

Nitric oxide and tumour necrosis factor alpha in the process of pseudoexfoliation glaucoma

Tatjana S. Sarenac Vulovic¹, Sladjana M. Pavlovic², Vladimir L.J. Jakovljevic³, Katarina B. Janicijevic⁴, Nemanja S. Zdravkovic²

¹Department of Ophthalmology, Clinical Center Kragujevac, Zmaj Jovina 30, Kragujevac 34000, Serbia

²Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac 34000, Serbia

³Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia

⁴Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia

Correspondence to: Tatjana S. Sarenac Vulovic. Department of Ophthalmology, Clinical Center Kragujevac, Zmaj Jovina 30, Kragujevac 34000, Serbia. tvoja.tanja@yahoo.com

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Abstract

• **AIM:** To establish the role of nitric oxide (NO), ascorbic acid and tumour necrosis factor α (TNF- α) in the pathogenesis of pseudoexfoliation glaucoma (XFG).

• **METHODS:** Our study included 120 patients who were referred for cataract surgery. All patients were divided into four groups according to clinical findings: XFG, early and late pseudoexfoliation syndrome (XFS), and cataract (without pseudoexfoliation). Serum and aqueous humour levels of the ascorbic acid, NO and TNF- α were measured. The concentrations of the ascorbic acid and NO were measured by an appropriate spectrophotometric method. Enzyme-linked immunosorbent assay (ELISA) was used to determine TNF- α level.

• **RESULTS:** Aqueous humour concentration of ascorbic acid was significantly lower in patients with late XFS (0.61 ± 0.11 mmol/L) and XFG (0.48 ± 0.15 mmol/L) compared to patients with early XFS (0.9 ± 0.15 mmol/L) and cataract (1.16 ± 0.22 mmol/L), while there was no difference in serum concentration in all examined groups. Aqueous humour concentration of NO was significantly higher in patients with XFG (77.7 ± 11.4 μ mol/L) compared to patients with early XFS (50.27 ± 9.34 μ mol/L) and cataract (49.77 ± 7.1 μ mol/L), while serum concentration was increased in the early stage of XFS (73.26 ± 8.29 μ mol/L). Aqueous humour level of proinflammatory cytokine TNF- α was increased in patients with XFS (early 460.04 ± 18.32 pg/mL; late 502.42 ± 53.23 pg/mL) and XFG (510.34 ± 43.07 pg/mL),

while there was no difference in serum level in all examined groups of patients.

• **CONCLUSION:** Reduced ascorbic acid and elevated NO and inflammation related cytokine TNF- α level in aqueous humour of the patients with developed XFG suggest that oxidative stress induces local inflammation.

• **KEYWORDS:** ascorbic acid; nitric oxide; pseudoexfoliation; tumour necrosis factor alpha

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INTRODUCTION

Pseudoexfoliation syndrome (XFS) is an age related disorder of the extracellular matrix (ECM), characterised by an intensive production of abnormal fibrous fibers and their accumulation in the eye^[1], but without the development of pseudoexfoliative glaucoma. It is called stress induced elastosis, because the main cause of its production is overproduction of reactive oxygen species^[2]. Oxidative stress in the body can be activated by different causes: ultraviolet radiation, ages, infection, *etc*^[3]. Liberated free radicals can activate cytokine secretion from different inflammatory cells. These cytokines can act as pro-inflammatory in this abnormal matrix process in XFS and pseudoexfoliation glaucoma (XFG)^[3]. Decreased antioxidative concentration provokes releasing of cytokines and accelerates pseudoexfoliation production^[4]. If the pseudoexfoliative material is deposited in iridocorneal angle with increased humour aqueous outflow resistance, glaucoma will be developed^[3].

Pseudoexfoliation production is in proportion with the age, because older population is more represented in groups with pseudoexfoliation (syndrome or glaucoma), which is in correspondence with the reachable date^[5].

Ascorbic acid is one the most important antioxidant and free radical scavenger in the body and can be reduced in patients with cataract, glaucoma, aged related macular disease, *etc*^[6]. Nitric oxide (NO) is short-lived gaseous molecule produced by a group of enzymes called nitric oxide synthases (NOS), using L-arginin to produce NO in the tissue^[7]. Inducible nitric

oxide synthases (iNOS), one of the three isoforms, is not primarily expressed, but can be induced in the macrophages by bacterial lipopolysaccharide or with secreted cytokines^[8]. NO as a potent vasodilator relaxes smooth muscles and it is a part of different pathological processes in the eye: cataract, uveitis, and glaucoma^[9].

TNF- α is a proinflammatory cytokine which plays a role in different physiological and immunological process^[10]. It can activate inflammation in the tissue, apoptosis mediated by mitochondria^[11], and, contrary to this, it can have neuroprotective role^[12]. Its role in the process of glaucoma development is still not clear.

In this study we have indicated the importance of oxidative stress in the development of XFG as well as the potential protective role of NO in this process.

SUBJECTS AND METHODS

Our study includes 120 patients, recruited for the cataract surgery. All patients were divided into four groups: XFS-early (diffuse precipitated white flakes on anterior lens capsule or on pupillary margin, mild pupillary dilatation followed by pigment dispersion and its deposition on anterior lens capsule and chamber angle, initial pupillary ruff atrophy) and late stage (massive pseudoexfoliation deposition on pupillary margin and anterior lens capsule with well developed clear zone without pseudoexfoliation material, heavy pigment deposition on lens capsule, chamber angle-specially Sampaolesi line with high restricted mydriasis) XFG and control group (with no other eye disease). Serum samples were collected before cataract surgery. After paracentesis was done, the samples of aqueous humour were collected during cataract surgery using 27 gauge needles. The samples of humour and sera were stored at -80°C until biochemical analysis. All patients underwent a complete ophthalmological examination. Pseudoexfoliation material can be found on corneal endothelial cells, iridocorneal angle, lens, pupillary margin, ciliary zonulas, vitreous, *etc.* and it was detected during detailed slit lamp examination in mydriasis. Aplanation tonometry was used for intraocular pressure measurements. Direct fundus examination was conducted for all patients to detect optic head changes. Based on intraocular pressure levels (IOP>22 mm Hg), optic head changes (disturbed C/D ratio) and reproducible visual field defects in computed perimetry, patients with pseudoexfoliation can be divided into two groups: XFS and XFG. Patients with the history of previous trauma, intraocular inflammation, diabetes mellitus, myopia, earlier laser photocoagulation, cryo therapy or intraocular surgery were excluded from the study. Control group was selected in terms of age and sex.

The regional Ethics Committee approved the study protocol and prior to the initiation a written informed consent was obtained from all subjects according to the Declaration of Helsinki.

Measurement of Ascorbic Acid Concentration in Aqueous Humour and Serum The determination of vitamin C in the samples was done by technique described by Rutkowski and Grzegorzczak^[13]. In the centrifugal test-tube we measured 0.5 mL of analysed liquid (aqueous humour-diluted 1/10 by distilled water), with 0.5 mL of the phosphotungstate reagent (PR). It was mixed thoroughly and left in a room temperature for 30min. The tubes were centrifuged (7000 rpm, 10min), and the whole of the separated supernatant was collected with a pipette. The supernatant is a test sample for spectrophotometric measurements. Standard sample was prepared as above (1 mL of standard solution was used instead of analysed solution), but with no centrifugation. We measured the absorbance of the test sample and of the standard sample at 700 nm against the mixture PR: 50 mmol/L solution of oxalic acid=1:1 (v/v) as a reference sample. Using the precise formula, we calculated the concentration of ascorbic acid in the analysed solution.

Measurement of Nitric Oxide Concentration in Aqueous Humour and Serum The NO concentration in the samples was measured using the technique described by Green *et al*^[14]. We pipetted 0.1 mL 3 mol/L perchloric acid-PCA, 0.4 mL 20 mmol/L ethylenediaminetetraacetic-EDTA and 0.2 mL of analysed solution into the Eppendorf tube (aqueous humour-diluted 1/10 by distilled water). The samples were incubated on -4°C for 10min, centrifuged for 4min on 1500 rpm. Supernatant of the analysed solution was dropped out, and the precipitate was mixed in Green's reagent. Green's reagent was prepared directly prior to the measurements. Distilled water was used for control measurements. In the test tube we put 0.1 mL of extracted plasma, 250 μ L of Green's reagent and 125 μ L of ammoniac buffer (pH 9)- mixed solutions of ammonia (1 mol/L) and ammonium chloride (1 mol/L). After stabilisation of the mixture, concentration of NO was measured by spectrophotometric method on 550 nm; and was compared to NO standards.

Enzyme-linked Immunosorbent Assay Sera (undiluted) and aqueous humour (diluted 1/10 by distilled water) were collected by single needle stick from cubital vein and from anterior chamber of the eye from selected patients; and stored at -80°C until thawed for assay. Serum and humour levels of cytokines were measured in a sample with high sensitivity enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, Minn, USA) specific for human cytokines. Labelling procedure was conducted according to R&D Systems instructions manual. All measurements were performed in triplicate.

Statistical Analysis The one-way ANOVA or Kruskal-Wallis tests were performed using SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL, USA). The results were expressed as the mean \pm SE. All *P* values were 2-sided and a *P* value <0.05 was considered statistically significant

(significance levels as indicated in figure legends). Spearman's correlation coefficients were calculated to assess the relationships between TNF- α , and NO. Exact P values and the number (n) of patients are given in the results section and figure legends of the respective study.

RESULTS

Aqueous humour concentration of ascorbic acid is significantly lower in patients with late XFS and XFG compared to patients with early XFS and cataract, while there was no difference in serum concentration in all examined groups. Ascorbic acid concentration in aqueous humour and serum in the control group (cataract), as well as in patients with XFG and XFS, are presented in Figure 1. The concentrations of ascorbic acid in the aqueous humour from patients with developed XFG (0.48 ± 0.15 mmol/L) and late stage of XFS (0.61 ± 0.11 mmol/L) were significantly lower ($P < 0.05$; $P < 0.001$) compared to patients with early stage (0.9 ± 0.15 mmol/L) of XFS and cataract (1.16 ± 0.22 mmol/L). When we analysed serum concentrations of ascorbic acid in patients with XFG (0.79 ± 0.12 mmol/L), XFS-early (1.05 ± 0.19 mmol/L) and late stage (1.0 ± 0.18 mmol/L) and cataract (1.19 ± 0.15 mmol/L), there was statistically significant decreased concentrations of ascorbic acid in patients with XFG compared with other groups of patients (XFS-early and late stage, and control group), $P < 0.05$ (Figure 1).

Aqueous humour concentration of NO is significantly higher in patients with XFG compared to patients with early XFS and cataract, while serum concentration was increased in the early stage of XFS. NO concentration in aqueous humour and serum in the control group (cataract), as well as in patients with XFG and XFS (early and late stage), are shown in Figure 2. We found that the concentration of NO in the aqueous humour from patients with developed XFG (77.7 ± 11.4 μ mol/L) was significantly increased ($P < 0.05$) in comparison to early XFS (50.27 ± 9.34 μ mol/L) and cataract (49.77 ± 7.1 μ mol/L) (Figure 2). In our study we demonstrated that there was a significant difference ($P < 0.05$) in NO serum concentrations between patients with early XFS (73.26 ± 8.29 μ mol/L) and cataract (45.73 ± 6.98 μ mol/L) and XFG (51.54 ± 8.23 μ mol/L) (Figure 2).

Aqueous humour level of proinflammatory cytokine TNF- α is increased in patients with XFS (early/late) and XFG, while there was no difference in serum level in all examined groups of patients. To study the participation of secreted TNF- α in XFS/XFG, we examined the level of this proinflammatory cytokine in aqueous humour and serum in the patients with cataract, XFG and XFS (Figure 3). Measurements of TNF- α in the aqueous humour indicated significant elevated ($P < 0.001$) level of this proinflammatory cytokine in XFG (510.34 ± 43.07 pg/mL) and XFS (early 460.04 ± 18.32 pg/mL; late

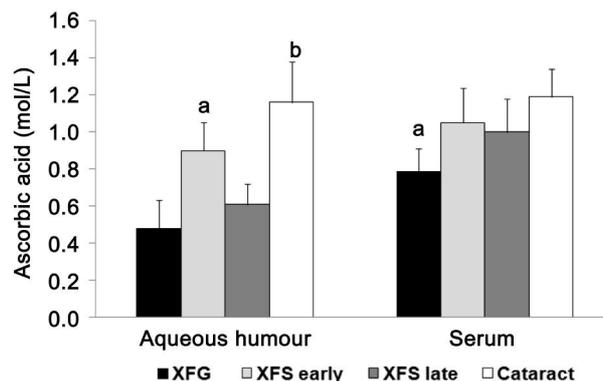


Figure 1 Aqueous humour and serum concentrations of ascorbic acid ($n=30$ patients per group) Measurements of concentrations of ascorbic acid in aqueous humour indicated significantly decreased ($P < 0.001$) level in patients with developed XFG and late XFS compared to patients of other two groups. There was no statistical significant difference in serum concentration of ascorbic acid in all examined groups. ^a $P < 0.05$, ^b $P < 0.001$.

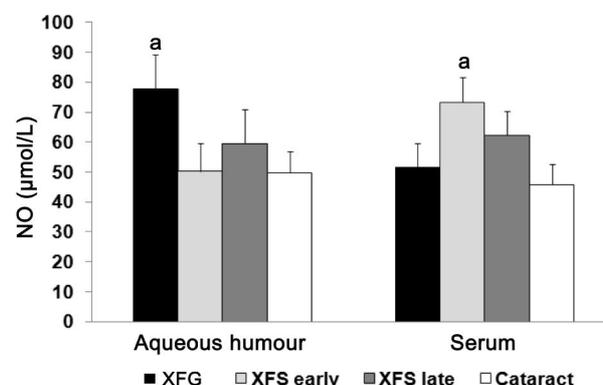


Figure 2 Aqueous humour and serum concentrations of NO ($n=30$ patients per group) NO concentration in aqueous humour was significantly increased ($P < 0.05$) in patients with developed XFG compared to patients with XFS-early and late stage and cataract. Serum concentration of the NO was significantly increased in early XFS when compared to other examined groups; ^a $P < 0.05$.

502.42 ± 53.23 pg/mL) in relation to the level in control (264.30 ± 16.4 pg/mL) group. Measurements of TNF- α in the serum of selected patients did not signify statistical differences ($P > 0.05$) between the selected groups. In XFG group the measured level was 41.5 ± 3.55 pg/mL, in early XFS group the level was 34.42 ± 2.98 pg/mL, in late XFS group it was 36.56 ± 3.08 pg/mL, and in the group with cataract 30.95 ± 2.6 pg/mL (Figure 3).

There was a strong positive correlation between aqueous humour levels of TNF- α and NO in XFS and XFG patients. We correlated all results, but we observed only a strong positive correlation between TNF- α and NO in XFS and XFG patients. The results of the relation between aqueous humour level of TNF- α in patients with XFS and the level of NO in patients with XFG (Figure 4) showed a strong positive correlation ($r = 0.7161$; $P < 0.001$). This result can be explained by the fact that TNF- α level was increased in all

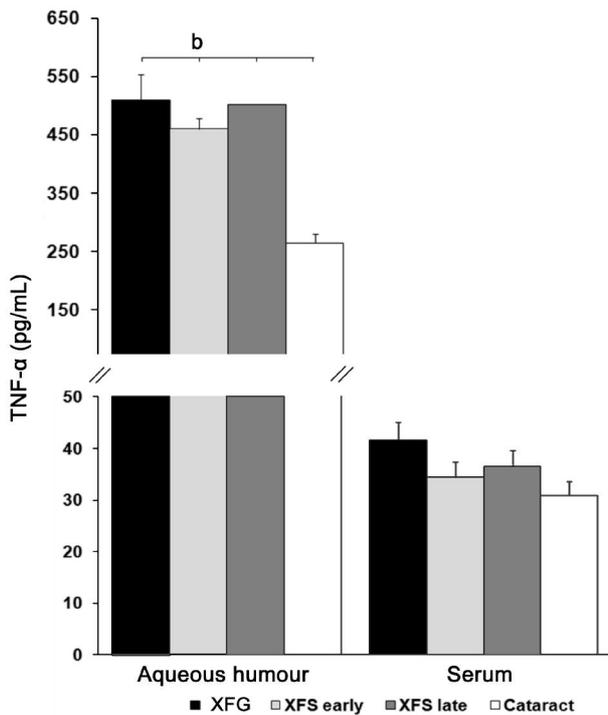


Figure 3 Aqueous humour and serum levels of the TNF- α ($n = 30$ patients per group) Aqueous humour level of proinflammatory cytokine TNF- α was significantly increased ($P < 0.001$) in patients with XFS (early/late) and XFG compared to patients with cataract. Measurements of TNF- α in the sera of all examined groups did not signify statistical differences. ^b $P < 0.001$.

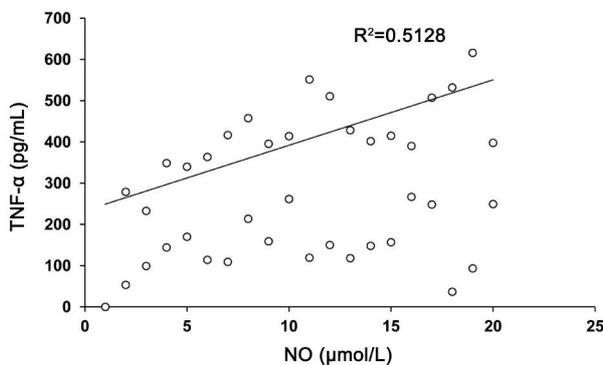


Figure 4 Correlation between the aqueous humour levels of TNF- α in patients with XFS and level of NO in patients with XFG signified a strong positive correlation ($r = 0.7161$; $P < 0.001$).

phases of XFG development, so it can maintain the inflammation in the tissue in the phase of XFG.

DISCUSSION

Our results indicated statistically significant higher concentration of ascorbic acid in the aqueous humour of patients with cataract in comparison with pseudoexfoliation groups (syndrome-late/early and glaucoma) patients. Koliakos *et al* [15] also established decreased concentrations of ascorbic acid in XFS, but they did not indicate for its concentrations in XFG. Using those results we have shown the protective role of ascorbic acid in the process of pseudoexfoliation production. Ascorbic acid is important in the proper function of the immune system [12].

Contrary to ascorbic acid, NO concentration was statistically significantly increased in aqueous humour in patients with developed XFG in comparison to patients with early XFS and cataract. These values indicated that in aqueous humour of patients with developed glaucoma ascorbic acid level was decreased with significantly higher level of NO. The endothelium of blood vessels uses NO to signal the surrounding smooth muscle to relax, thus resulting in vasodilatation and increasing blood flow in the tissue by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelium [16]. Phagocytes, as important cells in the immune response, can generate NO [17] by activating NOS using TNF- α as the second signal [18]. Our study indicated significantly higher level of this proinflammatory cytokine in aqueous humour of patients with pseudoexfoliation material, but not in the serum of patients with XFS/XFG. Also, we established a strong positive correlation between aqueous humour levels of TNF- α in XFS patients and NO levels in patients with XFG. All those results explained the fact that TNF- α is the second signal molecule in NO action, and that TNF- α can maintain the inflammation in the tissue in the phase of XFG. Moreover, this is the explanation for increased NO production only locally, in the eye microenvironment, as the final products of inflammatory reaction in the tissue [18].

Many different studies have assessed the systemic and local status of oxidative stress parameters of patients with XFG [5,19]. Oxidative status in the eye of patients with XFG is furthermore disturbed by increased intraocular pressure owing to accumulated pseudoexfoliation in the iridocorneal angle and decreased blood flow of the retinal blood vessel. Locally disrupted oxidative/antioxidative balance and releasing of its products initiated inflammatory reaction as the first step in pseudoexfoliation production in the tissue [20], with accumulation of different inflammatory cells (phagocytes, lymphocytes, *etc*) followed by TNF- α release [21]. Phagocytes in the inflamed tissue can produce NO to generate defense against disturbed homeostasis and to activate vasodilatation of local blood vessel, using TNF- α as a signaling molecule [9]. Dilatation of blood vessels can improve local oxidative status and can control inflammatory reaction [22].

Glaucoma is an ocular disease which is characterized by disturbed oxidative/antioxidative status activated by increased intraocular pressure and decreased blood flow in the retinal blood vessel and with the loss of retinal ganglion cells by apoptotic process [23-24]. Pressure-loaded glial cells produce TNF- α , as the second signal of NO action; and ascorbic acid is consumed in the struggle for ocular homeostasis [25]. The final result of TNF- α action can be the death of oligodendrocytes and retinal ganglion cells, as part of neurodegeneration [26-27].

The applicability of this manuscript is in the fact that disturbed oxidative parameters can provoke pseudoexfoliation production, so their improvement can stop this very complex process which can lead to a serious ocular disease with unknown outcome.

Patients with pseudoexfoliation can have disturbed oxidative status with decreased concentrations of antioxidants and increased levels of oxidants. Ascorbic acid concentrations decrease with the enhance of metabolism activities. There is a higher level of reactive oxygen species production in the eye with inflammation, while ascorbic acid concentrations are reduced as the part of it being used in the defense from inflammation. NO, as an endogenously generated gaseous molecule, has a protective role in the process of XFG development activating local blood vessel dilatation using TNF- α as a secondary signal molecule.

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