

# The role of conjunctival epithelial cell xanthine oxidoreductase/xanthine oxidase in oxidative reactions on the ocular surface of dry eye patients with Sjögren's syndrome

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**Summary.** Previous papers examined lipid peroxidase levels and myeloperoxidase activity as products of oxidative and inflammatory reactions in the tear fluid of patients suffering from dry eye. The aim of the present paper was to investigate whether the enzymes xanthine oxidoreductase/xanthine oxidase known to generate reactive oxygen species contribute to oxidative reactions on the ocular surface. Xanthine oxidoreductase/xanthine oxidase were examined immunohistochemically as well as histochemically in conjunctival epithelial cells of patients suffering from dry eye. Patients with verified autoimmune dry eye (Sjögren's syndrome) participated in our study; normal eyes served as controls. Conjunctival epithelial cells were obtained by the method of impression cytology using Millicell membranes. The results revealed a pronounced expression, as well as activity of xanthine oxidoreductase/xanthine oxidase in the conjunctival epithelium of dry eye. It is suggested that reactive oxygen species which are generated by this enzymatic system, contribute to oxidative reactions on the eye surface of patients with ocular manifestations of autoimmune disease (Sjögren's syndrome).

**Key words:** Dry eye, Impression cytology, Oxidative reactions

## Introduction

The ocular surface, tear film, lacrimal glands, and eye lids act as a functional unit to preserve the quality of the refractive surface of the eye and to resist injury and protect the eye against environmental conditions (Stern et al., 1998; Rolando and Zierhut, 2001). Dry eye syndrome is a chronic condition in which some components of the precorneal tear film are dysfunctional, leaving the patient with painful symptoms of dryness (e.g. Johnson and Murphy, 2004). The factors leading to abnormalities of the tear film are complex and may involve autoimmune disease (i.e. Sjögren's syndrome), loss of hormonal support, and glandular inflammation (Beauregard et al., 2003). Augustin et al. (1995) described oxidative reactions in the tear film of patients suffering from dry eye. Lipid peroxide levels and myeloperoxidase activity, as parameters for oxidative tissue damage and inflammatory activity, were determined in the tear fluid. Both parameters were elevated in dry eye. These authors suggested that free radicals of polymorphonuclear leukocytes and inflammation may be involved in the pathogenesis or the self-propagation of the disease.

Xanthine oxidoreductase/xanthine oxidase have been identified as a critical source of reactive oxygen species (Kelley et al., 2006). Xanthine oxidoreductase exists in two functionally distinct forms, xanthine dehydrogenase and xanthine oxidase, existing intracellularly primarily as dehydrogenase. Under various (patho)physiological conditions, reversible oxidation of critical cysteine residues (535 and 992) or limited proteolysis converts xanthine dehydrogenase to xanthine oxidase (Parks et

al., 1999), which reduces molecular oxygen to superoxide and hydrogen peroxide. However, according to Harris and Massey (1997), conversion to xanthine oxidase is not an absolute requirement for reactive oxygen species generation because xanthine dehydrogenase was also found to generate free radicals.

Xanthine oxidase was found to be present in polymorphonuclear leukocytes (e.g. Tubaro et al., 1980; Čejková et al., 2001). In ocular tissues, xanthine oxidoreductase/xanthine oxidase were described as being involved in oxidative damage to the cornea related to prolonged contact lens wearing (Čejková et al., 1998) or irradiation of the cornea with UVB rays (Čejková et al., 2001). Cekic et al. (1999) described xanthine oxidase as a source of reactive oxygen species in the lenses of alloxan-induced diabetic rats. Its activity was increased, suggesting increased oxidative stress to the lens. Fox and van Kuijk (1998) detected xanthine oxidase in the retina of the normal human eye and hypothesized that this enzyme may be involved in retinal ischemia-reperfusion injury. Čejková et al. (2002) described the expression as well as the activity of xanthine oxidoreductase/xanthine oxidase in the corneal epithelium and endothelium of normal human eyes. The authors suggested that during various pathological states, reactive oxygen species generated by this enzymatic system might be involved in oxidative eye injury.

In contrast to these papers, until now, the possible role of xanthine oxidoreductase/xanthine oxidase in oxidative reactions on the ocular surface of patients suffering from dry eye syndrome has not been investigated. Therefore, in this study xanthine oxidoreductase and xanthine oxidase were detected histochemically as well as immunohistochemically in conjunctival epithelial cells from normal and diseased eyes (patients suffering from dry eye). Conjunctival epithelial cells on Millicell membranes were obtained using the method of impression cytology which represents a minimally invasive diagnostic tool for ocular surface pathology (e.g. McKelvie, 2003; Calonge et al., 2004).

Patients with verified autoimmune disease (Sjögren's syndrome) participated in our study. Recently, particular attention has been devoted to this disease because it threatens the vision. Ocular symptoms of this autoimmune disease include dry or red eyes, foreign-body sensation, pruritus, photophobia, pain, visual changes, and even complete loss of vision (e.g. Patel and Lundy, 2002).

## **Material and methods**

### *Human subjects*

Both eyes of nine healthy controls (mean age 43.2±4.8 years; one man, eight women) and both eyes of nine subjects with dry eye and verified Sjögren's syndrome (mean age 43.8±5.4 years; one man, eight women) were examined in this study. The study

followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

The clinical state of the ocular surface was evaluated, using the fluorescein tear break-up time, the degree of corneal staining with fluorescein, the vital dye staining, and tear production with the Schirmer test. Control healthy volunteers did not wear contact lenses, had no symptoms of ocular irritation, had normal tear production as confirmed by a Schirmer test, and had no signs of ocular surface disease. Dry eye (in the group of patients with Sjögren's syndrome examined in this paper) was diagnosed according to Copenhagen criteria, i.e. at least two of the following tests were abnormal: Schirmer test < 10mm/5min, tear film break-up time (BUT) < 10s, rose bengal staining > 4 (van Bijsterveld, 1969; Manthorpe et al., 1986). Results of ophthalmologic tests (the mean Schirmer - 1 value, the mean value of BUT and the mean staining value of the rose bengal) for both dry eye patients and healthy subjects examined in this study are summarized in Table 1.

According to the severity of the symptoms, the dry eyes of the patients examined in our study were classified as grade 2 (moderate dry eye). (See classification of Murube et al., 2005, for details).

### *Conjunctival impression cytology, sample collection*

Conjunctival epithelial cells were obtained using the method of impression cytology with Millicell membranes (Millicell-CM, hydrophilic PTFE, Millipore Corporation, Billerica, MA 01821, USA). A single drop of 0.4% oxybuprocaine hydrochloride was first instilled to the eye. Impression cytology was carried out bilaterally on the upper (at the 11-12 and 12-13 o'clock positions) conjunctiva, 2 mm posterior from the limbus. Strips of Millicell membrane were gently pressed by a glass rod for 5 seconds onto the conjunctival surface to remove superficial epithelial cells. The membrane was gently removed and the specimens (conjunctival cells on the Millicells) were stored at -80°C until they were processed (histochemical and immunohistochemical examination).

### *Histochemical examination*

The Millicell membranes with conjunctival epithelial cells were fixed for 1 min in 0.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, 4°C, released from plastic holder, washed with cacodylate buffer and placed cell side up on round 12 mm coverslips. Then the specimens (Millicell membranes with conjunctival epithelial cells) were rinsed with distilled water and processed by cerium-DAB-CO-H<sub>2</sub>O<sub>2</sub> methods to detect xanthine oxidase activity (substrate: hypoxanthine) as described by Gossrau et al. (1989; 1990) and Nakos and Gossrau (1994). Incubation was carried out in media consisting of 0.1M Tris-HCl buffer, pH 7.6, 0.1M NaN<sub>3</sub>, 5 mM CeCl<sub>3</sub>, and 5-10 mM

## *Xanthine oxidoreductase/xanthine oxidase, dry eye, conjunctival epithelium*

substrate. Incubation varied from 15 to 30 min at 37°C. After rinsing the specimens with several changes of distilled water, visualization of the reaction products was performed in a medium consisting of 0.05M acetate buffer, pH 5.5, 1.4 mM 3,3-diaminobenzidine, 0.1M NaN<sub>3</sub> and 40mM CoCl<sub>2</sub> at 37°C for 25 min. After rinsing in tap water, the specimens were mounted in diluted Aquatex (Merck, Darmstadt, Germany) and immediately examined using an Orthoplan Leitz light microscope equipped with a Leica DC 500 digital camera.

Control reactions for xanthine oxidase were performed by incubation in either cerium-or substrate-free media. The specificity of the enzyme reactions was checked by using media to which 100 mM allopurinol was added as an inhibitor of xanthine oxidase (Gossrau et al., 1989, 1990).

### *Immunohistochemical examination*

The Millicell membranes with conjunctival epithelial cells were fixed in acetone for 1 minute, released from plastic holder, rinsed with PBS (Phosphate buffered saline tablets, Sigma), placed cell side up on round 12 mm coverslips and then (after rinsing with PBS) permeabilised with 0.2% triton (Triton X 100, Sigma) in PBS. Antibodies used for the incubation of specimens (Millicell membranes with conjunctival epithelial cells) included rabbit antiovine xanthine oxidase (Chemicon, Temecula, CA, USA), rabbit antihuman xanthine oxidase (Biogenesis, Poole, UK) and monoclonal mouse antihuman xanthine oxidoreductase/aldehyde oxidase AB-2 (LabVision, Fremont, CA, USA). The binding of the primary antibodies was demonstrated by the UltraVision detection system (antimouse and antirabbit) (LabVision, Fremont, CA, USA), following the instructions of the manufacturer. The UltraVision Detection systems contain a biotinylated secondary antibody that reacts with the primary antibody, enzyme-labeled streptavidin, and substrate-chromogen. Finally, nuclei were counterstained with haematoxylin. Conjunctival cells on Millicells in which the antibodies were omitted served as controls. The specimens were mounted in diluted Aquatex (Merck) and immediately examined.

For histochemistry and also immunohistochemistry,

the percentage of positive cells was determined. Two to three hundred cells were examined for each specimen.

Chemicals : All unspecified chemicals were obtained from Aldrich (Steinheim, Germany), Boehringer (Mannheim, Germany), Sigma (Munich, Germany) or Serva (Heidelberg, Germany) and were all of analytical, high or the highest purity grade.

### *Image analysis*

Image analysis was carried out on stored pictures acquired using an Orthoplan Leitz light microscope equipped with a Leica DC 500 digital camera with Leica Image Manager (Version 4.0). Luminance of all pixels in region of interest (ROI) was measured on image analysis system (NeuroLucida, MBF Bioscience, Williston, VT, USA) and the optical density counted as OD (optical density) = 255 (maximal luminance value) - actual luminance of ROI. For xanthine oxidase localised histochemically each group for measurement contained 12 clusters of cells, 9-14 cells in one cluster. In the immunohistochemical detection of xanthine oxidase and xanthine oxidoreductase, the nuclei were stained with haematoxylin and image analysis was done only in cytoplasm; 25 cells from normal and dry eye groups were used in each staining. For statistical analysis the one-way ANOVA with Tukey-Kramer multiple comparison tests was calculated using GraphPad InStat software (GraphPad Software, San Diego, CA, USA).

## **Results**

Clinical findings - results of ophthalmologic tests (the mean Schirmer - 1 value, the mean value of BUT and the mean staining value of the rose bengal) for dry eye patients and healthy subjects are summarized in Table 1.

### *Immunohistochemical and histochemical examination*

Xanthine oxidoreductase is more expressed in the conjunctival epithelial cells of dry eyes (Fig. 1a) than in the conjunctival epithelial cells of the normal eye (Fig. 1b). Also, the immunohistochemical demonstration of xanthine oxidase revealed a more pronounced activity of this enzyme in the conjunctival epithelial cells of

**Table 1.** Results of ophthalmologic test for dry eye patients and healthy subjects.

Healthy subjects mean values		
Schirmer - 1	BUT	the rose bengal staining
12.48 +/- 2.0 mm	14.23 +/- 2.40 seconds	1.89 +/- 0.70
Patients (dry eye) mean values		
Schirmer - 1	BUT	the rose bengal staining
1.42 +/- 0.37 mm	5.43 +/- 1.2 seconds	6.58 +/- 0.72

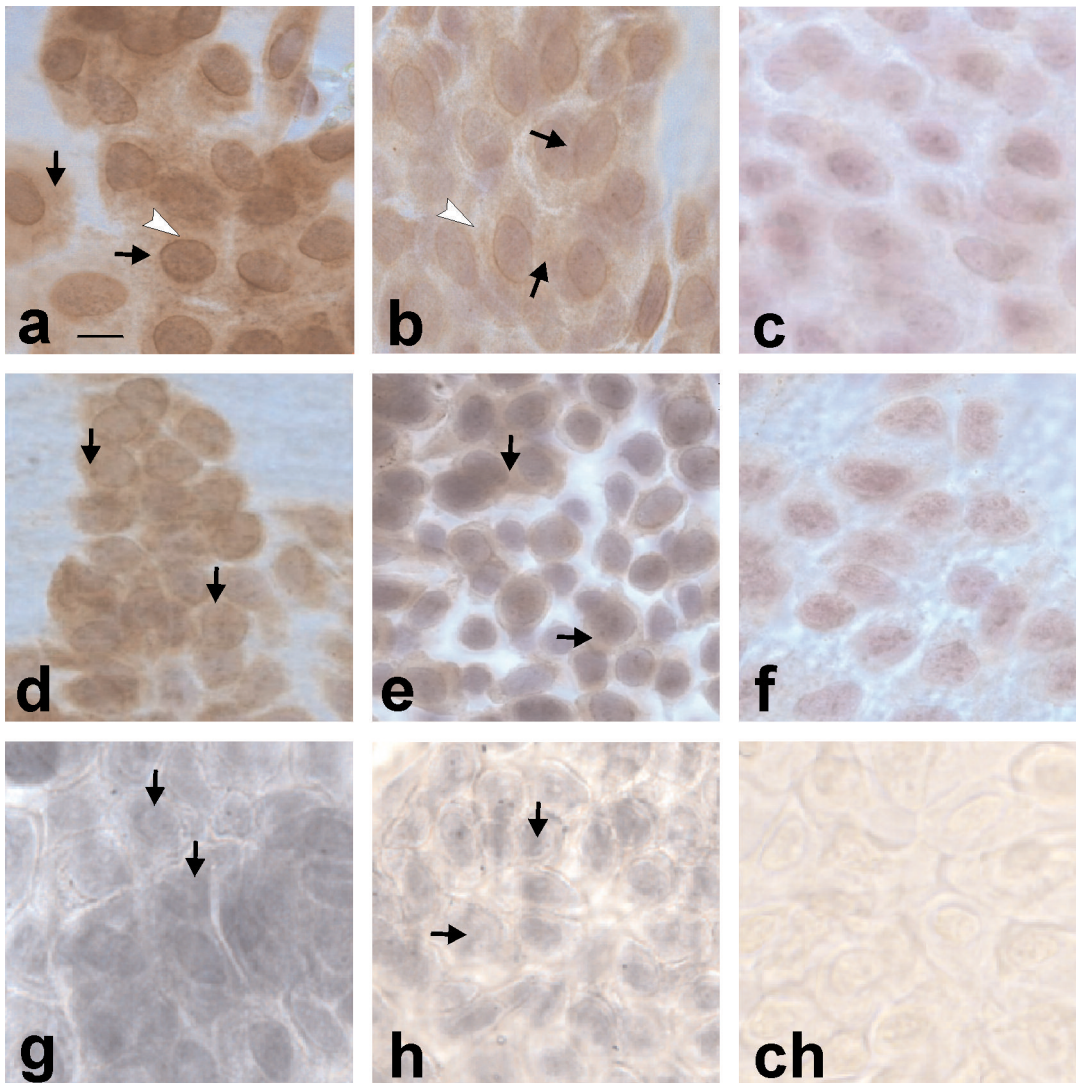
patients with dry eye (Fig. 1d) than in normal conjunctival epithelium (Fig. 1e). Very similar results were obtained from the histochemical demonstration of xanthine oxidase. In dry eyes (Fig. 1g) the activity of xanthine oxidase in conjunctival epithelial cells was more pronounced than in normal eyes (Fig. 1h). These results were consistent in all the dry eye patients examined as well as in all the eyes of the normal subjects. (For xanthine oxidoreductase and xanthine oxidase, dry eye, detected histochemically as well as immunohistochemically, the number of positive cells was 95% to 98%, in normal eyes 85% to 90%).

The results obtained by both antibodies employed for xanthine oxidase localization were similar. In Fig. 1d and Fig. 1e xanthine oxidase was detected with rabbit

antihuman xanthine oxidase antibody (Biogenesis).

In our figures xanthine oxidase and xanthine oxidoreductase show diffuse cytoplasmic labeling (black arrows); however, the immunohistochemical detection of xanthine oxidoreductase also shows perinuclear labeling (white arrows). The antibody used (monoclonal mouse antihuman xanthine oxidoreductase/aldehyde oxidase AB-2 antibody) to detect xanthine oxidoreductase recognizes aldehyde oxidase together with both forms of xanthine oxidoreductase. Previously, we employed the AB-2 antibody to detect human corneal xanthine oxidoreductase and discussed that the perinuclear labeling was very probably caused by the participation of aldehyde oxidase (Čejková et al., 2002).

Control specimens for histochemistry treated in



**Fig. 1.** Xanthine oxidoreductase and xanthine oxidase in conjunctival epithelial cells of a normal human eye and a dry eye (Sjögren's syndrome). Xanthine oxidoreductase and xanthine oxidase detected immunohistochemically are more expressed in dry eye (xanthine oxidoreductase – a. xanthine oxidase – d) than in the normal eye (xanthine oxidoreductase – b. xanthine oxidase – e). Also, xanthine oxidase localised histochemically is more pronounced in dry eye (g) as compared to the normal eye (h). Controls for the immunohistochemistry of xanthine oxidoreductase (c) and xanthine oxidase (f) in which the primary antibodies were omitted in the incubation media. Only the nuclei are stained with haematoxylin. ch. control for xanthine oxidase activity (conjunctival cells on Millicells were treated in incubation media without substrate). No staining appears. Remark: In our figures black arrows show cytoplasmic localization of xanthine oxidase and xanthine oxidoreductase. In the immunohistochemical detection of xanthine oxidoreductase (Fig. 1a and Fig. 1b) white arrows show perinuclear

labeling. This labeling is caused very probably by aldehyde oxidase; the antibody employed to detect xanthine oxidoreductase recognizes both forms of xanthine oxidoreductase together with aldehyde oxidase. Scale bar: 10  $\mu$ m

*Xanthine oxidoreductase/xanthine oxidase, dry eye, conjunctival epithelium*

incubation media without substrate (Fig. 1ch) or in the incubation media with substrates to which allopurinol was added, did not show staining. Also, control samples for immunohistochemistry (primary antibodies omitted) did not show staining (Fig. 1c - control for xanthine oxidoreductase, Fig. 1f - control for xanthine oxidase); (In Fig. 1c and Fig. 1f only nuclei are stained with haematoxylin).

#### Image analysis

The increase in xanthine oxidoreductase/xanthine oxidase expression and activity in conjunctival epithelium of dry eyes (SS) as compared to the normal eyes is shown in Fig. 2 and Fig. 3.

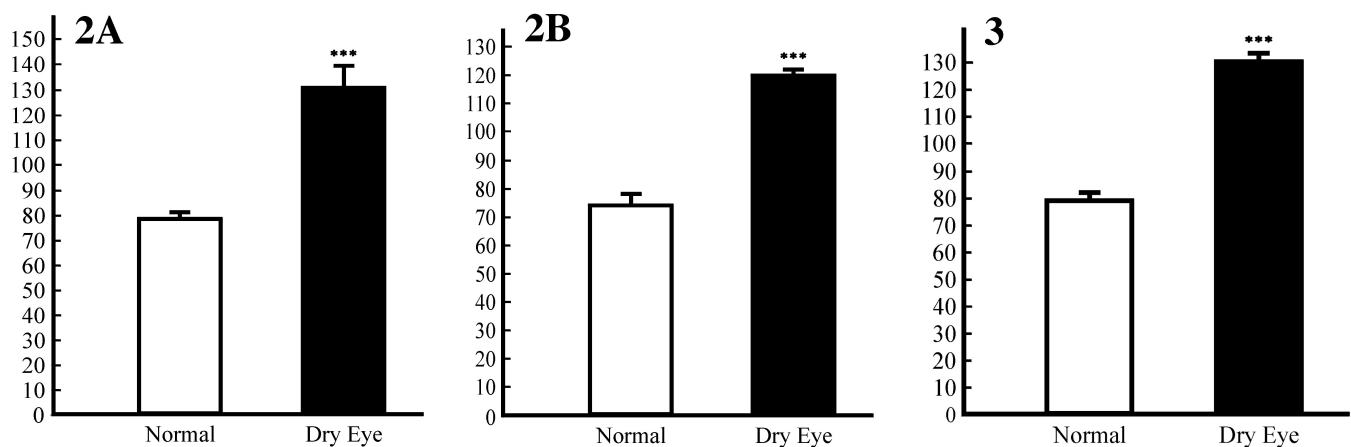
#### Discussion

This study demonstrates for the first time the expression and activity of xanthine oxidoreductase/xanthine oxidase in conjunctival epithelial cells of normal human eye and dry eye (Sjögren's syndrome). Results show that the enzymes are more pronounced in dry eye as compared to the normal eye.

Xanthine oxidoreductase/xanthine oxidase is an important component of an integrated inflammatory response in organ dysfunction (Harrison, 2002). Xanthine oxidoreductase exists in two interconvertible forms, xanthine dehydrogenase and xanthine oxidase. These enzymes are members of the molybdenum hydrolase flavoprotein family and represent different

forms of the same gene product. During the past ten years, evidence has mounted to support a role for xanthine oxidoreductase in the pathophysiology of inflammatory diseases as well as its previously determined role in ischemia-reperfusion injury (reviewed by Pritsos, 2000).

It has been described in various organs and tissues that xanthine oxidoreductase present in epithelial as well as endothelial cells can be induced by inflammatory products (pro-inflammatory cytokines, proteases) (Page et al., 1998; Komaki et al., 2005), hypoxia (Cote et al., 1996; Kelley et al., 2006), mechanical stress (Abdulnour et al., 2006) or traumatic injury (Solaroglu et al., 2005). All these circumstances may be involved in dry eye disease. The lacrimal glands of patients with autoimmune dry eye (Sjögren's syndrome) produce increased levels of pro-inflammatory cytokines, such as interleukin-1 beta, interferon  $\gamma$ , and tumor necrosis factor- $\alpha$ , which are secreted into the tear fluid (Robinson et al., 1998; Rosenbaum et al., 1998; Pflugfelder et al., 1999). Luo et al (2004) described that experimental dry eye stimulates the expression of pro-inflammatory cytokines and also the expression of matrix metalloproteinase-9 and activates mitogen-activated protein kinase signaling pathway on the ocular surface. According to Pflugfelder et al. (1999) the severity of dry eye disease (Sjögren's syndrome) increases as the level of epidermal growth factor in the tear fluid decreases and levels of pro-inflammatory cytokines in the conjunctival epithelium increase. Pflugfelder et al. (2004) pointed out the role of pro-inflammatory



**Fig. 2.** Quantitative estimation of xanthine oxidase histochemical staining (A) and xanthine oxidase immunohistochemical staining (B) in conjunctival epithelium of normal eye and dry eye (SS) using NeuroLucida image analysis. Optical density was measured as a luminance values and counted as described in Methods. Differences between normal eye vs. dry eye are highly significant (\*\*\*:  $P < 0.001$ ). For statistical analysis the one-way ANOVA with Tukey-Kramer multiple comparison tests was calculated using GraphPad InStat software.

**Fig. 3.** Image analysis of immunohistochemical staining of xanthine oxidoreductase in conjunctival epithelium of normal eye and dry eye (SS). The optical density was measured as a luminance values and counted as described in Methods. For statistical analysis the one-way ANOVA with Tukey-Kramer multiple comparison tests was calculated using GraphPad InStat software. The expression of xanthine oxidoreductase in dry eye is much more pronounced as compared to the normal eye. The difference between normal and dry eye is highly significant (\*\*\*:  $P < 0.001$ ).

cytokines and proteases in the tear fluid of dry eye in the development of inflammation and the importance of anti-inflammatory therapy for treating dry eye disease. According to Solomon et al. (2001), conjunctival cells appear to be one source of the increased concentration of interleukin-1 beta in the tear fluid. The authors suggested that an increased concentration of matrix metalloproteinase-9 (a physiological activator of interleukin-1 beta) on the ocular surface may be one mechanism by which precursor interleukin-1 beta is cleaved to the mature, biologically active form.

Abdulnour et al. (2006) found that mechanical stress activates xanthine oxidoreductase through the mitogen-activated protein kinase-dependent pathway. In dry eye, decreased tear secretion, decreased tear turnover, and desiccation result in mechanical irritation and promote inflammation on the ocular surface. Increased xanthine oxidoreductase levels may contribute to the development of inflammatory processes. Injuries to the ocular surface of dry eye produced by mechanical irritation evoke hypoxic conditions in the cells (cellular hypoxia) because damaged cells cannot utilize oxygen normally; the consumption of oxygen is impaired (Scheufler, 2004). According to Kelley et al. (2006), moderate hypoxia also increases both xanthine oxidoreductase immunoreactive protein levels as well as xanthine oxidoreductase activity. Hypoxia increases the generation of xanthine oxidoreductase-dependent reactive oxygen species and the cellular export of the active enzyme. Cote et al. (1996) found in the lung tissue that exposure to hypoxia produced a significant increase in xanthine oxidase activity and an increase in the ratio of xanthine oxidase to xanthine dehydrogenase.

In conclusion, our results show that the enzymes xanthine oxidoreductase/xanthine oxidase, examined both as proteins and as activities, are much more pronounced in the conjunctival epithelium of dry eye as compared to the normal eye. Factors leading to the induction of this enzymatic system in dry eye may be complex, involving inflammatory mediators, mechanical stress and hypoxia. Xanthine oxidoreductase/xanthine oxidase, when present in greater amount in the conjunctival epithelium, may be released into the tear fluid and contribute to oxidative reactions on the ocular surface due to reactive oxygen products generated by these enzymes.

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*Acknowledgements.* This study was supported by a grant from the Ministry of Health of the Czech Republic No. NR/8828-3 and by a grant from the Grant Agency of the Czech Republic No. 304/06/1379. The study was performed as part of a research project of the Institute of Experimental Medicine No. AV0Z50390512 and as a part of a research project of the Czech Ministry of Education, Youth and Sports, No. 0021620806/20610011.

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Accepted March 9, 2007