP and the resultant decrease of oxygen flow into the tissue may lead to an increase in intracellular calcium ions concentration, which may activate proteolysis and lead to conversion of xanthine reductase into xanthine oxidase which produces superoxide anion and a compensatory increase in superoxide dismutase activity (Tezel & Yang, 2004).

### 3.2 Experimental glaucoma model

Elevated IOP is the most important risk factor in the progression of glaucomatous damage. All animals, without any exception, responded with an increase in IOP after the development of the experimental glaucoma surgery. No differences in the IOP of control eyes were detected during the experimental period. A significantly increase in IOP compared to control eyes was observed at 7 days after the surgery.

Chemiluminescence is the emission of radiation resulting from a chemical reaction. The organ chemiluminescence is a method to evaluate signals of oxidative metabolism. This assay is specific and non-invasive for the organ and provides a time course evaluation of peroxidative breakdown of lipids. The termination reaction of peroxy radicals and singlet oxygen yield excited states and chemiluminescence in parallel with malondialdehyde production and conjugated lipid dienes.

An analysis of spontaneous eye surface luminescence at 0, 7, 15, 30, 60 days was showed in Figure 3. During the first days the luminescence showed a decreased in the eye photoemission by 22 %, 35 % and 27 %, respectively at 7, 15 and 30 days after the surgery. An increase of 22 % in light emission was observed at 60 days. Chemiluminescence at 0 days was considered as 100 % of light emission and results were relative to this value. At the first 30 days we observed a decrease in the relative chemiluminescence; this situation may be due in part because of the consumption of non-enzymatic antioxidants. Up to 30 days the marked increase in the spontaneous chemiluminescence of the eye appears to indicate an increase in the steady state levels of oxidant species, suggesting the occurrence of oxidative stress. Increased eye chemiluminescence was associated with the development of cell injury after oxidative stress that could not be compensated by the antioxidant defenses.

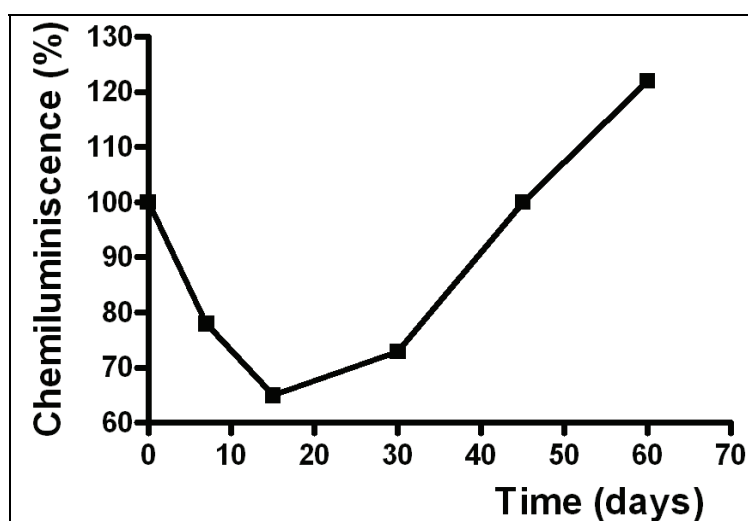


Fig. 3. Analysis of spontaneous chemiluminescence percentage at different times in the eye surface. Experimental glaucoma model was performed at time 0.

Aqueous humor samples were compared regarding the alterations in the levels of non-enzymatic antioxidants measuring the total reactive antioxidant potential. A significant decrease in levels of non-enzymatic antioxidants was found in the aqueous humor of hypertensive eyes since 15 days after surgery. A significant decrease in TRAP values was observed at 30 days compared to 15 days; on the other hand the levels of this parameter did not change compared to 45 days. At 60 days TRAP levels were significantly higher than at 30 or 45 days, whereas, these levels were significantly lower than at 7 days of treatment (Figure 4).

Figure 5 shows the average of TRAP values in the vitreous humor of rats at 0, 7, 15, 30, 45, and 60 days after surgery. A significant decrease in levels of non-enzymatic antioxidants was found in the vitreous humor of hypertensive eyes since 7 days after surgery. No significant changes were observed in the levels of TRAP at 7 days compared to 15 days.

Since 15 until 60 days there was a significant and progressive decrease in levels of non-enzymatic antioxidants at all over the experimental period. The percent of decrease was 42 % for 15 days, and 78% for 60 days compared to control eyes.

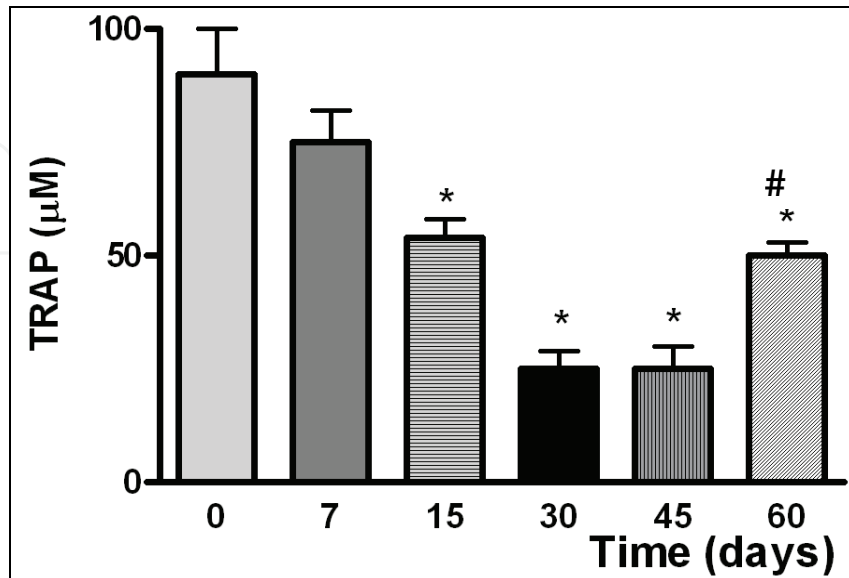


Fig. 4. TRAP in the aqueous humor at different times. Data are mean  $\pm$  SEM. \* $p < 0.05$  versus time 0, #  $p < 0.05$  versus time 30 and 45 days. Experimental glaucoma model was performed at time 0.

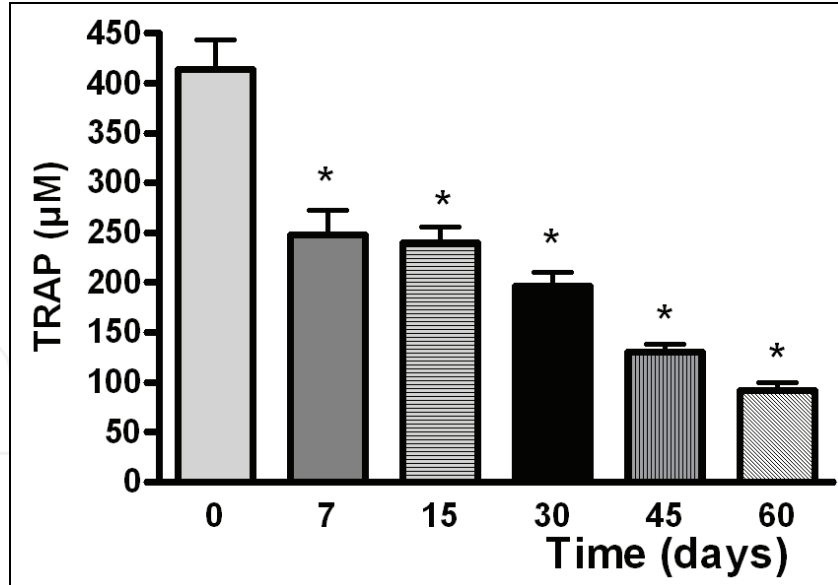


Fig. 5. TRAP in the vitreous humor at different times. Data are mean  $\pm$  SEM. \* $p < 0.001$ . Experimental glaucoma model was performed at time 0

TBARS levels as an index of lipid peroxidation were assessed in the optic nerve head from the hypertensive rats or from sham rats for the same periods (Figure 6). At 30 days of surgery, TBARS levels were significantly higher (200 %) in hypertensive rats than in sham ones ( $6.24 \pm 0.42$  nmol/ mg protein). A further significant increase was observed at 45 and

60 days (300 and 600%). No significant changes were observed at 7 and 15 days compared to control value ( $6.24 \pm 0.42$  nmol/ mg protein).

The retina exhibits a distinct susceptibility to oxidative stress due to its enhanced metabolic rate with high levels of oxygen demand, and higher lipid content in its membranes. The TBARS levels support this situation; the increase in them was time-dependent and correlated with high IOP. Figure 6 shown, a significant increase of lipid peroxidation occurred through 15 days after IOP elevation.

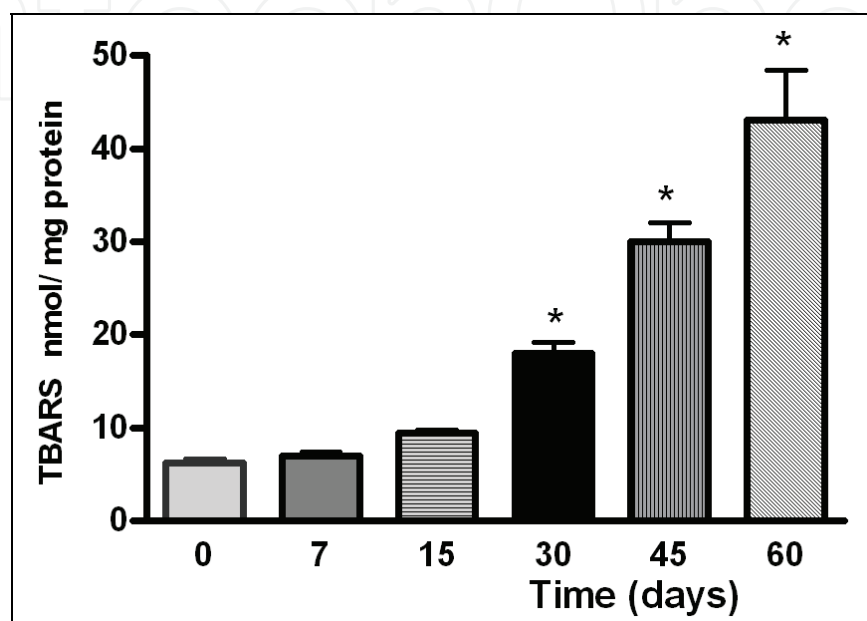


Fig. 6. Optic nerve head TBARS levels at different times. Data are mean  $\pm$  SEM. \* $p < 0.001$ . Experimental glaucoma model was performed at time 0.

Negative correlation between TRAP and TBARS levels was found (Ferreira et al., 2010). The levels of lipid peroxidation products are increased meanwhile the levels of non-enzymatic antioxidants are decreased in the optic nerve head of rats with glaucoma. The oxidation of endogenous antioxidants reflects an increase in tissue oxidants that is assessed by the decrease in the total level of antioxidants. These molecules prevent or reduce the extent of the oxidative destruction of biomolecules.

The nitrite levels in the optic nerve head were represented at different times of surgery in Figure 7. A significant increase of nitrite concentration in the optic nerve head was found since 7 days of surgery and this increase was kept up until 60 days of treatment. There was no significant difference between nitrite levels at 7, 15, 30 and 45 days. Nanning et al have demonstrated the release of nitric oxide (NO) by superoxide. This would result in the formation of peroxynitrite that leads to cytotoxic effects in the surrounding cells (Manning et al., 2001). Therefore, NO may be a mediator in ganglion cells death. Different studies showed that NO levels were increased in retinas after IOP elevation (Siu et al., 2002), and increased levels of NOS isoforms in the optic nerve head were reported (Neufeld et al., 1997). NOS inhibitor provided protection for retinal ganglion cells neurodegeneration (Neufeld et al., 1999). Oral administration of an inducible nitric oxide synthase (iNOS) inhibitor did not protect the optic nerve in a rat model (Kasmala et al., 2004), on the other hand another report documented iNOS activity is not elevated in an experimental glaucoma

model (Morrison et al., 2003). The capacity of NO to induce apoptosis have been documented in astrocytes (Hu et al., 1996) and neuronal cells (Heneka et al., 1998). Further detailed studies are required to elucidate and clarify the role of NO in glaucoma.

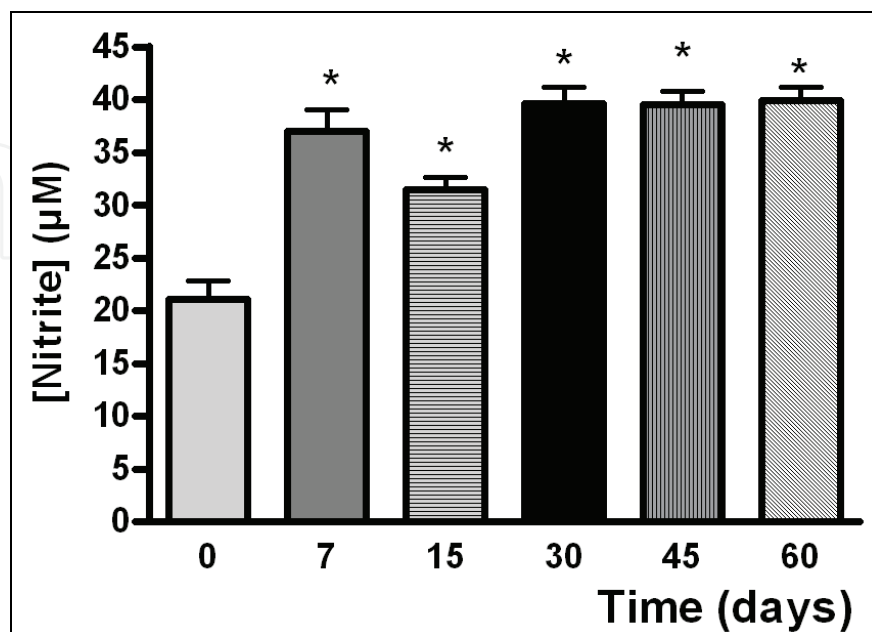


Fig. 7. Nitrite concentration in the optic nerve head at different times. Data are mean  $\pm$  SEM. \* $p < 0.001$ . Experimental glaucoma model was performed at time 0

#### 4. Conclusion

The defense against the harmful effects of free radicals is achieved by endogenous antioxidant compounds and antioxidant enzymes present in the aqueous humor. The antioxidant status of aqueous humor might therefore provide an important insight into the factors involved in the development of glaucoma. If oxygen active species are implicated in trabecular meshwork cell damage, an associated antioxidant mechanism should be present to repair the oxidative injury.

The changes observed in the antioxidant defenses in the aqueous humor suggest a possible role of the active oxygen species in the tissue damage and the pathogenesis of XFG. Our findings suggest that free radicals action with a depleted endogenous non-enzymatic defense system play a role in the worse damage observed in XFG than in POAG eyes. At any level of IOP, damage in XFG is worse than in POAG, prognosis is also worse, progression is more rapid, response to medical therapy is worse, needs surgery more often and blinds more people. Our results would suggest that further research regarding the use of antioxidants as an adjunct therapy in glaucoma may be indicated. An important goal of future research in glaucoma is therefore to understand the mechanisms of retinal ganglion cell (RGC) death and to develop new ways to treat them. Progressive loss of retinal ganglion cells leads to optic nerve atrophy and visual fields defects in glaucoma patients. Oxidative stress caused by increased generation of reactive oxygen and nitrogen species has been implicated RCG death. Recent in vitro studies using primary culture of RGC have also provided evidence that different glaucomatous stimuli involves increased of reactive oxygen species generation and antioxidant treatment provides additional protection. However, although the involvement of

free radicals in the pathophysiology of pseudoexfoliation glaucoma has not been confirmed yet, we suggest their likely role in this disease.

Oxidative stress is thought to contribute to the pathophysiology of many neurodegenerative diseases. The retina contains large amounts of polyunsaturated fatty acids and thus could be susceptible to oxidation by free radicals. Furthermore elevated intraocular pressure or vascular diseases altered blood flow. The consequence decrease in perfusion of the retina and optic nerve head can cause ischemia and this affects retinal ganglion cells survival. Reactive oxygen species are involved in signalling retinal ganglion cells death by acting as a second messenger and/ or modulating protein function. Oxidative stress induces dysfunction of retinal ganglion cells and may contribute to spreading neuronal damage. Chronic elevation of IOP demonstrated significant loss of retinal ganglion cells and has a measurable effect on the redox status.

Spontaneous organ chemiluminescence evaluates the redox status of the tissues in real time in several pathologies, including glaucoma. Spontaneous chemiluminescence of the eye in rats with experimental glaucoma and in control ones was measured for the first time in order to be a useful tool for studying the time course change of oxidative stress.

Reactive oxygen and nitrogen species were increased in glaucoma; this could be evidenced by the increase in chemiluminescence, nitrite levels and the increase in lipid peroxidation.

The glaucoma model was used to elucidate the role of oxidative stress and allows demonstrating the changes found in previous studies using human subjects with glaucoma.

The increased chemiluminescence observed at a later stage could be due to a decrease in the antioxidant defenses, evidenced by TRAP decrease in aqueous and vitreous humor. The levels of lipid peroxidation products and nitric oxide levels increased during the development of glaucomatous optic neuropathy.

According to several investigation lines, there is increase evidence that oxidative stress may be involved in the development and/ or progression of glaucomatous damage

Cells usually tolerate mild oxidative stress that results in up regulation of antioxidant defense system, in order to restore the antioxidant- oxidant balance.

It seems possible that the decrease in non-enzymatic antioxidant may overcome the ability of cells to resist oxidative damage. The increased levels of lipid peroxidation products and in vivo chemiluminescence demonstrate this damage.

The relationship between oxidative stress and neurodegeneration is not completely clear. Free radicals can act directly as neurotoxic, or may also function as secondary messengers to spread the damage. Our results would suggest that further research regarding the use of antioxidants as an adjunct therapy in glaucoma may be indicated. Treatment interventions to reduce in vivo oxidative stress may be important in patients with this disease. Future studies will improve our knowledge on the mechanisms of damage in glaucoma and thus devise more effective treatments, in addition to IOP reduction.

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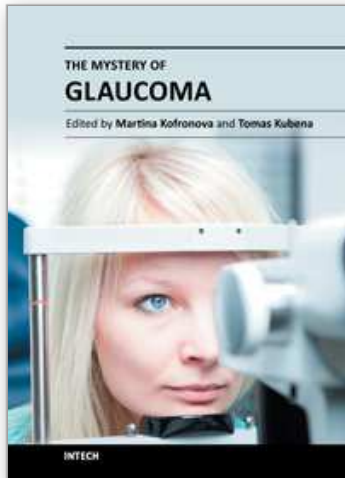
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## **The Mystery of Glaucoma**

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