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Review

Ocular surface injuries in autoimmune dry eye. The severity of microscopical disturbances goes parallel with the severity of symptoms of dryness

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Summary. Autoimmune dry eye (Sjögren's syndrome, SS) is a chronic systemic disease characterized by salivary and lacrimal gland inflammation and tissue damage leading to keratoconjunctivitis sicca and xerostomia. In this review attention has been devoted to the cause of the development of oxidative injuries of the ocular surface of patients suffering from SS. It was shown that lacrimal glands and diseased conjunctival epithelium reveal increased expression of pro-inflammatory cytokines which are released into the tear fluid. A high amount of pro-inflammatory cytokines highly induce the elevated expression and activity of enzymatic systems that generate reactive oxygen and nitrogen species. An abundant amount of these toxic products leads to a decrease in antioxidants and to the formation of cytotoxic related oxidants, such as peroxynitrite. All these factors, together with reactive oxygen species from polymorphonuclear leukocytes, contribute to the development of oxidative injuries at the ocular surface. From the clinical point of view it is important that the level of severity of the above described microscopical disturbances found in conjunctival epithelial cells goes parallel with the level of severity of dry eye symptoms.

Key words: Autoimmune dry eye, Oxidative injuries, Pro-inflammatory cytokines, Reactive oxygen and nitrogen species, Antioxidant enzymes

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Introduction

Dry eye syndrome is a chronic condition in which some components of the preocular tear film are dysfunctional, leaving the patient with painful symptoms of dryness. The factors leading to abnormalities of the tear film are complex and may involve autoimmune disease (i.e. Sjögren's syndrome) (e.g. Connolly, 2001; Fox, 2005), loss of hormonal support, and glandular inflammation (Beauregard et al., 2003; Alvarez, 2008).

Sjögren's syndrome (SS) is a chronic, systemic, autoimmune inflammatory disorder characterized by lymphocytic infiltration of exocrine glands, most notably the salivary and lacrimal glands. The exocrinopathy can be encountered alone (primary Sjögren's syndrome) or in association with other autoimmune disorders, such as rheumatoid arthritis, systemic lupus erythematosus, and progressive systemic sclerosis (secondary Sjögren's syndrome) (Srinivasan and Slomovic, 2007). Activated lymphocytes in patients with autoimmune diseases appear to have selective homing into lacrimal and salivary glands leading to tissue damage (Tabbara and Vera-Cristo, 2000).

Ocular symptoms of autoimmune dry eye disease (SS) 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). In patients suffering from SS, the clinical state of the ocular surface is 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. Dry eyes in patients with Sjögren's syndrome are

usually diagnosed according to Copenhagen criteria, i.e. at least two of the following tests are abnormal: Schirmer test ≤ 10mm/5min, tear film break-up time (BUT) ≤ 10s, rose bengal staining ≥ 4 (van Bijsterveld, 1969; Manthorpe et al., 1986). According to the severity of symptoms of dryness, Murube et al. (2005) divided patients with dry eye into three groups: grade one, mild (symptoms without slitlamp signs), grade two, moderate (symptoms with reversible slitlamp signs) and grade three, severe (syptoms with permanent slitlamp signs).

Augustin et al. (1995) found oxidative reactions in the tear film of patients suffering from dry eye. These authors described elevated lipid peroxide levels and myeloperoxidase activity, as parameters for oxidative tissue damage and inflammatory activity, in the tear fluid of drye eye. It was suggested that free radicals of polymorphonuclear leukocytes and inflammation may be involved in the pathogenesis or the self-propagation of the disease. Čejková et al. (2007a) described diseased conjunctival epithelium of eye with SS as the source of reactive oxygen species. The conjunctival epithelium revealed pronounced expression, as well as activity, of xanthine oxidoreductase/xanthine oxidase that generate reactive oxygen species. 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, in the conjunctival epithelial cells of eyes with SS an increased expression of nitric oxide synthases that generate nitric oxide was found (Čejková et al., 2007b). According to these authors both enzymatic systems were induced in eyes with SS by pro-inflammatory cytokines. A high amount of reactive oxygen species at the ocular surface resulted in a decrease in enzymatic antioxidants in conjunctival epithelium (Čejková et al., 2008). In these studies conjunctival epithelial cells of patients suffering from SS were collected by means of the method of impression cytology using Millicell membranes. This method was developed for these purposes. Immunohistochemical results (enzyme expression levels and pro-inflammatory cytokine expression levels) were determined by image analysis and statistical evaluation.

Pro-inflammatory cytokines in SS

The diseased lacrimal gland of an eye suffering from autoimmune disease (SS) produces highly increased levels of pro-inflammatory cytokines, such as interleukin-1ß, interferon-γ and tumor necrosis factor-α, which are secreted into the tear fluid (Robinson et al., 1998; Rosenbaum et al., 1998; Pflugfelder et al., 1999). Solomon et al. (2001) suggested that conjunctival cells of dry eye are another source of the increased levels of interleukin-1ß in the tear fluid. According to these authors the elevated levels of matrix metalloproteinase-9 (a physiological activator of interleukin-1ß) on the ocular surface may be one mechanism by which precursor interleukin-1ß is cleaved to the mature, biologically active form. According to Pflugfelder et al. (1999) the severity of dry eye disease is more pronounced as the levels of pro-inflammatory cytokines in the conjunctival epithelium increase and the level of epidermal growth factor in the tear fluid decreases. Pflugfelder (2004) pointed out that pro-inflammatory cytokines together with destructive proteases in the tear fluid of dry eye play a very important role in the development of inflammation. Luo et al. (2004) described that experimental dry eye stimulated the expression of pro-inflammatory cytokines and also the expression of metalloproteinase-9 and activated mitogen-activated kinase signalling pathway on the ocular surface. According to Čejková et al. (2007b) increased levels of pro-inflammatory cytokines found in conjunctival epithelium of eyes with SS (Fig. 1, see Čejková et al., 2007b for details) stimulate the expression and activity of enzymes that generate reactive oxygen and nitrogen species.

Nitric oxide synthases in SS

Nitric oxide synthases (NOS) can be divided into two major groups, constitutive NOS (cNOS) and indicible NOS (iNOS, NOS2). cNOS are calcium-dependent and two isoforms are present, the neuronal form (nNOS, NOS1) and the endothelial form (eNOS, NOS3) (Bredt and Snyder, 1994). Constitutively expressed NOS has been shown to be important in normal physiology. In contrast, NOS2 is a calcium-independent enzyme, producing nitric oxide during inflammation. NOS2 is induced in a number of cell types, such as fibroblasts and macrophages, especially after stimulation by pro-inflammatory cytokines and liposaccharides (e.g. Yoshida et al., 1994).

Beauregard et al. (2003) incubated cultured immortalized rabbit lacrimal gland acinar cells with pro-inflammatory cytokine (interleukin-1ß) and found that these cells were able to produce iNOS in response to interleukin-1ß. The authors hypothetized that pro-inflammatory cytokines, such as interleukin-1ß, cause a marked increase in nitric oxide production via induction of iNOS in lacrimal gland epithelial cells and that this may be a significant pathophysiological pathway of dry eye syndrome. Čejková et al. (2007b) showed that conjunctival epithelial cells of patients suffering from dry eye (SS) reveal pronounced expression of NOS-2 as well as NOS-3 as compared to the conjunctival epithelium of normal eye. The expression of both NOS increased along with the severity of dry eye symptoms from less evident symptoms of dryness to much more pronounced symptoms (Fig. 2) (see Čejková et al., 2007b for details). These authors discussed that the more pronounced expression of nitric oxide synthases found in conjunctival epithelial cells of dry eye was caused by the influence of pro-inflammatory cytokines (such as mature

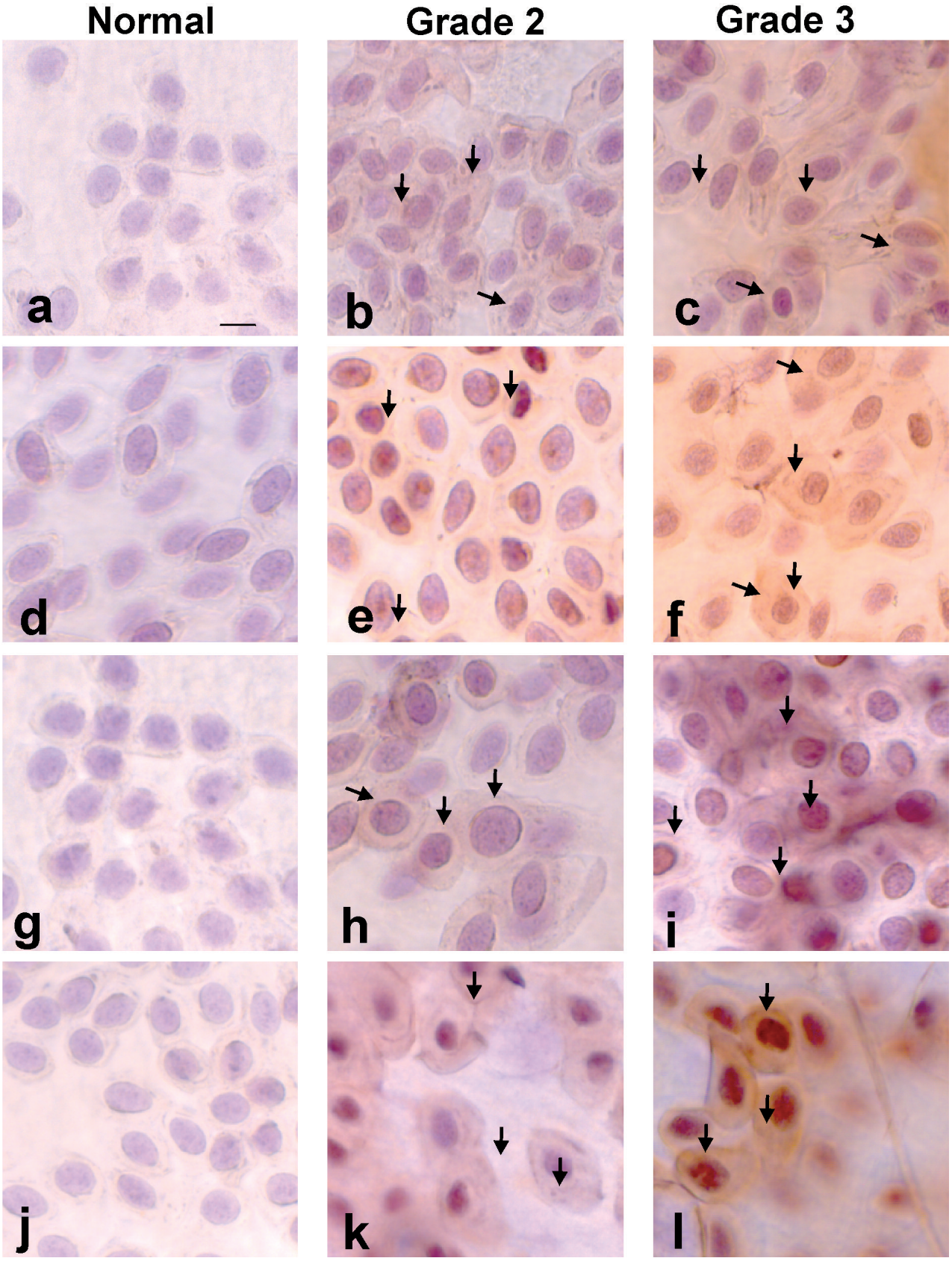

Fig. 1. Immunohistochemical staining of pro-inflammatory cytokines in the conjunctival epithelium of eyes with SS. Normal conjunctival cytology samples did not reveal cytokine staining or the staining was very weak (IL 1-ß, **a**; IL-6, **d**; IL-8, **g**; TNF α, **j**). Only nuclei were stained with haematoxylin. However, all cytokine studied were already expressed in the conjunctival epithelium of dry eye (SS) grade 2, moderate symptoms of dryness (IL1-ß, **b**; IL-6, **e**; IL-8, **h**; TNF-α **k**) and the expression increased in the conjunctival epithelium of dry eye (SS) - grade 3, severe symptoms of dryness (IL-1 ß **c**; IL-6, **f**; IL-8, **i**; TNF-α, **l**). Arrows point to cytokine expression. Scale bar: 10 μM.

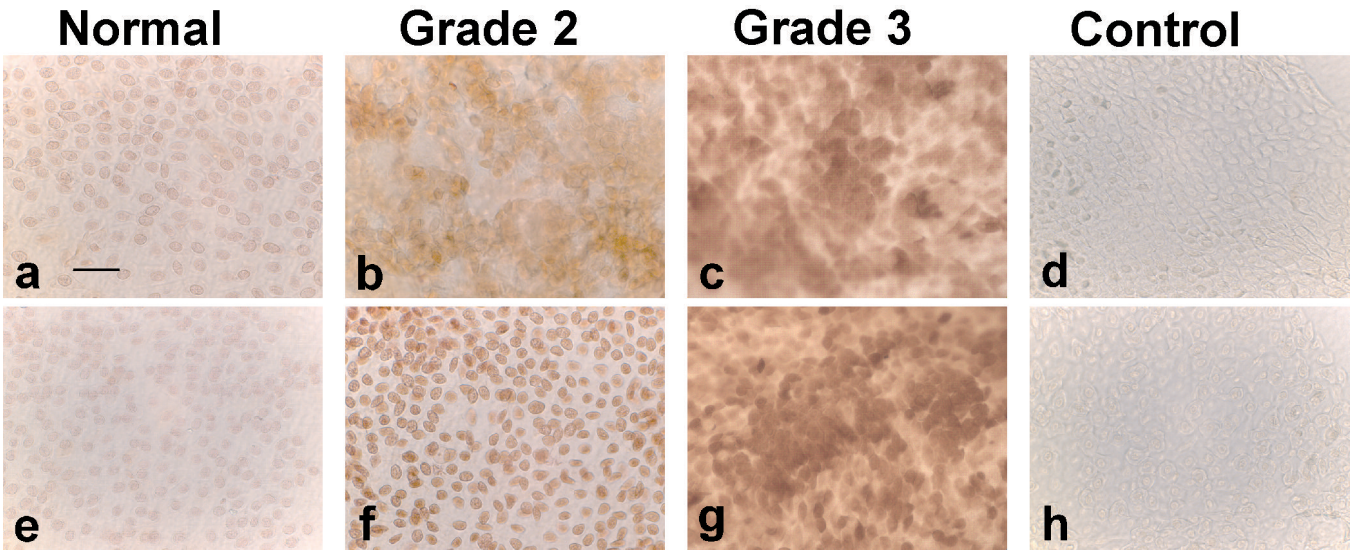

Fig. 2. Immunohistochemical demonstration of NOS3 and NOS2 in the conjunctival epithelium of dry eye (SS). In the conjunctival epithelium of dry eye NOS3 was gradually expressed along with the severity of dry eye symptoms (**b**, dry eye moderate symptoms of dryness, grade 2; (**c**), dry eye with severe symptoms of dryness, grade 3). This is in contrast to the conjunctival epithelium of normal eye (**a**), where NOS3 is only slightly expressed. In the control sample for NOS3 (primary antibody omitted) no staining appears (**d**). Very similar results were obtained with NOS2. Also, the staining of NOS2 in the conjunctival epithelium of dry eye increases together with the more profound expression of dryness symptoms (**f**, grade 2; **g** grade 3). In the normal eye (**e**) NOS2 is only slightly expressed in the conjunctival epithelium. **h** - control (primary antibody omitted) specimen for NOS2; no staining appears. Scale bar: 10 μM.

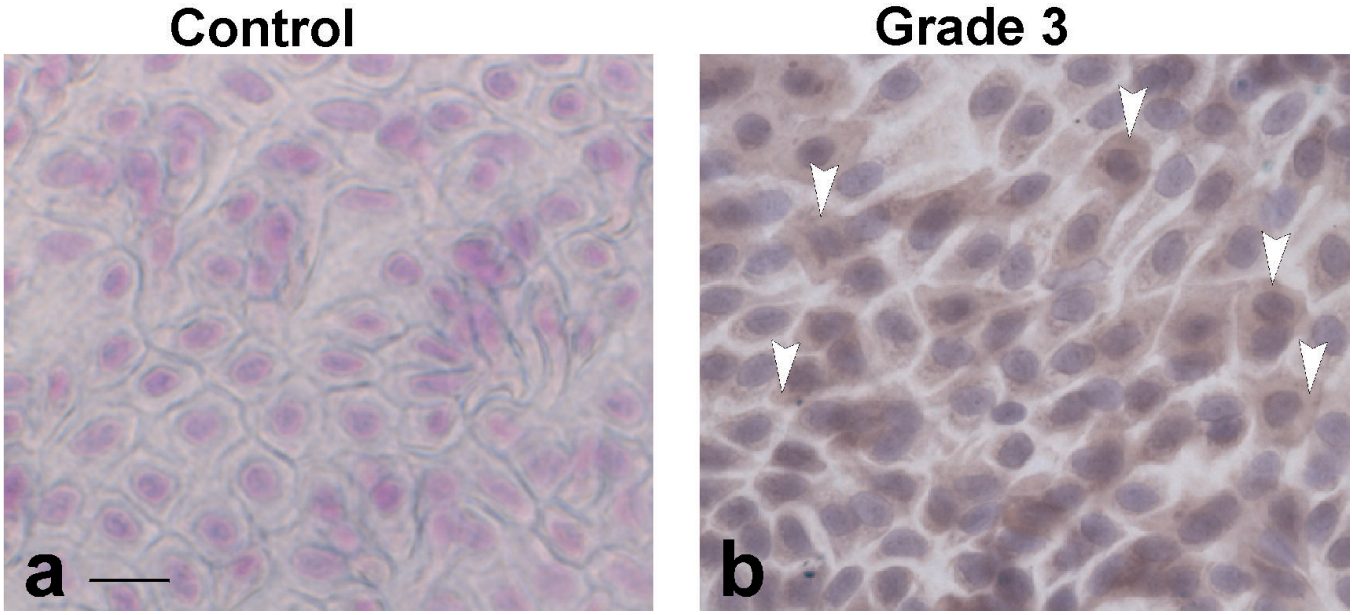

Fig. 3. Peroxynitrite formation in conjunctival epithelium of dry eye demonstrated by nitrotyrosine residues; nuclei were counterstained with haematoxylin. **a.** In control for nitrotyrosine where primary antibody was incubated with 10 mM nitrotyrosine, no staining appears; only nuclei are stained. **b.** In dry eye (SS, grade 3, severe symptoms of dryness) nitrotyrosine is highly expressed (arrows). Scale bar: 10 μM.

interleukin-1 beta, interleukin 6, interleukin 8, interferon gamma and tumor necrosis factor alpha) released in the tear fluid in elevated levels 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).

It is suggested that NOS expression in dry eye disease (SS) is highly involved in dry eye injury (and pronounced symptoms of dryness), perhaps through the formation of nitrogen-related oxidants, such as peroxynitrite (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 through lipid peroxidation of photoreceptors in experimental autoimmune uveitis. Lipid peroxidation is an important biological consequence of oxidative damage of cell membranes and the formation of cytotoxic aldehydes. Increased levels of malondialdehyde, the toxic aldehyde byproduct of lipid peroxidation, were also found in conjunctival epithelium of patients with SS (Čejková et al., 2007b). These authors described that peroxynitrite formation (demonstrated by nitrotyrosine residues) (Fig. 3) and lipid peroxidation (demonstrated by increased malondialdehyde staining) can be seen in conjunctival epithelium of dry eye (SS), grade 3, severe symptoms of dryness.

Xanthine oxidoreductase/xanthine oxidase in SS

Xanthine oxidoreductase/xanthine oxidase have been identified as a critical source of reactive oxygen species

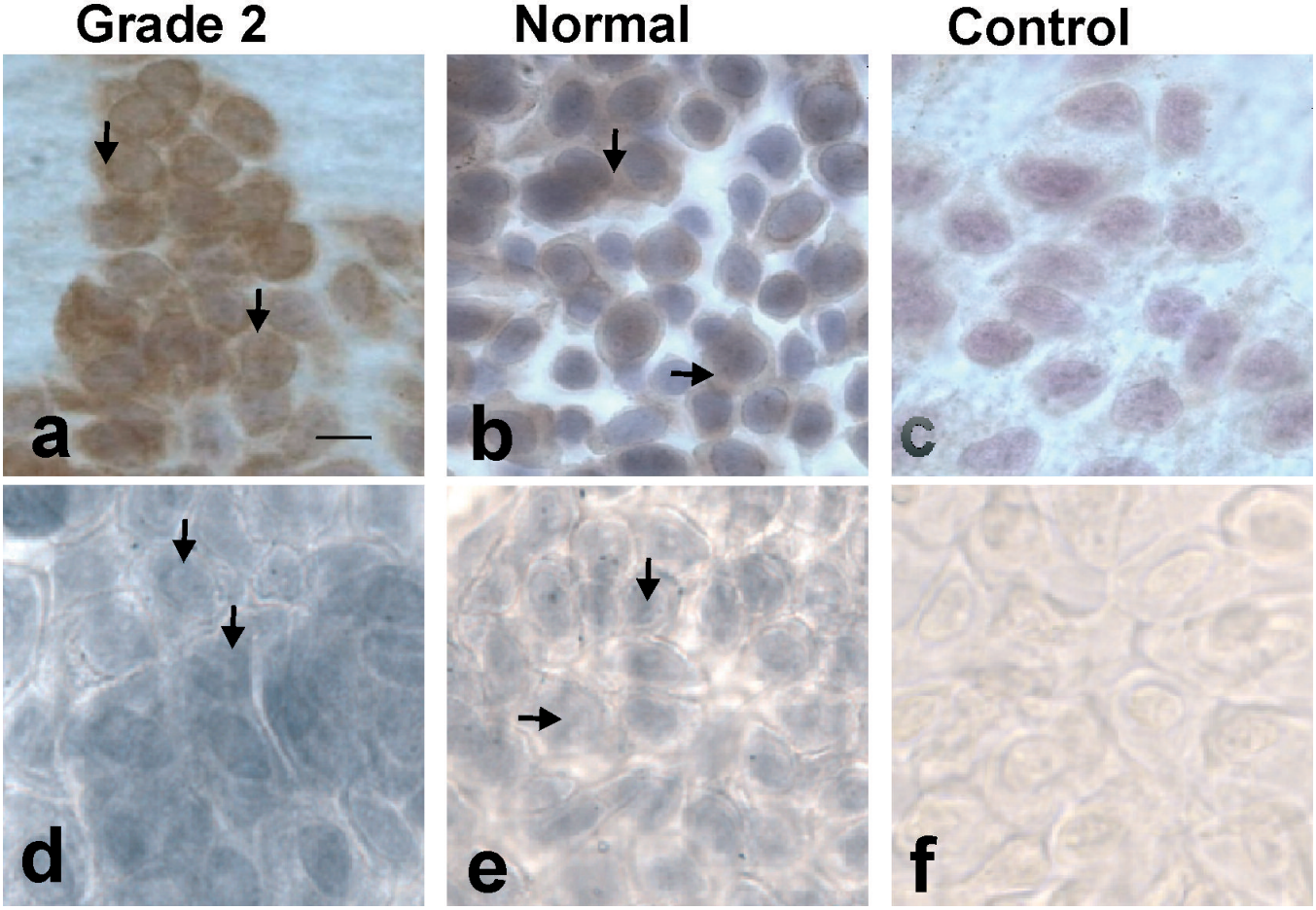

Fig. 4. Xanthine oxidase in conjunctival epithelial cells of a normal human eye and a dry eye (Sjögren's syndrome). Xanthine oxidase detected immunohistochemically is more expressed in dry eye (**a**) than in the normal eye (**b**). Also, the activity of xanthine oxidase (localised histochemically) is more pronounced in dry eye (**d**) as compared to the normal eye (**e**). Controls for the immunohistochemistry of xanthine oxidase (**c**) in which the primary antibodies were omitted in the incubation media. Only the nuclei are stained with haematoxylin. **f.** Control for xanthine oxidase activity (conjunctival cells on Millicels were treated in incubation media without substrate). No staining appears. Scale bar: 10 μM.

(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, Harris and Massey (1997) pointed out that conversion to xanthine oxidase is not absolutely necessary for reactive oxygen species generation because xanthine dehydrogenase was also found to generate free radicals.

In conjunctival epithelium of dry eye (SS), xanthine oxidoreductase/xanthine oxidase is more pronounced than in normal eye (histochemistry and immunohistochemistry of xanthine oxidase see Fig. 4) (Čejková et al., 2007a for details). 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 (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. As mentioned above, the 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) release pro-inflammatory cytokines into the tear fluid. 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 dessication 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.

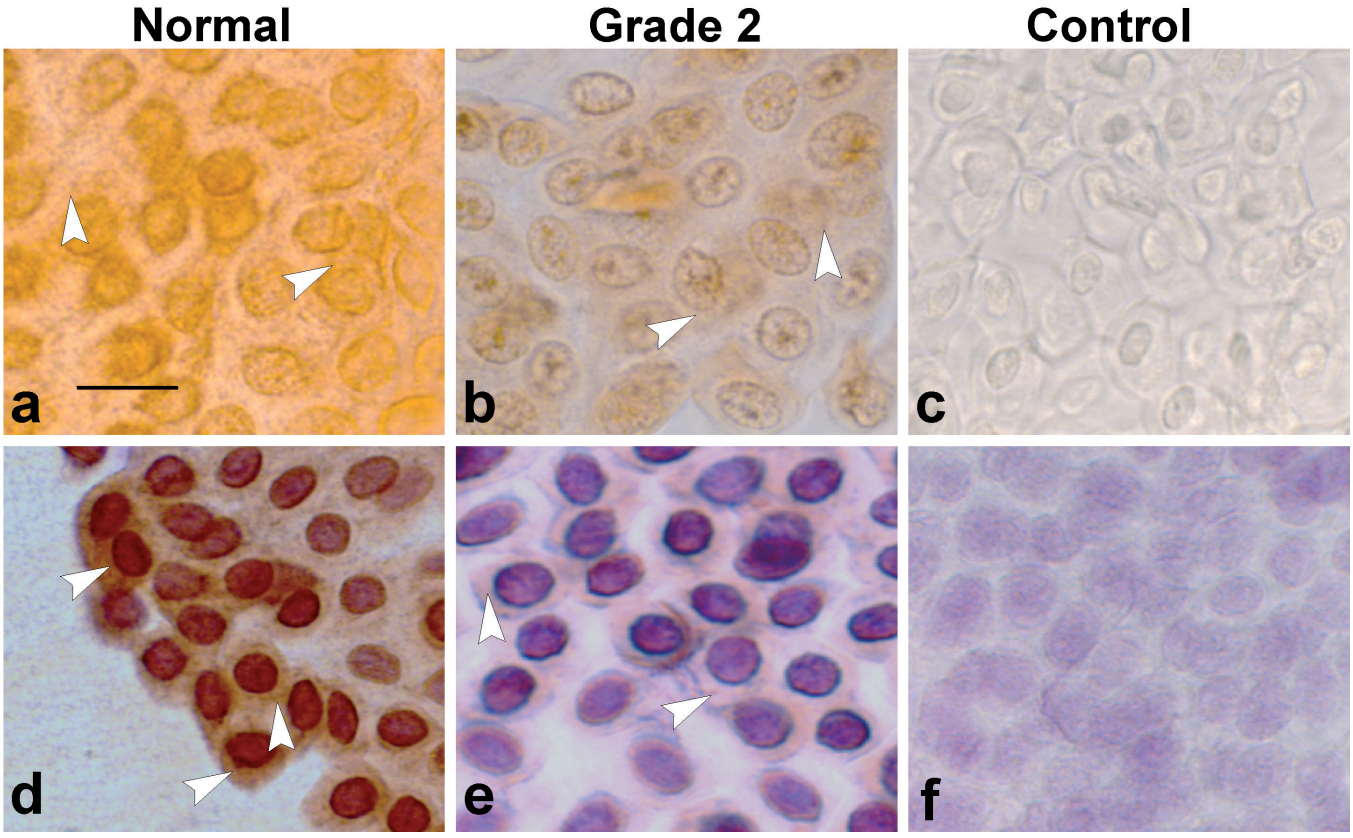

Fig. 5. Immunohistochemical staining of catalase in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with less pronounced symptoms of dryness). Catalase staining is more expressed in the normal eye (**a** - without counterstaining, **d** - specimens counterstained with haematoxylin), than in dry eye (**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 μM

According to Kelley et al. (2006), moderate hypoxia also increases both xanthine oxidoreductase immunoreactive protein levels as well as xanthine oxidoreductase activity.

Antioxidant enzymes in SS

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) that play a key role in protecting the eye against oxidative damage. Superoxide dismutase catalyses the dismutation of superoxide to hydrogen peroxide and molecular oxygen. This enzyme protects the ocular tissues against superoxide radicals. Glutathione peroxidase, together with catalase, are very important scavengers of hydrogen peroxide.

Čejková et al. (2008) showed that the expression of antioxidant enzymes studied immunohistochemically is less pronounced in the conjunctival epithelium of dry eye (SS) than in the conjunctival epithelium of the normal eye. The decrease in enzyme expression was dependent on the severity of the dry eye symptoms. In grade 2 (patients with moderate symptoms of dryness) catalase and glutathione peroxidase were significantly decreased (Figs. 5, 6), while superoxide dismutase expression was very similar to the normal eye. However, in the case of grade 3 (patients with severe symptoms of dryness) the expression of superoxide dismutase was also reduced (Fig. 7).

Čejková et al. (2008) suggested that the reduced expression of antioxidant enzymes may contribute to the development of oxidative injuries at the ocular surface of dry eye (SS). The antioxidant enzymes might be overwhelmed by the large amount of reactive oxygen species present at the ocular surface. It was further suggested that the topical treatment of dry eye (SS) with antioxidant enzymes (superoxide dismutase combined with catalase, a potent scavenger of hydrogen peroxide) might improve the recovery of ionic imbalance and thus result in the prevention, or at least a decrease, in the

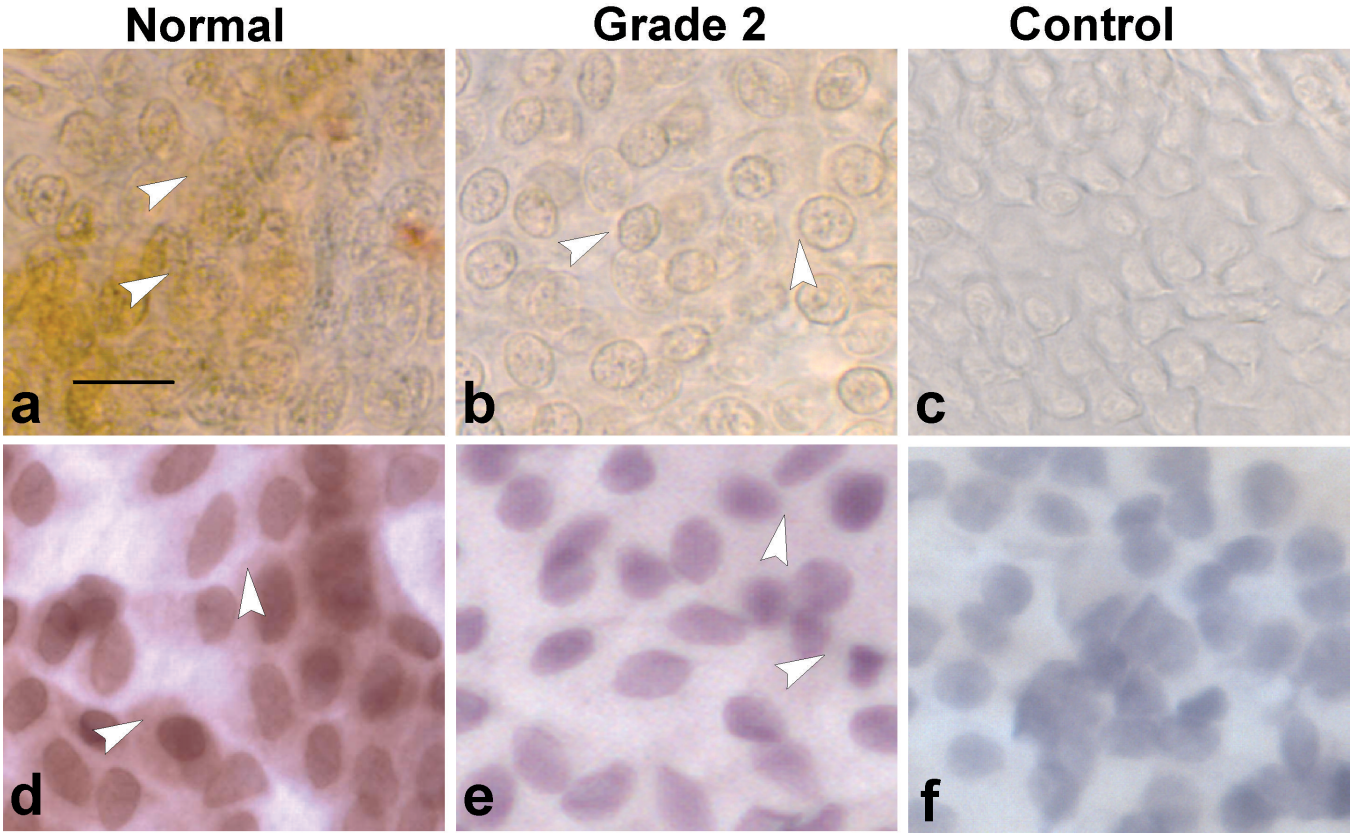

Fig. 6. Immunohistochemical staining of glutathione peroxidase in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with moderate symptoms of dryness). The expression of glutathione peroxidase in the normal eye (**a** - without counterstaining, **d -** with counterstaining) 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 μM.

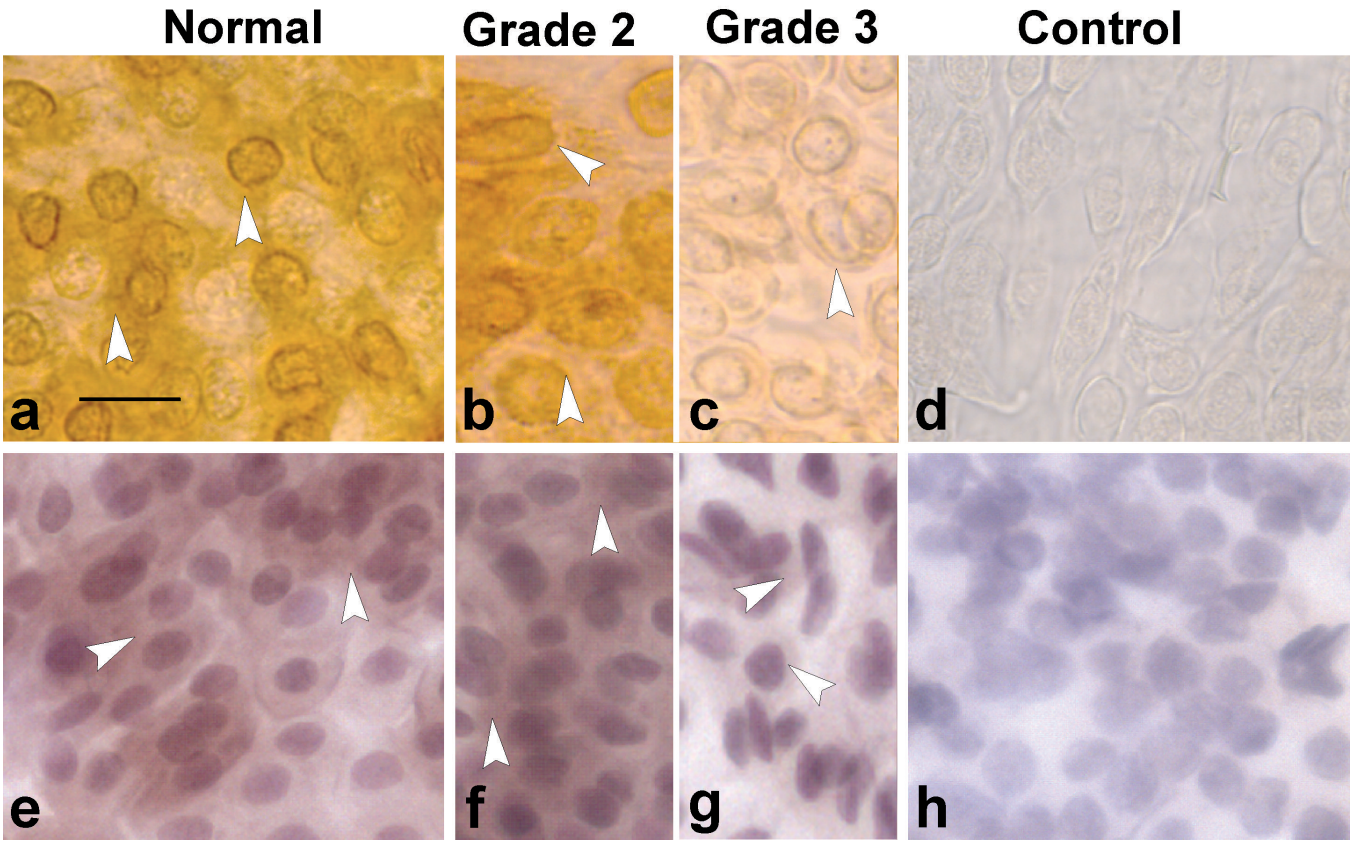

Fig. 7. Immunohistochemical staining of superoxide dismutase in the conjunctival epithelial cells of normal eyes and dry eye (SS) (grade 2, patients with moderate symptoms of dryness; grade 3, patients with severe symptoms of dryness. SOD staining in normal and dry eye (grade 2) does not substantially 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 severe 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 μM.

occurrence of oxidative injuries. The authors recommended to combining superoxide dismutase with catalase 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.

Concluding remarks

Although great effort has been exerted towards understanding the etiopathogenesis and immunopathology of SS, recent modes of therapy remain insufficient in many cases. Systemic therapy is aimed at the use of steroidal and non-steroidal drugs and disease-modifying agents. Topical treatment includes aqueous enhancement therapy and anti-inflammatory drugs. In this review it was shown that the severity of microscopical disturbances in conjunctival epithelial cells (the increase of pro-inflammatory cytokine expression, increased expression of enzymes that generate reactive oxygen and nitrogen species, decreased expression of enzymes that cleave toxic oxygen products) corresponded to the severity of dry eye symptoms. It is suggested that new insights into the involvement of oxidative injuries may lead to the development of novel therapies for eyes with SS.

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