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Review

The role of oxidative stress in corneal diseases and injuries

Jitka Čejková and Čestmír Čejka

Laboratory of Eye Histochemistry and Pharmacology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Summary. In various corneal injuries (such as chemical burns or irradiation of corneas with UVB radiation) and ocular diseases (e.g. dry eye disease, keratokonus, bullous keratopathy, Fuchs' endothelial dystrophy), the expressions of malondialdehyde (a marker of lipid peroxidation) and nitrotyrosine (a marker of oxidative stress) appeared in cells of individual corneal layers and conjunctival cells (dry eye disease). This is in contrast to healthy corneas in which negligible levels of malondialdehyde and no expressions of nitrotyrosine are present. The injured or diseased corneas reveal decreased capacity of antioxidants (enzymatic as well as non-enzymatic), whereas the levels of pro-oxidants (e.g. oxidases that generate reactive oxygen species) remain at physiological levels or even increase, leading to the antioxidant/prooxidant imbalance and oxidative stress. Oxidative stress in the cornea stimulates generation of pro-inflammatory cytokines, proteolytic enzymes and enzymes that generate nitric oxide (nitric oxide synthases). An abundant amout of reactive oxygen species and nitric oxide lead to the formation of toxic reactive products contributing to tissue damage. This review aims to summarize immunohistochemical changes in severe corneal injuries and diseases in which oxidative stress has been proved.

Offprint requests to: Associate Prof. Jitka Čejková, MD, PhD, DSc, Head, Department of Eye Histochemistry, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220, Prague 4,CR. e-mail : cejkova@biomed.cas.cz
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Introduction

The cornea is a transparent avascular tissue that is regularly exposed to sunlight (and ultraviolet light) and atmospheric oxygen. UV exposure is an important environmental stress factor that generates free radicals and reactive oxygen species dangerous to most cells and tissues (Wenk et al., 2001). The cornea absorbs the majority of UV radiation (mainly UVB radiation) entering the eye, suggesting that the cornea would be highly susceptible to damage from reactive oxygen species. In healthy corneas, a number of antioxidant protective mechanisms are present to minimize and reduce this risk (Buddi et al., 2002). Indeed, 20-40% of the soluble protein content of the cornea is an isoenzyme of aldehyde dehydrogenase (ALDH3), which directly absorbs UV and removes cytotoxic aldehydes produced by lipid peroxidation induced by UV radiation (Abedinia et al., 1990). Furthermore, the cornea reveals antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase scavenging reactive oxygen species. Thus, the cornea may be disturbed by toxic oxygen products under circumstances when their protective mechanisms are overhelmed. This takes place due to the excessive amount of reactive oxygen species and/or due to the decrease in antioxidants (Čejková et al., 2000). The aim of this review was to summarize microscopical observations dealing with ocular diseases or injuries in which oxidative stress is involved.

Human corneal diseases: keratoconus, Fuchs' endothelial dystrophy and bullous keratopathy

Keratoconus is a progressive, non-inflammatory disease appearing in young adults, leading to a variable decrease in the quality of vision and ocular discomfort (Krachmer et al., 1998). The molecular pathogenesis of keratoconus is poorly understood. Recently, it has been suggested that oxidative stress may be involved in keratoconus (Behndig et al., 2001; Buddi et al., 2002; Arnal et al., 2011; Toprak et al., 2014). Immunohistochemically, Buddi et al. (2002) described that keratoconus corneas reveal the expression of nitrotyrosine (a marker of oxidative stress), malondialdehyde (a marker of lipid peroxidation) and expression of endothelial nitric oxide synthase and inducible nitric oxide synthase in individual corneal layers, particularly in the corneal epithelium. This is in contrast to the healthy corneas, where endothelial nitric oxide synthase and malondialdehyde are present in very slight expressions, whereas inducible nitric oxide synthase, as well as nitrotyrosine expressions, are completely absent (Čejková et al., 2005).

Fuchs endothelial corneal dystrophy is a multifactorial corneal disease caused by degeneration of the corneal endothelium. Loss of endothelial cell density is associated with edema leading to the loss of corneal clarity. According to Jurkunas et al. (2010) oxidative DNA damage and apoptosis of endothelial cells are involved in this disease. Corneal dysfunction is manifested by the decrease in visul acuity (Klintworth, 2003). In Fuchs dystrophy corneas nitrotyrosine, malondialdehyde, endothelial nitric oxide synthase and inducible nitric oxide synthase are highly expressed in the corneal endothelium and less in the epithelium (Buddi et al., 2002).

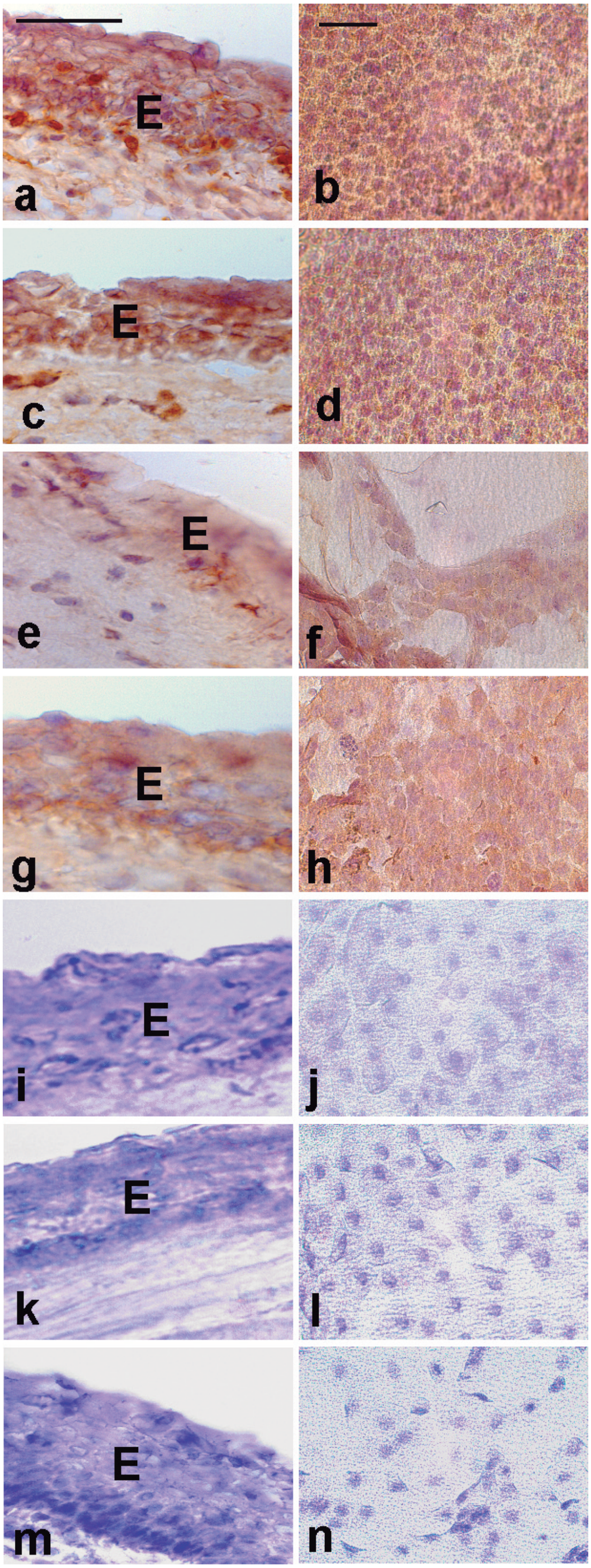
Bullous keratopathy is a pathological condition in which small vesicles, or bullae, are formed in corneas due to the endothelial dysfunction. This leads to increased corneal hydration and impaired vision. Buddi et al. (2002) described that these corneas display byproducts of lipid peroxidation but not peroxynitrite.

Dry eye syndrome

Dry eye disease is a chronic condition in which some components of the preocular tear film are dysfunctional, leaving the patient with painful symptoms of dryness. Tear fluid hyperosmolarity may be involved in a series of corneal disorders (e.g. Parra et al., 2014). 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). 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). Augustin et al. (1995) described oxidative reactions in the tear film (elevated lipid peroxide levels and myeloperoxidase activity) of patients suffering from dry eye. It was suggested that free radicals of polymorphonuclear leukocytes and inflammation may be involved in the pathogenesis of this disease. According to Čejková et al. (2007a, 2008) diseased conjunctival epithelium of severe dry eye may be a source of reactive oxygen species because the conjunctival epithelium reveal pronounced expression and also activity of enzymes that generate reactive oxygen species (e.g. xanthine oxidoreductase/ xanthine oxidase). Moreover, the conjunctival epithelial cells of patients with severe dry eye reveal an increased expression of nitric oxide synthases that generate nitric oxide (Čejková et al., 2007b). It is suggested that nitric oxide synthase expressions in dry eye disease is highly involved in injuries of the ocular surface and pronounced symptoms of dryness, perhaps through the formation of nitrogen-related oxidants, such as peroxynitrite. Peroxynitrite is a potent oxidizing, nitrating and hydroxylating agent, resulting from the reaction of nitric oxide with superoxide. Moreover, increased levels of malondialdehyde, the toxic aldehyde byproduct of lipid peroxidation, were also found at the ocular surface in patients with dry eye syndrome (Čejková et al., 2007b). Lipid peroxidation is an important biological consequence of oxidative damage of cell membranes and the formation of cytotoxic aldehydes.

The enzymatic systems that generate reactive oxygen and nitrogen species might be induced in dry eye by proinflammatory cytokines (Čejková et al., 2009). The diseased lacrimal gland of an eye suffering from dry eye (mainly in autoimmune disease) produces highly increased levels of pro-inflammatory cytokines which are secreted into the tear fluid (Robinson et al., 1998; Rosenbaum et al., 1998; Pflugfelder et al., 1999). Solomon et al. (2001) also suggested that diseased conjunctival cells of dry eye might be the source of the increased levels of pro-inflammatory cytokines (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. Luo et al. (2004) described that experimental dry eye stimulated the expression of pro-inflammatory cytokines and also the expression of metalloproteinases (metalloproteinase-9) and activated mitogen-activated kinase signalling pathway on the ocular surface. Brejchova et al. (2009) described the participation of a wide range of metalloproteinases in severe (autoimmune) dry eye disease. According to these authors these enzymes are involved in corneal melting leading to serious consequences such as corneal perforation and vision loss.

A number of approaches have been developed for the treatment of dry eye syndrome with the aim to restore ocular surface integrity, suppress the inflammatory response, proteolytic expressions and



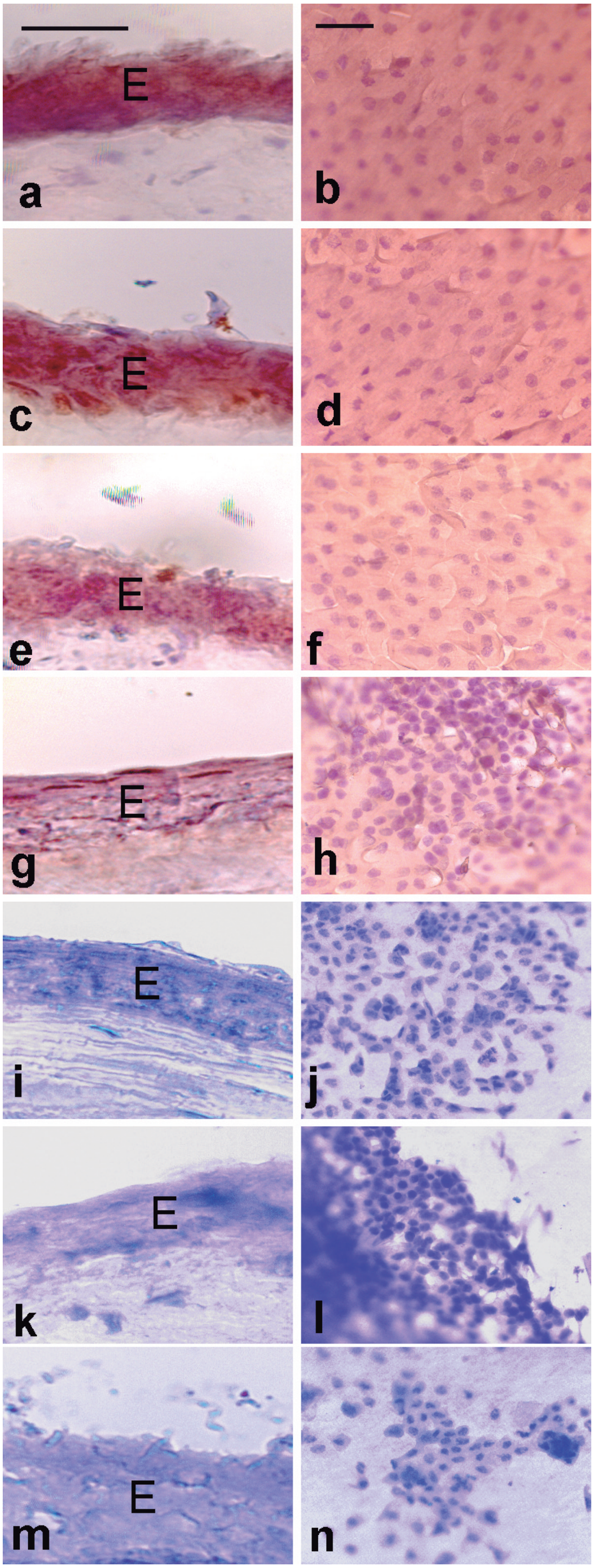
decrease oxidative stress in the cornea. The important therapeutic approaches include eye drops containing trehalose (e.g. Li et al., 2012), autologous serum eye drops (e.g. Celebi et al., 2014; Jirsova et al., 2014), cyclosporine A (e.g. Schultz, 2014) and sodium hyaluronate (e.g. Hwang et al., 2014).

Corneal oxidative injuries evoked by alkalis or irradiation with UVB rays

Alkali burns

Alkali injury to the cornea often leads to severe ocular damage resulting in the partial or total loss of vision. Highly concentrated alkalis are dangerous to the cornea due to the extensive destruction of all its layers, although less concentrated alkalis also pose a threat to vision because they evoke oxidative stress in the cornea (Kubota et al., 2011). Immediately after corneal alkali injury enhanced reactive oxygen species production appeared in the mouse cornea (Kubota et al. (2011): superoxide production and increased levels of nuclear factor kappa-light-chain-enhancer of activated B cells, a protein complex that controls the transcription of DNA. Also, monocyte chemoattractant protein-1 and vascular endothelial growth factor (VEGF) were significantly enhanced, pointing to corneal angiogenesis. Čejková et al. (2013) described that alkali injury leads to the decreased expression of aldehyde dehydrogenase3A1 in the epithelium of the injured cornea. Mammalian corneal epithelial cells express high levels of this enzyme (Piatigorsky, 2000), which protects (together with other

Fig. 1. Staining of nitrotyrosine, (counterstained with haematoxylin) in the rabbit corneal epithelium irradiated with UVB radiation and treated with antioxidant trehalose. Comparison of the expression of nitrotyrosine in cryostat sections of the cornea and in corneal epithelial cells collected by the method of impression cytology. a, b. Expression of nitrotyrosine in corneal epithelium irradiated four times with UVB rays (312 nm, daily dose 0.5 J/cm^2). Nitrotyrosine staining is clearly visible in the corneal epithelium (**a.** cryostat section, **b.** impression cytology sample). **c, d.** Irradiated cornea treated with buffered saline for one week after irradiation. Staining for nitrotyrosine is highly pronounced in the corneal epithelium (**c.** cryostat section, **d.** impression cytology sample). **e, f.** Irradiated cornea treated with trehalose for one week after the last irradiation procedure. Low levels of nitrotyrosine are present in the epithelium (**e.** cryostat section, **f.** impression cytology sample). **g, h.** Irradiated cornea on which buffered saline was applied for two weeks after the last irradiation. Nitrotyrosine staining is present in the corneal epithelium (**g.** cryostat section, **h.** impression cytology sample). **i, j.** Irradiated cornea treated with trehalose drops for two weeks after the end of the irradiation procedure. No positive staining is seen in the cornea (**i.** cryostat section, **j.** impression cytology sample). **k, l.** Normal cornea. Nitrotyrosine staining is absent in the corneal epithelium. The epithelium is counterstained only (**k.** cryostat section, **l.** impression cytology sample). **m, n.** Control section (**m**), control impression cytology sample (**n**). No positive staining appears when the primary antibody is omitted from the incubation medium. Corneal cryostat sections: a, c, e, g, i, k, m. Corneal impression cytologies: b, d, f, h, j, l, n. E: corneal epithelium. Čejková et al., 2012, Histol. Histopathol. 27, 1029-1040. Scale bar: 10 µm.



antioxidant enzymes) the cornea against oxidative damage (Downes et al.,1993; Manzer et al., 2003). Pappa et al. (2005) described that aldehyde dehydrogenase3A1 may protect corneal epithelial cells against oxidative stress not only through its metabolic function, but also by prolonging the cell cycle. Moreover, decreased antioxidant status in alkali injured cornea may lead to the increased expressions of pro-inflammatory cytokines associated with the development of corneal inflammation and corneal neovascularization and with increased expression of nitric oxide synthases, which generate nitric oxide (e.g., Sotozono et al., 1997, 1999; Čejková et al., 2013). High levels of reactive oxygen species and nitric oxide resulted in peroxynitrite formation. The demonstration of peroxynitrite (by the expression of nitrotyrosine), serves as an important marker of free radical damage (Ceriello, 2002; Chirino et al., 2006). Furthermore, malondialdehyde appears in alkali-injured corneas (Čejková et al., 2013). Because in the normal cornea staining for nitrotyrosine and malondialdehyde is absent or present at negligible levels, the expression of malondialdehyde in damaged burned corneas serves as a sensitive marker of oxidative damage.

To save vision after an alkali injury, a number of therapies has been investigated, including epidermal growth factor (Gonul et al., 1995), fibronectin (Phan et al., 1991), ascorbate (Levinson et al., 1976; Pfister and Paterson, 1980; Pfister et al., 1982), citrate (Pfister et al., 1981; Haddox et al., 1989; Pfister et al., 1991), metalloproteinase inhibitors (Schultz et al., 1992;

Fig. 2. Malondialdehyde staining (counterstained with haematoxylin) in the rabbit corneal epithelium irradiated with UVB radiation (312 nm, daily dose 0.5 J/cm^2) and treated with antioxidant trehalose. Comparison of the expression of malondialdehyde (a marker of lipid peroxidation) in cryostat sections of the cornea and in corneal epithelial cells collected by the method of impression cytology. **a, b.** Cornea irradiated with UVB radiation for four days. Malondialdehyde staining is strong in the corneal epithelium (**a.** cryostat section, **b.** impression cytology sample). **c, d.** Irradiated cornea treated for one week with buffered saline. Malondialdehyde staining is stll strong in the corneal epithelium (**c.** cryostat section, **d.** impression cytology sample). **e, f.** Irradiated cornea on which trehalose was dropped for one week after the last irradiation. The staining for malondialdehyde is less pronounced. Compare with buffered saline treatment (**c.** cryostat section, **d.** impression cytology sample). **g, h.** Cornea treated with buffered saline for two weeks after irradiation. Malondialdehyde staining is present in the corneal epithelium (**g.** cryostat section, **h.** impression cytology sample). **i, j.** Cornea treated with trehalose for two weeks following irradiation. Malondialdehyde staining is absent in the corneal epithelium (**i.** cryostat section, **j.** impression cytology sample). **k, l.** Normal cornea. Malondialdehyde staining is not present in the corneal epithelium (**k.** cryostat section, **l.** impression cytology sample). **m, n** - Negative control. The primary antibody was omitted from the incubation medium. No positive staining is present in the epithelium (**m.** cryostat section, **n.** impression cytology sample). Corneal cryostat sections: a, c, e, g, i, k, m. Corneal impression cytologies: b, d, f, h, j, l, n. E: corneal epithelium. Čejková et al., 2012, Histol. Histopathol. 27, 1029-1040. Scale bar: 10 µm.

Wentworth et al., 1992; Paterson et al., 1994; Pfister et al., 1997; Sotozono et al., 1999), bovine lactoferrin (Pattamatta et al., 2009), bevacizumab (Mello et al., 2011) and hydrogen (H2) irrigation solution (Kubota et al., 2011). Recently, oxidative stress in the cornea after alkali injury was highly supppressed by antioxidant therapy with H2-enriched irrigation solution (Kubota et al., 2011) or by mesenchymal stem cells transferred on the damaged corneal surface on nanofiber scaffolds (Čejková et al., 2013). The antioxidant therapies were effective. They accelerated corneal healing and suppressed corneal neovascularization. Chronic corneal ulcerative processes after alkali burns were effectively healed by matrix regenerating agent (RGTA, CACICOL20) (supplied by Laboratories Thea, Clermont-Ferrand, France) - a biopolymer mimicking heparan sulfates (Čejková et al., 2014).

Irradiation of the cornea with UVB radiation

The eye and particularly the cornea are directly exposed to sunlight and, due to the thinner ozone layer, to increased amounts of UV radiation (Norval et al., 2011). Photokeratitis, intraocular corneal inflammation, is evoked by UVB radiation (Young, 2006). The initial *in vivo* (clinical) signs of photokeratitis are due to the lost or damaged corneal epithelial cells, with other signs resulting from this primary response (Cullen, 2002). According to Pitts et al. (1977) the threshold radiant exposure of rabbit corneas rises very rapidly from 0.022 J/cm^2 at 300 nm to 10.99 J/cm^2 at 335 nm. Radiant exposures exceeding twice the threshold resulted in irreversible corneal damage. Doughty and Cullen (1990) described that the endothelial ultraviolet damage threshold was approximately 0.125 J/cm^2 (at the anterior corneal surface).

The cornea plays the key role in protecting the inner eye against oxidative injury caused by UVB radiation, known to induce reactive oxygen species generation. The cornea absorbs 92% of UVB and 60% of UVA radiation and is most sensitive to UVB damage (Zigman, 1995). The aqueous humor, containing ascorbic acid, proteins and some aminoacids (tyrosine, phenylalanine, cysteine, tryptophane), is also responsible for UVB absorption so that only a small number of UV rays reach the intraocular lens (Ringvold, 1998). The lens acts to filter light between 300-400 nm from reaching the retina. Under physiological conditions, the antioxidant enzymes (aldehyde dehydrogenase3A1, superoxide dismutase, catalase, glutathione peroxidase) protect the cornea against oxidative stress (summarized by Čejková et al., 2000). Although a number of antioxidants are present in the cornea, it may be damaged by oxidative stress when the production of damaging reactive oxygen species overwhelms the antioxidants. Under experimental conditions this occurs in the cornea after repeated irradiation with UVB rays (Čejková et al., 2000, 2001, 2004). UVB rays (and the reactive oxygen species generated by them) cause morphologic disturbances in the cornea. Already a single irradiation of the cornea with UVB rays was sufficient to block the proliferation of epithelial cells. Higher doses of UVB rays resulted in a considerable reduction in epithelial thickness (Koliopoulos and Margaritis, 1979). Along with morphological disturbances, a decrease of both UV absorption and removal of reactive oxygen species by cornea and aqueous humor appeared. It was shown that this decrease was closely dependent on UV wavelength, dose and frequency of irradiation (single or repeated irradiation). Čejková et al. (2000) reported that UVB rays (not UVA rays) caused a decrease of antioxidant enzymes (mainly catalase and glutathione peroxidase) in the corneal epithelium of rabbits after 4 days of repeated irradiation. Löfgren and Söderberg (2001) found a decrease in lactate dehydrogenase in the corneal epithelium after irradiation of the rat eye with UVB rays. Together with morphologic and enzymatical disturbances in individual corneal layers, the mechanism maintaining the physiological level of corneal hydration is disturbed, leading to corneal swelling. Corneal light absorption is increased and corneal transparency together with visual acuity decreased. The oxidant/antioxidant imbalance in the cornea caused by reduced levels of antioxidants and abundant amount of

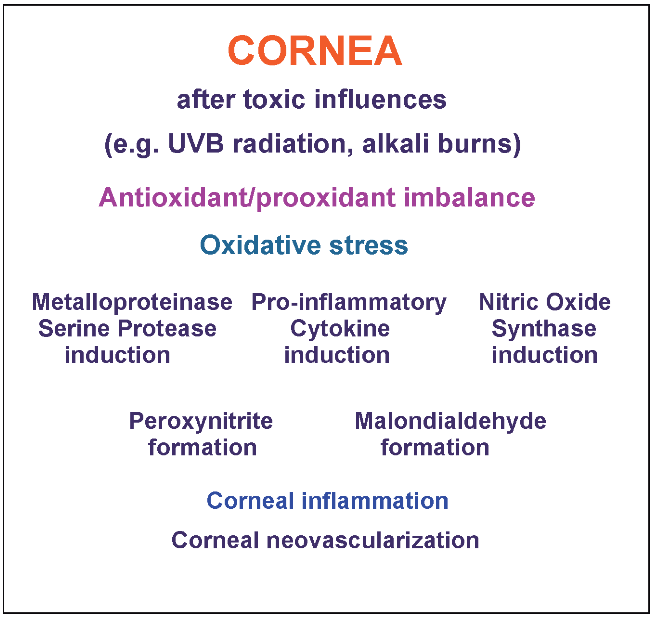


Fig. 3. Disturbances of the cornea after the influence of various toxic agents, such as UVB radiation or alkali burns. In the corneal epithelium the antioxidant enzymes became decreased, whereas the prooxidant enzymes remain at physiological levels or even increase. This leads to the antioxidant/prooxidant imbalance and oxidative stress in the cornea. Oxidative stress is associated with the induction of pro-inflammatory cytokines, metalloproteinases, serine proteases and nitric oxide synthases. Peroxynitrite formation appears, a reaction product between superoxide and nitric oxide. Malondialdehyde, a marker of lipid peroxidation is present in the cornea. This leads to the development of abundant corneal inflammation and/or corneal neovascularization.

reactive oxygen species produced primarily by UVB radiation and secondarily by enzymes, e.g. by xanthine oxidase which generates reactive oxygen species, leads to the development of intracorneal inflammation and corneal neovascularization.

The studies on oxidative stress in the cornea evoked by irradiation of corneas with UVB radiation in experimental animals serve for the development of novel antioxidant therapies for the treatment of corneal diseases or injuries in which oxidative stress is involved. Of these drugs, trehalose in eye drops (supplied by Laboratoires Thea, Clermont-Ferrand, France) had very effective anti-oxidative, anti-inflammatory and anti stressive properties in UVB-damaged cornea (Čejková et al., 2010, 2011, 2012) (Figs. 1, 2). Hyaluronic acid was described to be effective in suppression of oxidative stress in corneas irradiated with UVB rays (Pauloin et al., 2009). For prevention of UVB radiation from sunlight in humans, besides spectacles or contact lenses with effective UV filter, actinoquinol alone or combined with hyaluronic acid was found to be safe in protecting eyes against the damaging effect of UVB radiation (Čejka et al., 2010).

Conclusion

In conclusion, summarized data suggest a strong relationship between the accumulation of oxidative stress in the cornea, the increase in oxidative stress markers, changes in antioxidant mechanisms and the development of corneal disorders (Fig. 3). The management of oxidative stress may provide a new approach for the prevention and treatment of various corneal diseases or injuries.

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