Evaluation of Antioxidant Enzymes in Keratoconus

ALINA CANTEMIR[1], ANISIA IULIANA ALEXA[1]*, ALIN CIOBICA[2,3], IOANA MIRUNA BALMUS[2], IULIA ANTIOCH[2], BOGDAN STOICA[1], DORIN CHISELITA[1], DANUT COSTIN[1]

[1]Grigore T. Popa University of Medicine and Pharmacy of Iasi, 16 Universitatii Str., 700115, Iasi, Romania

[2] Alexandru Ioan Cuza University of Iasi, Faculty of Biology, 11 Carol I Blvd., 700506, Iasi, Romania

[3]Center of Biomedical Research of the Romanian Academy, Iasi Branch, 8 Carol I Blvd, 700506, Iasi, Romania

Studies performed in keratoconus have generally suggested the presence of a compromised antioxidant system, but this is not always consistent with specific observed parameters, which on the whole showed clear evidences of dysregulation. The aim of the present report is to evaluate the serum specific activity of some peripheral antioxidant defenses like superoxide dismutase (SOD) and glutathione peroxidase (GPX) in keratoconus patients, when compared with age and sex-matched healthy subjects. We found a very significant decrease in both antioxidant enzymes (superoxide dismutase and glutathione peroxidase) in keratoconus patients, as compared to the controls. However, further research is necessary in order to elucidate the effects of this disorder on antioxidant enzymes or the possible interventions at the oxidative stress level in keratoconus patients.

Keywords: oxidative stress, keratoconus, superoxide dismutase, glutathione peroxidase

Keratoconus is a significant ophthalmological disorder, manifested most of the times as a protrusion and thinning of the cornea [1]. It is considered a very serious and progressive disorder, occurring during the teen years and advancing even until the 4th decade of life [2, 3].

However, what is more important in the present context is the fact that the molecular pathogenesis of keratoconus is not completely understood up to this date.

Keratoconus is considered to be a multifactorial disease with several different aspects contributing to its pathogenesis and progression, such as biochemical, genetic, environmental and biomechanical risk factors [3-5].

It also seems that in the manifestations of this very complex disease, inflammation and oxidative stress could play an important role [6-8].

Although there are very few studies in this area of research, the few authors working in this area reported various modifications for some oxidative stress markers in keratoconus patients, suggesting that oxidative stress might play an important role in the development and progression of this severe disorder [2, 3, 9].

The importance of oxidative stress is also suggested by the fact that the cornea absorbs approximately 80% of the incident ultraviolet B light, which results in an increased potential for generating significant amounts of free radicals or reactive oxygen species [2]. Even more than that, it was demonstrated that ultraviolet light, a light which is the main component of solar UV, could actually induce oxidatively-modified clustered DNA lesions [3, 10].

It is known that the potential toxicity of free radicals is counteracted by a number of cytoprotective enzymes and antioxidants from the cornea, which could limit the damage. Moreover, this protective mechanism functions cooperatively in a chain reaction which includes various antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPX) [11, 12].

Still, there are many controversies regarding the implications of the oxidative stress status in the keratoconus pathology, as demonstrated in the few studies involving this area of research. For example, a decreased specific activity for the extracellular superoxide dismutase (which catalyses the conversion of superoxide radicals to hydrogen peroxide, which is then converted into water by GPX and catalase) was reported several times in the literature [12-15] in keratoconus patients. On the other side, similarly designed studies by *Nature* group journals, such as the one conducted by Toprak et al. [6] showed that, when keratoconus and control groups are compared, serum total antioxidant status is not significantly modified; the same group found higher levels of serum oxidant status in patients with keratoconus [6].

In a recent study performed by the Kilic et al. [16], the authors did not observe any increase in any of the oxidative stress markers that they determined in keratoconus patients. Moreover, the same group failed to find any significant relationship between keratoconus and serum total antioxidant capacity and total oxidant status [16].

It seems that the implications of the oxidative stress status in keratoconus are still not completely understood. It is still not known if oxidative stress represents a consequence of the disorder or a possible cause for it [3]. In this context, there is a serious need for further studies to be conducted on the involvement of oxidative stress in the pathogenesis of keratoconus.

The aim of the present paper is to evaluate the specific activity of some serum enzymatic antioxidant defences (superoxide dismutase and glutathione peroxidase) in patients with keratoconus, as compared with a group of age-matched healthy subjects.

Experimental part

Methods

The subjects of this study (n = 40) consisted of 20 patients (15 males and 5 females; age 27.1± 1.45 years) with keratoconus, recruited from the Oftaprof Clinic of Iasi, Romania, and 20 healthy control age- and gender-matched subjects (15 males and 5 females; age 29.3 ± 1.09 years)

* *email: alexa_anisia@yahoo.com*

with normal ophthalmological examination and enrolled mainly from the hospital staff.

Demographic data of the controls were chosen in order to match with the patients with keratoconus. The analysis of covariance showed that patients with keratoconus did not differ significantly from healthy control subjects with respect to age, gender and smoking habits. The smoker percent was also considered in the selection of the controls, considering the relevance of nicotine in the oxidative stress status [17, 18]. Four out of the 20 keratoconus patients (20%) and 4 out of the 20 controls (20%) were smokers. In addition, none of the subjects studied were taking antioxidant or anti-inflammatory supplements. Also, subjects with other corneal pathology or surgery or acute comorbidities were excluded.

The study was conducted according to provisions of the Helsinki Declaration and was also approved by the local ethics committee.

All the patients signed the informed consent for the participation in this study. Blood samples were collected in the morning, before breakfast, and afterwards allowed to clot and centrifuged immediately. Serum was aliquoted into Eppendorf tubes and stored at -40°C until measurement.

Biochemical estimations

Determination of superoxide dismutase

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD Assay Kit (FLUKA, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide anions) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Determination of glutathione peroxidase

The activity of glutathione peroxidase (GPX) was measured using the GPX cellular activity assay kit CGP-1 (SIGMA). This kit uses an indirect method, based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is an indicator of GPX activity [17-21].

Data analysis

The levels of superoxide dismutase and glutathione peroxidase were statistically analyzed by using one-way analysis of variance (ANOVA). All results are expressed as mean ± SEM. F values for which $p<0.05$ were regarded as statistically significant.

Results and discussions

The first enzyme that we determined was superoxid dismutase, which is the first enzymatic antioxidant in the way of the free radical cascade and transforms superoxide radicals to hydrogen peroxide. We observed a very significant decrease ($F(1,38) = 53$, $p < 0.0001$) of its enzymatic activity in the serum of patients with keratocnus, as compared to the age and sex-matched control group (fig. 1), suggesting an increase in the oxidative stress status.

When we determined the enzymatic activity of the second enzyme from our study, glutathione peroxidase,

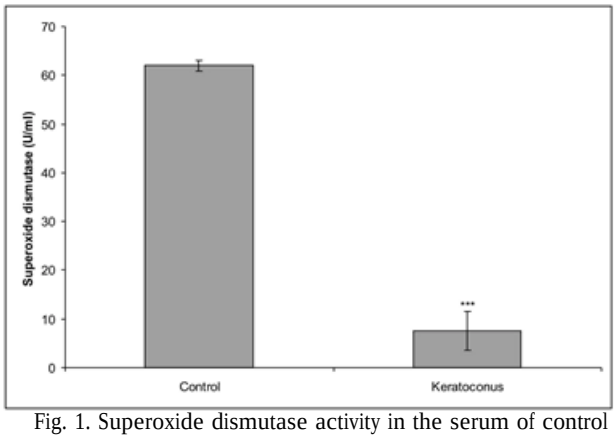

Fig. 1. Superoxide dismutase activity in the serum of control subjects and keratoconus patients. The values are mean±SEM. (n = 20 in control, and n=20 in keratoconus group)
***p < 0.0001

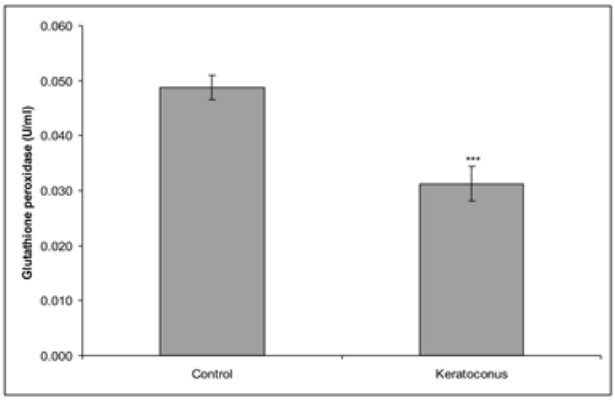

Fig. 2. Gluathione peroxidase activity in the serum of control subjects and keratoconus patients. The values are mean±SEM. (n = 20 in control, and n=20 in keratoconus group).
***p < 0.0001

which transforms hydrogen peroxide into water together with the catalase, we could additionally observe a very significant decrease ($F(1,38) = 20$, $p < 0.0001$) of its activity in the keratoconus group, as compared to the healthy controls (fig. 2), suggesting again an increased in the oxidative stress status in keratoconus.

Our results provide additional evidence for increased oxidative stress in keratoconus pathology, as expressed by altered antioxidant enzyme activity for both the antioxidant enzymes that we determined in our study (superoxide dismutase and glutathione peroxidase) in patients with keratoconus, as compared to a control group.

Three decades ago, keratoconus was considered to be a non-inflammatory disease [22] but recent studies clearly demonstrated that inflammation and oxidative stress might be quite important in the pathology of this disorder [23, 24].

One of the possible explanations for the implications of the oxidative stress in keratoconus is represented by the fact that the eye is in direct contact with various stressful external agents such as UV, ionizing radiations or air pollution, as we already mentioned before. This could also explain the increased relevance for the oxidative stress modifications in various other ophthalmological disorders such as macular degeneration, cataract, uveitis, keratoconus and Fuchs endothelial corneal dystrophy [3].

Mechanistically speaking, the depleted antioxidant systems fail to protect against the oxidative damage and, in a *vicious cycle*, the patients with keratoconus could have an inadequate antioxidant enzymatic activity that is incapable of responding to a very possible increase of free

radical production, which could lead to the development of the pathological alterations observed in this disease.

It is considered that, besides the antioxidants decrease, in keratoconus patients we could find elevated level of lipid peroxidation markers, such as malondialdehyde and 4-hydroxy-2-nonenal. These aldehydes are quite important, especially considering that lipid peroxidation processes are occurring in response to elevated levels of reactive oxygen species, while also generating serious damages in the membranes of lysosomes, which in turn release proteolytic enzymes [2].

In fact, MDA represents the most frequently used way to asses the thiobarbituric acid reactive substances and has been very widely employed for the indication of the oxidative stress status in clinical studies [25]. Another very important product of lipid oxidation is 4-HNE, which is a highly cytotoxic reactive α,β-aldehyde that is generated during various physiological and pathophysiological conditions based on the production of ROS [26].

The group of Arnal et al. [2], referring to the relations between keratoconus and oxidative stress status showed that HNE can modify proteins on cysteine, lysine, and histidine residues and such modifications can impair protein function and promote protein aggregation [2]. They stated that these aldehydes are highly reactive and can covalently interact with proteins and DNA to form adducts that alter signal transduction, gene expression and proliferation.

It also seems that the reactive nitrogen species, especially those containing oxygen, might contribute to the aforementioned stressful effects in cornea [3].

In fact is it known that corneal cells are capable of expressing isoforms of the nitric oxide synthase, especially since the nitric oxide is produced physiologically in the cornea [2].

This could be quite important, since our group have previously demonstrated that oxidative stress, together with nitrosative and carbonilic stress, may constitute a central process where other factors of vulnerability meet, while their interactions could have an important impact in many modern disorders [27, 28].

All these aspects could be also relevant on both the metabolic and the functional level. It was shown that physiological concentration of nitric oxide contributes to the neutralization of the superoxide and derived reactive species, while increased pathological concentrations are generating the alteration of cell membranes, structural proteins and active phospholipids, by also triggering the degradation of the nuclear DNA, mitochondria, and lysosomes [28]. Nitric oxide has multiple bimodal effects, depending on concentration, since in low concentrations it contributes to the regulation of local vasodilatation, for example, and has additional antiproliferative and antibacterial effects. Increased concentrations of nitric oxide become toxic mainly due to the activation of protein nitration and nitrosation that form nitrosothiols, which oxidize essential amino acids, such as tyrosine, cysteine, methionine and glutathione [29].

In their referential paper, the aforementioned group of Arnal et al. [2] demonstrated more or less similar effects at the cornea level, since very increased concentrations of nitric oxide synthase and nitrotyrosine were found in the cornea of keratoconus patients, as compared to healthy controls. The relevance of nitric oxide in the cornea is also increased considering its implications inflammation, angiogenesis or in preventing corneal edema and maintaining normal thickness, which is affected in keratoconus [2].

In this way, it seems that various elements of the oxidative stress status such as the activity of the main antioxidant enzymes, lipid peroxidation markers or related nitrosative stress are actually quite important for the corneal thinning in keratoconus [30]. From a genetic point of view, it was previously shown that there is actually a decrease in SOD 1 expression (the gene encoding the enzyme which catalyses the conversion of superoxide radicals to hydrogen peroxide) in keratoconus corneas [31]. However, there are also some controversial results in this genetic field, since other studies did not find any keratoconus-related mutations in the SOD1 gene [32].

It was recently demonstrated by the group of Abu-Amero et al. [33] that keratoconus is associated with an increased copy number of mitochondrial DNA, the authors also stating that this increased mitochondrial DNA may suggest some connections with mitochondrial respiratory chain defects and oxidative stress related-events.

Other groups recently searched for connections that might appear between some metabolic defects and modifications of the oxidative stress status in keratoconus. In this way, they looked for the alterations of some endogenous metabolites in the tears of keratoconus patients and their relations with oxidative stress, founding clear correlations between the metabolic alterations, the specific treatment and oxidative processes in keratoconus [34].

In addition, other authors demonstrated that the relevance and the implications of the oxidative stress status in the keratoconic pathology can be demonstrated also *in vitro*. In this way, the American group lead by Karamichos et al. [35] revealed in a recent study the increased oxidative stress status in cell cultures of human corneal keratocytes, fibroblasts, and keratoconus cells.

Conclusions

Our results provide additional evidences that oxidative stress damage occurs in patients with keratoconus. This was mainly demonstrated by observing a very significant decreased activity of the main antioxidant enzymes that we determined: superoxide dismutase and glutathione peroxidase. However, further research is necessary in order to better understand and elucidate the connections between the pathology of keratoconus and the oxidative stress status, as well as the possible use of antioxidant supplementation as a therapeutic strategy against this disorder.

References

1.RABINOWITZ, Y.S., Keratoconus. Surv. Ophthalmol., **42**, 1998, p. 301

2.ARNAL, E., PERIS-MARTINEZ, C., MENEZO, J.L., JOHNSEN-SORIANO, S., ROMERO, F.J., Invest Ophthalmol Vis Sci,. **52**, No. 12, 2011, p. 8594

3.WOJCIK, K.A., KAMINSKA, A., BLASIAK, J., SZAFILIK, J., SZAFILIK, J.P., Int. J. Mol. Sci., **14,** No. 9, 2013, p. 19301

4.ROMERO-JIMENEZ, M., SANTODOMINGO-RUBIDO, J., WOLFFSOHN, J.S., Cont. Lens. Anterior Eye, **33**, 2010, p. 160

5.BURDON, K.P., VINCENT, A.L., Clin. Exp. Optom., **96**, 2013, p. 150

6.TOPRAK, I., KUCUKATAY, V., YILDIRIM, C., KILIC-TOPRAK, E., KILIC-ERKEK, O., Eye, **28**, No. 3, 2014, p. 287

7.BOARIU, M., MARINESCU, A., KRALEV, A., CLONDA, C., NEGRUTIU, M.L., SINESCU, C., Rev. Chim. (Bucharest), **65,** no**.** 12, 2014, p. 1477.

8.MOLDOVAN, L., GASPAR, A., TOMA, L., CRACIUNESCU, O., SAVIUC, C., Rev. Chim. (Bucharest), **62,** no**.** 3, 2011, p. 299.

9.GREINERT, R., VOLKMER, B., HENNING, S., BREITBART, E.W., GREULICH, K.O., CARDOSO, M.C., RAPP, A., Nucleic Acids Res., **40**, 2012, p. 10266

10.CIOBICA, A., PADURARIU, M., DOBRIN, I., STEFANESCU, C., DOBRIN, R., Psychiatr. Danub., **23**, No. 3, 2011, p. 240
11.MOCANU, M., BADESCU, M., HANCIANU, M., BADULESCU, O.V., Rev. Chim. (Bucharest), **66**, no. 7, 2015, p. 997.
12.TULCAN, C., IGNA, V., AHMADI, M., ZARCULA, S., MOTOC, M., BORZA, C., Rev. Chim. (Bucharest), **64,** no**.** 2, 2013, p. 195.
13.BEHDING, A., KARLSSON, K., JOHANSSON, B.O., BRANNSTROM, T., MARKLUND, S.L., Invest. Ophthalmol., **42,** 2001, p. 2295
14.BROWN, D.J., LIN, B., CHWA, M., ATILANO, S.R., KIM, D.W., KENNEY, M.C., Mol. Vis., **10**, 2004, p. 285
15.OLOFSSON, E.M., MARKLUND, S.L., PEDROSA-DOMELLOF, F., BEHNDIG, A., Mol. Vis., **13**, 2007, p. 1287
16.KILIC, R., CUMURCU, T., SANCAKTAR, E., EVLIYAOGLU, O., SEZER, H., Curr. Eye Res., **19**, 2015, p. 3
17.HRITCU, L., CIOBICA, A., GORGAN, L., Cent. Eur. J. Biol., **4**, No. 3, 2009, p. 338
18.CIOBICA, A., PADURARIU, M., HRITCU, L., Psychiatr. Danub., **24**, No. 2, 2012, p. 196
19.STEFANESCU, C., CIOBICA, A., Affect. Disord., **143**, No. 1-3, 2012, p. 36
20.SCROBOTA, I., ALB, C., CALNICEANU, H., BACIUT, G., NEAGOE, I.B., ONISEI, D., POPOVICI, A.R., BUZATU, R., BOLFA, P., Rev. Chim. (Bucharest), **66,** no**.** 9, 2015, p. 1467.
21.CONSTANTIN, M.M., CORBU, C., IONITA, G., Rev. Chim. (Bucharest), **61,** no**.** 5, 2010, p. 495.
22.KRACHMER, J.H., FEDER, R.S., BELIN, M.W., Surv. Ophthalmol., **28,** 1984, p. 298
23.LEMA, I., DURAN, J.A., Ophthalmology, **112**, 2005, p. 656
24.GONCU, T., AKAL A., ADBELLI, F.M., ÇAKMAK, S., SEZEN, H., YLMAZ, Ö.F., Cornea, **34**, No. 9, 2015, p. 1020
25.PADURARIU, M., CIOBICA, A, DOBRIN, I., STEFANESCU, C., Neurosci. Lett., **479**, No. 3, 2010, p. 319
26.SCHAUR, R.J., Mol. Aspects Med., **24**, 2003, p. 150
27.BILD, W., CIOBICA, A., PADURARIU, M., BILD, V., Journal of Physiology and Biochemistry, **69**, No. 1, 2013, p. 150
28.ARCAN, O., CIOBICA, A., BILD, W., DOBRIN, R., PETRARIU, F.D., COJOCARU, D., Rev. Med. Chir., **116**, No. 3, 2012, p. 870
29.ATOCHIN, D.N., HUANG, P.L., Pflugers Arch., **460**, p. 969
30.WOJCIK, K.A., BLASIAK, J., KUROWS, A.K., SZAFILIK, J., SZAFILIK, J.P., Klin. Oczna., **115,** No. 4, p.313
31.UDAR, N., ATILANO, S.R., BROWN, D.J., Invest. Ophthalmol. Vis. Sci., **47**, 2006, p. 3347
32.STABUC-SILIH, M., STRAZISAR, M., RAVNIK-GLAVAC, M., HAWLINA, M., GLAVAC, D., Alp. Panonica Adriat., **19**, 2010, p. 7.
33.ABU-AMERO, K.K., KONDKAR, A.A., AZAD, T.A., SULTAN, T., KALANTAN, H., Al-MUAMMAR, A.M., Mol. Vis., **27**, No. 20, 2014, p. 1205.
34.KARAMICHOS, D., JESPER, H., KUTCHEON, A., ASARA, J. A., ZIESKE, J. D., Investigative Ophthalmology, **55**, 2014, p. 1001.
35.KARAMICHOS, D., HUTCHEON, A.E., RICH, C.B., TRINKAUS-RANDALL, V., ASARA, J.M., ZIESKE, J.D., Sci Rep., **4,** 2014, p. 4608.

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