

Decreased expression of antioxidant enzymes in the conjunctival epithelium of dry eye (Sjögren's syndrome) and its possible contribution to the development of ocular surface oxidative injuries

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Summary. Previous studies have described elevated lipid peroxidase, myeloperoxidase and xanthine oxidoreductase/xanthine oxidase levels on the ocular surface of patients suffering from autoimmune dry eye (Sjögren's syndrome, SS). Reactive oxygen species generated by various enzymatic systems may be dangerous to the eye if they are not sufficiently cleaved by antioxidants. Because antioxidants have not been investigated in dry eye, the aim of this study was to examine the expression of antioxidant enzymes that cleave reactive oxygen species and play a key role in antioxidant protection. Conjunctival epithelial cells of dry eye (SS) patients were obtained by the method of impression cytology using Millicell membranes. Normal eyes served as controls. In the conjunctival epithelium superoxide dismutase, catalase and glutathione peroxidase were examined immunohistochemically. The enzyme expression levels were determined by image analysis and statistical evaluation. In contrast to normal eyes, where antioxidant enzymes were highly expressed in the conjunctival epithelium, in dry eye their expression was much less pronounced in correlation with the increasing severity of dry eye symptoms. Our study suggests that the decreased expression of antioxidant enzymes in dry eye disease (SS) contributes to the development of anterior eye surface oxidative injuries.

Key words: Superoxide dismutase, Catalase, glutathione peroxidase, Conjunctival epithelium

Introduction

Dry eye syndrome is a chronic condition in which some components of the precorneal tear film are dysfunctional. The factors leading to the tear film abnormalities are complex and may involve autoimmune disease (i.e. Sjögren's syndrome, SS), loss of hormonal support, and glandular inflammation (Stern et al., 1988; Beauregard et al., 2003). Sjögren's syndrome is a chronic autoimmune disorder of the exocrine glands with associated lymphocytic infiltration of the affected glands. The exocrinopathy can be encountered alone (primary Sjögren's syndrome) or in association with other autoimmune disorders, the three most common ones being rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis (secondary Sjögren's syndrome) (Srinivasan and Slomovic, 2007).

In the tear film of patients suffering from dry eye (SS) elevated lipid peroxide levels and myeloperoxidase activity were found in the tear fluid (Augustin et al., 1995). Lipid peroxide levels and myeloperoxidase activity were taken as parameters for oxidative tissue damage and inflammatory activity. Free radicals of polymorphonuclear leukocytes have been suggested to be involved in oxidative reactions. Čejková et al. (2007a) described another source of reactive oxygen species in dry eye disease (SS). These authors found the pronounced expression, as well as activity of reactive oxygen species generating xanthine oxidoreductase/xanthine oxidase in diseased conjunctival epithelium. It was proposed that xanthine oxidoreductase/xanthine oxidase, when present in a large amount in the conjunctival epithelium, may be released in the tear fluid and contribute to oxidative stress of the ocular surface due to reactive oxygen species generated by them.

Moreover, it was found that conjunctival epithelial cells of eyes with SS revealed an increased expression of nitric oxide synthases (Čejková et al., 2007b). Both enzymatic systems were induced in eyes with SS by pro-inflammatory cytokines (such as mature interleukin-1 beta, interleukin 6, interleukin 8, interferon gamma and tumor necrosis factor alpha) released in the tear fluid from diseased lacrimal glands (Robinson et al., 1998; Rosenbaum et al., 1998) and conjunctival epithelium (Pflugfelder et al., 1999; Solomon et al., 2001; Luo et al., 2004; Čejková et al., 2007b). The increased expression of nitric oxide synthases generated nitric oxide, together with the increased expression and activity of reactive oxygen species generating xanthine oxidoreductase/xanthine oxidase, is dangerous to the eye due to the formation of toxic peroxynitrite (which was found in SS conjunctival epithelial cells by the staining of nitrotyrosine residues, Čejková et al., 2007b). Peroxynitrite is a potent oxidising, nitrating and hydroxylating agent, resulting from the reaction of nitric oxide with superoxide. Wu et al. (1997) described that peroxynitrite caused oxidative damage of the retina, due to lipid peroxidation of photoreceptors in experimental autoimmune uveitis. Lipid peroxidation is an important biological consequence of oxidative damage to cell membranes and the formation of cytotoxic aldehydes. Increased levels of malondialdehyde, the toxic aldehyde byproduct of lipid peroxidation, were found in the conjunctival epithelium of dry eye (SS) (Čejková et al., 2007b).

Under normal conditions, in various tissues and fluids, antioxidants are balanced with the formation of reactive oxygen species at a level at which these compounds can play their physiological role without any toxic effects (Halliwell, 1991). Also, in the normal eye there exists a prooxidant/antioxidant balance at the ocular surface. The danger to the eye appears when this balance is disturbed. This may occur due to elevated levels of reactive oxygen species and/or a decrease in antioxidants. The above-mentioned papers described increased amounts of toxic oxygen and nitrogen products in dry eye (SS). In contrast to the extensive findings dealing with prooxidants in dry eye, the antioxidants have not been examined in the literature to date. Therefore, we decided to study this problem. In the conjunctival epithelium of dry eye (SS) (and normal eye as a control), obtained by the method of impression cytology, antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) that cleave reactive oxygen species were detected immunohistochemically. The expression of these antioxidant enzymes was measured by means of image analysis.

Material and methods

Human subjects

Both eyes of nine healthy controls (mean age 46.2±3.1 years; two men, seven women) and both eyes

of nine subjects with dry eye and verified Sjögren's syndrome (mean age 41.5±5.8 years; two men, seven women) were examined in this study.

The ocular surface of all subjects was evaluated using the fluorescein tear break-up time, the degree of corneal staining with fluorescein, vital dye staining, and tear production with the Schirmer test. 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 the 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). In this study the following mean values were found for healthy subjects (controls) and for patients with dry eye (SS): In healthy eyes the mean Schirmer-1 value was 13.40±1.20 mm, the mean value of BUT was 12.40±2.30 seconds, and the mean staining value of rose bengal was 1.80±0.50. In dry eyes (SS) the mean Schirmer-1 value was 34.20±0.30 mm, the mean value of BUT was 5.30±1.20 seconds, and the mean staining value of rose bengal was 6.50±0.30.

According to the severity of the symptoms, the dry eyes of the patients with SS examined in this study were classified as grade 2 (moderate dry eye; two men, five women) and as grade 3 (severe dry eye; two women). (See classification of Murube et al., 2005). The group of patients with moderate dry eye had less pronounced symptoms of dryness, while the two women with severe dry eye had highly pronounced symptoms of dryness.

Conjunctival impression cytology, sample collection

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

Conjunctival epithelial cells were obtained using the method of impression cytology with Millicell membranes (Millicell-CM, hydrophilic PTFE, Millipore Corporation, Billerica, MA 01821, USA). First, 0.4% oxybuprocaine hydrochloride (single drop) was instilled in 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. To remove superficial epithelial cells, strips of Millicell membrane were gently pressed for 5 seconds onto the conjunctival surface. After the removal of the membrane the specimens (conjunctival cells on the Millicells) were stored at -80°C until they were employed for immunohistochemical examination.

Immunohistochemistry

The Millicell membranes with conjunctival epithelial cells were fixed in acetone for 1 minute, released from the plastic holder, rinsed with PBS

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(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 x100, Sigma) in PBS. Antibodies used included rabbit antiovine catalase, rabbit antirat superoxide dismutase 1 and sheep antihuman glutathione peroxidase (Abcam, Cambridge, UK).

Subsequently, for catalase and superoxide dismutase detection, an anti-rabbit HRP/DAB Ultravision Detection System (Lab Vision, Fremont, CA, USA) was employed as recommended by the manufacturer: hydrogen peroxide block (10 min), ultra V block (5 min), primary antibody incubation (60 min), biotinylated goat anti-rabbit/mouse antibody incubation (10 min), and streptavidin peroxidase incubation (10 min). Visualization was performed using a freshly prepared DAB substrate/chromogen solution.

For glutathione peroxidase detection Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was subsequently employed. Staining procedure was equivalent to Vectastain Elite ABC protocol: quenching of endogenous peroxidase activity (0.3% H_2O_2 , 20 min), normal blocking serum incubation (20 min), primary antibody incubation (60 min), biotinylated secondary

antibody incubation (30 min), Vectastain Elite ABC reagent incubation (30 min), DAB incubation (2 min).

Negative controls included the omission of the primary antibody. Some samples were counterstained with Mayers' hematoxylin (Sigma). After staining procedure, the samples were immediately examined using an Orthoplan Leitz light microscope equipped with a Leica DC 500 digital camera.

Image analysis

Image analysis was performed using stored images of the immunohistochemical staining for superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) in the conjunctival epithelium of normal and dry eyes (SS, grade 2, patients with less pronounced symptoms of dryness). Images were acquired using an Orthoplan Leitz light microscope equipped with a Leica DC 500 digital camera with Leica Image Manager (Version 4.0). Specimens without the primary antibodies were used as a control for the specificity of each staining and were termed Blank. The luminance of all pixels in the region of interest (ROI) was measured using Neurolucida image analysis software (MBF Bioscience,

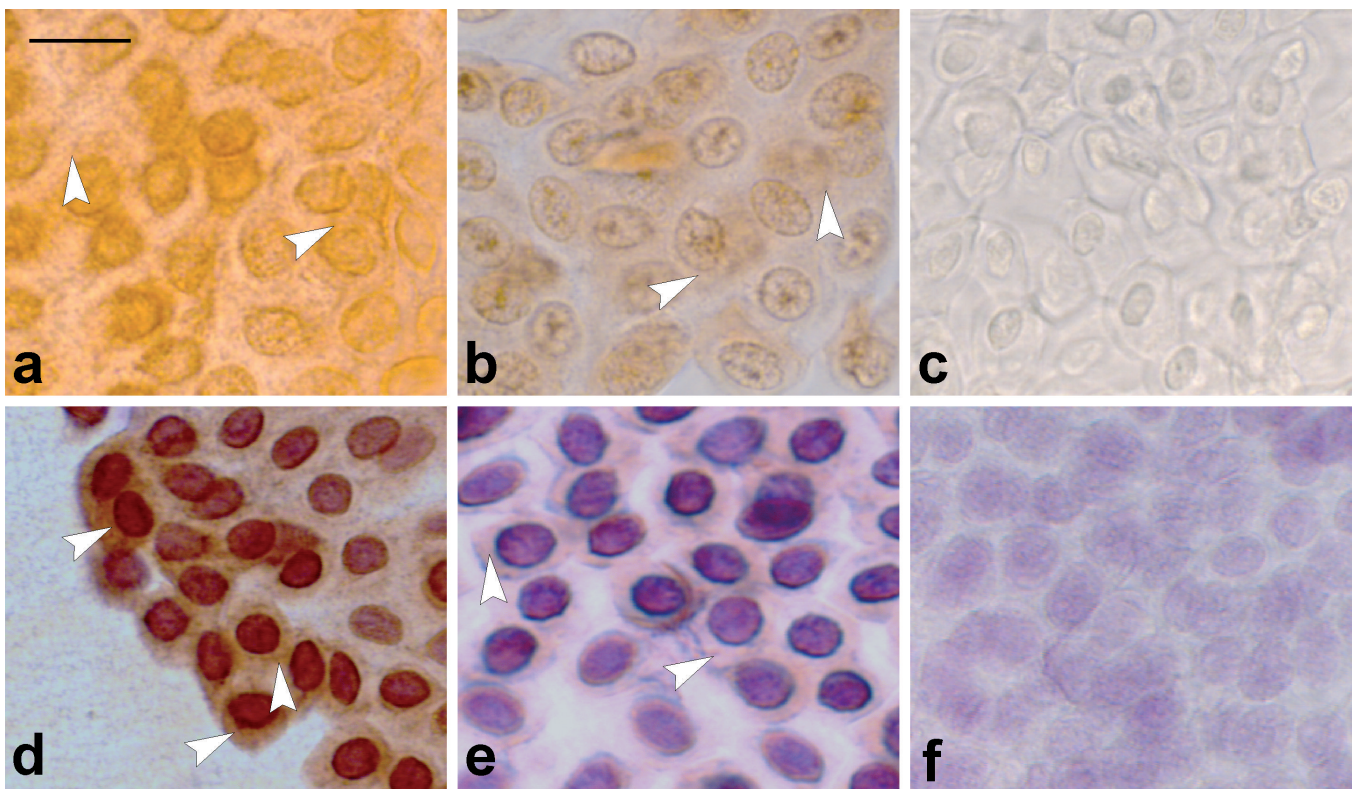


Fig. 1. Immunohistochemical staining of catalase (CAT) in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with less pronounced symptoms of dryness). CAT staining is more expressed in the normal eye (**a** - without counterstaining, **d** - Specimens counterstained with haematoxylin), than in dry eye (grade 2) (**b** - without counterstaining, **e** - nuclei counterstained with haematoxylin). Controls (primary antibody omitted): **c** - without counterstaining; no staining is seen; **f** - specimen counterstained with haematoxylin; only nuclei are stained. Scale bar : 10 μ m

Williston, USA), and the optical density was determined as OD (optical density) = 255 (maximal luminance value) - actual luminance of the ROI. In OD measurements, each experimental group contained 12 groups of cells, 2-3 cells in each group.

Statistical analysis

For statistical analysis a one-way ANOVA with Tukey-Kramer multiple comparison tests was employed using GraphPad InStat software (GraphPad Software, San Diego, CA, USA).

Results

Immunohistochemistry of conjunctival cells

Catalase (CAT) was much more expressed in the normal eye (Fig. 1a,d) than in dry eye, grade 2, patients with less pronounced symptoms of dryness (Fig. 1b,e). Also, glutathione peroxidase (GPX) was more expressed in the normal eye (Fig. 2a,d) than in dry eye (grade 2) (Fig. 2b,e). In contrast, the expression of superoxide

dismutase (SOD) was very similar in the normal eye (Fig. 3a,e) and in dry eye (grade 2) (Fig. 3b,f). In patients with highly pronounced symptoms of dryness (grade 3), the expression of SOD in dry eye is reduced (Fig. 3c,g).

Controls (the primary antibodies were omitted in the incubation media) for the immunohistochemistry of CAT are shown in Fig. 1c and Fig. 1f, controls for GPX in Fig. 2c and Fig. 2f and controls for SOD are shown in Fig. 3d and Fig. 3h. In counterstained specimens only nuclei are stained. In other specimens no staining appears.

Image analysis

The results of optical density measurements (dry eyes with less pronounced symptoms of dryness, grade 2) are shown in Figs. 4-6. No significant differences were found between superoxide dismutase (SOD) expression in the conjunctival epithelium of normal and dry eyes (Fig. 4). In contrast, the expression of glutathione peroxidase (GPX) and catalase (CAT) was significantly decreased in dry eye as compared with the

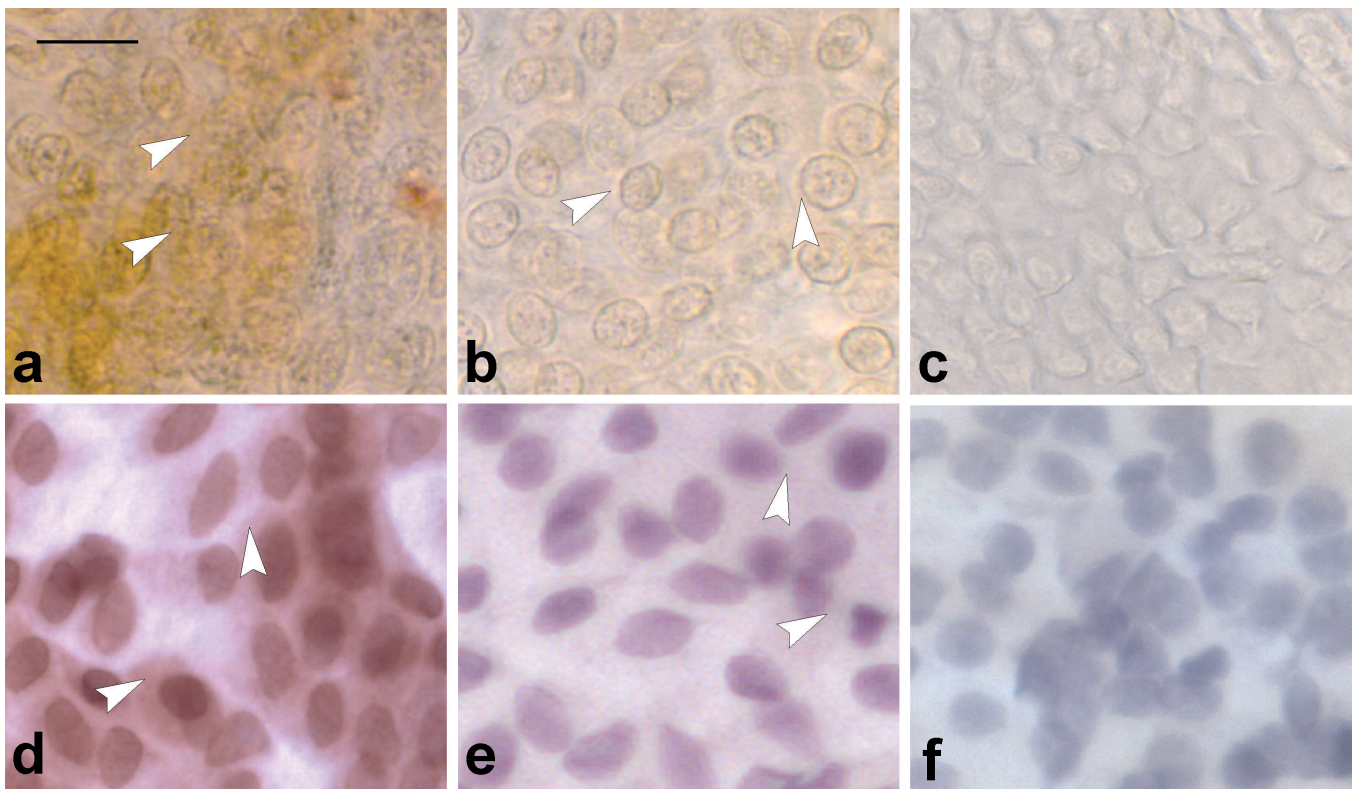


Fig. 2. Immunohistochemical staining of glutathione peroxidase (GPX) in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with less pronounced symptoms of dryness). The expression of GPX in the normal eye (**a** - without counterstaining, **d** - with counterstaining) is more pronounced than in dry eyes (grade 2) (**b** - without counterstaining, **e** - with counterstaining). Controls (primary antibody omitted): **c** - Specimen without counterstaining; no staining appears; **f** - specimen counterstained with haematoxylin; only nuclei are stained. Scale bar : 10 μ m

normal eye (Figs. 5, 6).

Discussion

This study examines for the first time antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) in dry eye disease. The results of immunohistochemistry, as well as image analysis, show that the expression of these enzymes is less pronounced in the conjunctival epithelium of dry eye (SS) as compared to the conjunctival epithelium of normal eyes. The decrease in enzyme expression was dependent on the severity of the dry eye symptoms. In grade 2 (patients with less pronounced symptoms of dryness) catalase and glutathione peroxidase were significantly decreased, while superoxide dismutase expression was very similar to the normal eye. However, in the case of grade 3 (patients with highly pronounced symptoms of dryness) the expression of all antioxidant enzymes studied (including superoxide dismutase) was reduced. In this connection it must be pointed out that we had few patients with grade 3. Therefore, conjunctival cells from

these eyes were evaluated only immunohistochemically (not with image analysis).

The eye contains low molecular weight antioxidants (such as ascorbic acid, glutathione and alpha-tocopherol), as well as high molecular weight antioxidant enzymes (such as catalase, superoxide dismutase and glutathione peroxidase), which play a key role in protecting the eye against oxidative damage. Superoxide dismutase catalyses the dismutation of superoxide to hydrogen peroxide and molecular oxygen. Thus, this enzyme protects the ocular tissues against superoxide radicals. Glutathione peroxidase, together with catalase, are very important scavengers of hydrogen peroxide. In the normal eye there exists a balance between prooxidants and antioxidants. The danger to the eye appears under various conditions and diseases, such as dry eye, when the antioxidant/prooxidant balance is disturbed. Previous papers suggested the involvement of prooxidants in the development of this imbalance. An increased amount of reactive oxygen species generated by polymorphonuclear leukocytes (Augustin et al., 1995) and by xanthine oxidoreductase/xanthine oxidase

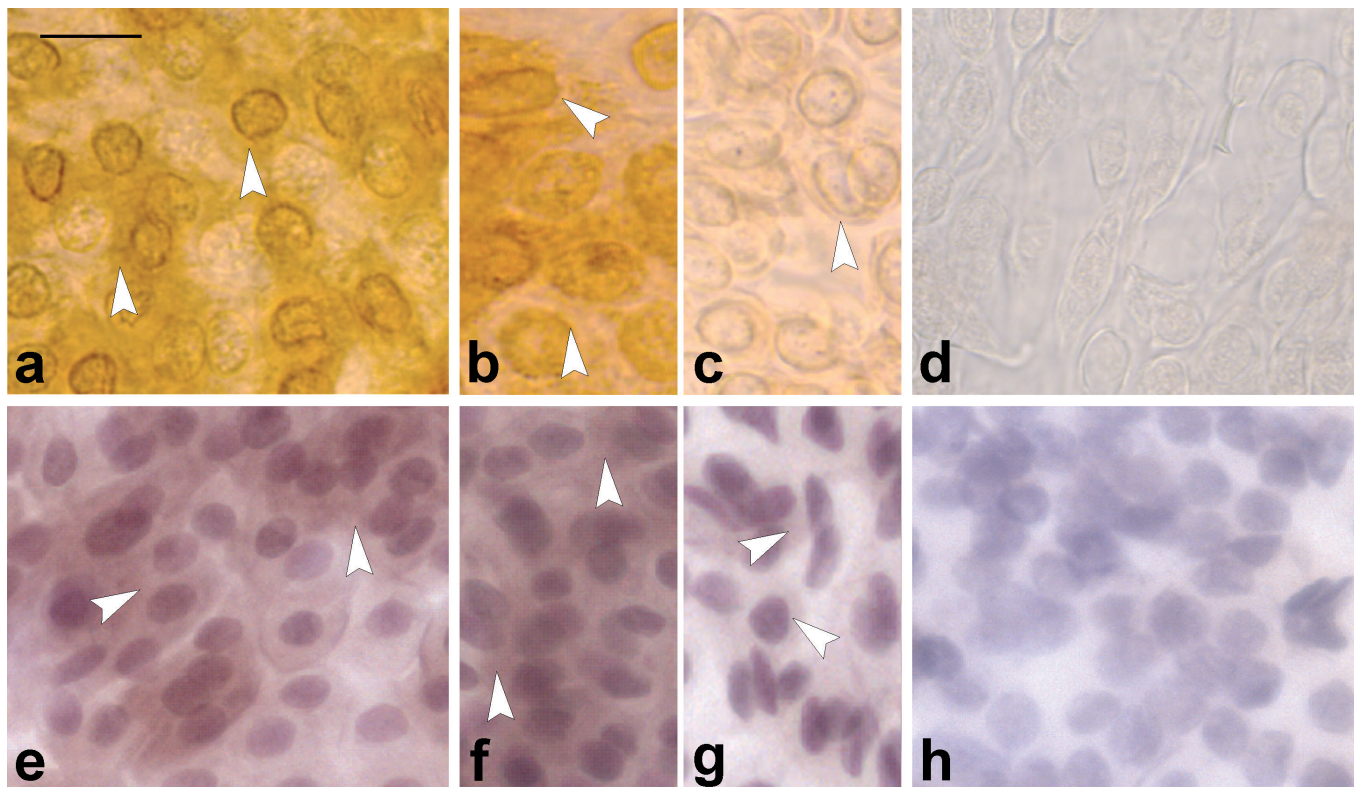


Fig. 3. Immunohistochemical staining of superoxide dismutase (SOD) in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with less pronounced symptoms of dryness; grade 3, patients with highly pronounced symptoms of dryness). SOD staining in normal and dry eye (grade 2) does not substantially differ (**a** - normal eye, without counterstaining, **e** - normal eye, with counterstaining; **b** - dry eye, without counterstaining, **f** - dry eye, with counterstaining). In dry eye with highly pronounced symptoms of dryness (grade 3), the expression of SOD is reduced (**c** - dry eye, specimens without counterstaining, **g** - dry eye, nuclei counterstained). Controls (primary antibody omitted): **d** - without counterstaining; no staining is seen; **h** - specimen in which the nuclei were counterstained with haematoxylin; only nuclei are stained. Scale bar : 10 μ m

released from the diseased conjunctival epithelium (Čejková et al. 2007a) were described in dry eye (SS). Xanthine oxidoreductase/xanthine oxidase, which generate reactive oxygen species, may be induced by pro-inflammatory cytokines (Page et al., 1998; Komaki et al., 2005), which have also been found in dry eye disease (Robinson et al., 1998; Rosenbaum et al., 1998; Pflugfelder et al., 1999; Solomon et al., 2001; Luo et al., 2004; Čejková et al., 2007b). The results of our study point to the suggestion that the reduced expression of antioxidant enzymes contributes to the imbalance between prooxidants and antioxidants. The decreased expression of antioxidants, elevated levels of reactive oxygen species, together with the increased expression of nitric oxide synthases generated nitric oxide may result in the formation of cytotoxic nitrogen-related oxidants and oxidative tissue damage (Čejková et al., 2007b).

In conclusion, we suggest that the reduced expression of antioxidant enzymes contributes to oxidative injuries of dry eye (SS). The antioxidant enzymes might be overwhelmed by the large amount of reactive oxygen species present at the ocular surface. It is further suggested that the topical treatment of dry eye (SS) with antioxidant enzymes (superoxide dismutase combined with catalase, a potent scavenger of hydrogen peroxide) may improve the recovery of ionic imbalance and thus might result in the prevention or at least a decrease of oxidative injuries. To combine superoxide

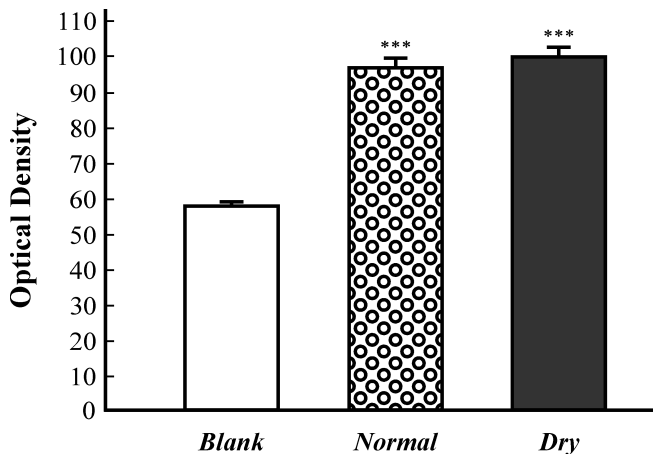


Fig. 4. Comparison of superoxide dismutase (SOD) staining optical densities among individual samples of the conjunctival epithelium (shown in columns). Columns: Blank - Specimen without primary antibodies used as a control for the specificity of the staining. Normal - normal human eye, Dry - dry eye (SS, grade 2, patients with less pronounced symptoms of dryness). Mean and standard error of the mean (S.E.M.) are shown in the Figure. Variation among the column medians (ANOVA) is significantly greater than expected by chance, $P < 0.0001$. Statistically significant differences between the columns according to the Tukey-Kramer Multiple Comparison Test are indicated by symbols above the columns : *** $P < 0.001$ compared with the group Blank.

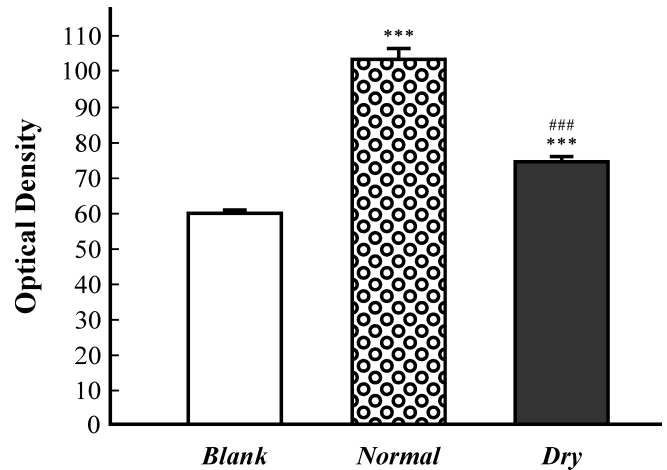


Fig. 5. Differences in glutathione peroxidase (GPX) staining optical densities among individual samples of the conjunctival epithelium (same as Fig.4). Columns: Blank - Specimen without primary antibodies used as a control for the specificity of the staining. Normal - normal human eye, Dry - dry eye (SS, grade 2, patients with less pronounced symptoms of dryness). Mean and standard error of the mean (S.E.M.) are shown in the Figure. Variation among the column medians (ANOVA) is significantly greater than expected by chance, $P < 0.0001$. Statistically significant differences between the columns according to the Tukey-Kramer Multiple Comparison Test are indicated by symbols above the columns in the diagram : *** $P < 0.001$ compared with the group Blank , ### $P < 0.001$ compared with the group Normal.

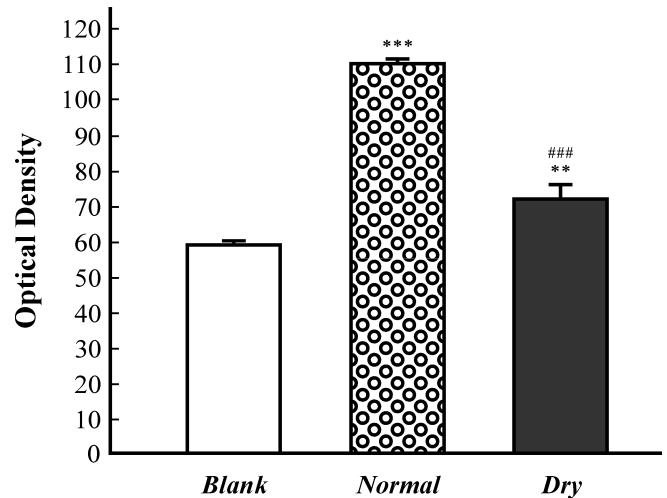


Fig. 6. Differences in catalase (CAT) staining optical densities among individual samples of the conjunctival epithelium (same as Fig. 4). Columns: Blank - Specimen without primary antibodies used as a control for the specificity of the staining. Normal - normal human eye, Dry - dry eye (SS, grade 2, patients with less pronounced symptoms of dryness). Mean and standard error of the mean (S.E.M.) are shown in the figure. Variation among the column medians (ANOVA) is significantly greater than expected by chance, $P < 0.0001$. Statistically significant differences between the columns according to the Tukey-Kramer Multiple Comparison Test are indicated by symbols above the columns : ** $P < 0.01$ compared with group Blank , *** $P < 0.001$ compared with the group Blank , ### $P < 0.001$ compared with the group Normal.

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dismutase with catalase is absolutely necessary, because superoxide dismutase produces hydrogen peroxide from superoxide and catalase removes hydrogen peroxide. Because the expression of catalase is highly reduced in dry eye (SS) and hydrogen peroxide is very toxic to the eye, catalase supplementation is required. To examine the effectiveness of this therapeutic approach is the aim of our next investigation.

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