Summary. Trehalose, a nonreducing disaccharide of glucose, produced and stored in many lower and higher organisms, although not in mammals, is synthetized as a stress responsive factor when cells are exposed to various environmental stress conditions. Recently, trehalose has been implicated in various situations in mammals. The aim of this paper was to examine whether trehalose might decrease the damage of the rabbit cornea evoked by UVB rays. During irradiation with UVB rays, consisting of a daily dose of 0.5 J/cm$^2$ for four days, trehalose was applied in eye drops on the right eye and buffered saline on the left eye. One day after the end of irradiation the animals were sacrificed and the corneas examined spectrophotometrically for light absorption. Another group of corneas similarly treated were examined morphologically and immunohistochemically. Corneal thickness (hydration) was measured using a Pachymeter. The results show that compared to buffered saline, trehalose treated corneas displayed fewer corneal disturbances during UVB irradiation. The increases in corneal hydration and light absorption were less pronounced and intracorneal inflammation and corneal neovascularization were suppressed. Nitric oxide synthases that generate nitric oxide were less expressed in the cornea, and formation of cytotoxic peroxynitrite (demonstrated by nitrotyrosine residues) was decreased. The expression of the antioxidant aldehyde dehydrogenase3A1 was less inhibited in the corneal epithelium, and apoptotic corneal epithelial cell death (detected by immunostaining for active caspase-3) was greatly diminished. In conclusion, trehalose reduced UVB-induced damage caused by reactive oxygen and nitrogen species and decreased changes in the corneal optics.

Keywords: UVB rays, Rabbit cornea, Trehalose treatment

Introduction

Trehalose is a nonreducing disaccharide of glucose that is produced and stored in many lower and higher forms of organisms, including bacteria, yeast, fungi, insects, intervertebrates and plants (e.g. Chen and Haddad, 2004). It does not occur in mammalian cells, although humans have the enzyme trehalase in intestinal villae cells and in kidney brush border cells, probably to handle ingested trehalose (Elbein et al., 2003). Trehalose is synthetized in lower organisms as a stress responsive factor when cells are exposed to environmental stress conditions such as heat, cold, dessication and oxidation. When these organisms are exposed to stress, they adapt by synthesizing huge amounts of trehalose, which helps them to retain the cellular integrity. This is thought to occur by prevention of denaturation of proteins by trehalose, which would otherwise degrade under stress (Jain and Roy, 2009).

Recently, our understanding of the role of trehalose has expanded, and it has been implicated in various situations in mammals. Trehalose was considered as a cryoprotectant for the cryopreservation of human oocytes (Eroglu et al., 2002). Trehalose protected both
Drosophilla cells and also mammalian cells from hypoxic and anoxic injury (Chen and Haddad, 2004) and inhibited the proinflammatory phenotype in a transgenic mouse model of ocularpharyngeal muscular dystrophy: it reduced aggerage formation and delayed the pathology of this disease (Davies et al., 2006). According to Béranger et al. (2008), trehalose significantly protected prion-infected cells from induced oxidative damage in cellular models of Alzheimer’s and Huntington’s diseases, suggesting that this compound has a therapeutic effect.

In ophthalmic research trehalose was found to protect corneal epithelial cells from drying (Matsuo, 2001), while Matsuo et al. (2002) reported that trehalose is effective in the treatment of moderate to severe dry eye syndrome. Chen et al. (2009) documented in experimental murine dry eye that trehalose could improve the appearance of ocular surface epithelial disorders due to dessication through the suppression of apoptosis. Izawa et al. (2006) compared the effect of trehalose on the ocular surface with the effect of salinemaltose or sucrose treated surfaces using atomic force microscopy. The indicator for surface roughness was significantly lower after trehalose treatment. The authors suggested that one effect of trehalose on the ocular surface is to keep the corneal epithelial surface smooth under drying conditions.

According to our knowledge, no information exists about the effect of trehalose in a UVB irradiation model. Therefore, we decided to study this problem in order to fill this gap.

Materials and methods

Animals and experimental injuries

Adult New Zealand white rabbits (2.5-3.0 kg) were used in our experiments. The investigation was conducted according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rabbits were anesthetized by an i.m. injection of Rometar (Xylazinum hydrochloricum, 2%, Spofa, 0.2 ml/kg body weight) and Narkamon (Ketaminum hydrochloricum, 2%, Spofa, 0.2 ml/kg body weight). The open eyes of anesthetized rabbits were irradiated (both eyes of each rabbit) with a UVB lamp (Bioblock Scientific, Illkirch Cedex, France; 312 nm wavelength, 6W) with a dose per day of 0.5 J/cm² for four days. The UV lamp stand - with an exactly determined distance between the lamp and eye of the animal - was employed for irradiation. The plane of the lamp was parallel to the tangential plane of the eye (rectangular to the optical axis of the eye). The intensity of irradiation was regularly measured with a UVB sensor connected to a radiometer (both instruments manufactured by Cole-Parmer Inc., Vernon Hills, Illinois, USA). The total dose of irradiation was also checked using these devices. Although the used source of UVB emits only UVB rays peaking at 312 nm (according to the irradiation spectrum given by the manufacturer), a UVC sensor (Cole-Parmer Inc.) was employed to check for the potential emission of UVC light. Only the corneas were irradiated; the rest of the eye surface was protected from UV rays. During the irradiation, trehalose was dropped on the right eye and buffered saline on the left eye. In addition to their application during irradiation, buffered saline and trehalose were applied once in the morning and twice in the afternoon. During the whole experiment, the central corneal thickness (hydration) was measured using a Pachymeter. Every day a microbial examination was performed on all eyes, and only sterile eyes or eyes with non-pathogenic microbes were used. After finishing the experiments (day 4 of repeated irradiation), the animals were left without any treatment and on day 5 they were sacrificed under thiopental anesthesia (Thiopental, Spofa). The eyes were photographically documented. The excised corneas were employed for spectrophotometrical or immunohistochemical and morphological examinations. Normal corneas served as controls.

Ophthalmic solutions

Trehalose (87.6 mM) preservative-free eye drops were supplied by Laboratoires Thea, Clermont-Ferrand, France. Trehalose (anhydrous) was dissolved in an aqueous vehicle containing sodium chloride for tonicity adjustment (315 mosm/kg) and Tris buffer (pH 7.0). Buffered saline (0.01 M) was used as the negative control.

The measurement of corneal thickness (hydration)

The central corneal thickness of anesthetized rabbits was measured (using an Ultrasonic Pachymeter SP-100, Tomey Corporation, Noritake-shinmachi, Nishi-ku, Nagoya, Japan) every day before the irradiation procedures and one day after the end of irradiation before sacrificing the animals.

Spectrophotometry of the whole corneas (the measurement of corneal light absorption)

Immediately after sacrificing the animals, the corneas were examined spectrophotometrically using a method described previously (Čejka et al., 2007). Briefly: The corneas were excised from the sclera and from the center of each cornea a circle of 6 mm diameter was cut out using a special circular knife. Each of these circles was placed into a 6 mm diameter hole in a stainless steel sheet insert, covered on both sides with quartz glass, and the whole assembly was placed between two halves of an insert (made of acryl glass) designed to fit inside a standard quartz cuvette. The insert also contained a 6.0 mm hole that coincided with the measuring light beam of the spectrophotometer, and the instrumental light entered the measured piece of...
cornea from the epithelial side, i.e. from the same direction as light entered the cornea in situ.

The stainless steel sheets were manufactured in advance in an increasing range of thicknesses (0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3 mm). For the spectrophotometrical measurements, an insert with the same thickness as the thickness of the normal or irradiated cornea or the nearest higher thickness sheet insert was used.

Before the spectrophotometrical measurements, the corneas (with the whole assembly described above) were submersed into 340 mOsm kg⁻¹ PBS in the measuring cuvette. The reference spectrophotometrical measurements (with metal sheets for normal and irradiated corneas) were conducted with the same assembly bathed in the same solution (only without the samples). The absorbance readings were made over a range of wavelengths 300-650 nm using a HELIOS b 84021 v4.55 scanning spectrophotometer (Spectronic Unicam, Cambridge, UK) with a wavelength resolution (step size) of 1 nm. The obtained data were expressed either as the spectrum of transmittance $T = T(\lambda)$ or absorbance $A = A(\lambda)$.

For the absorbance the following formula is valid: $A = \alpha \cdot d$, where $\alpha$ [mm⁻¹] is the coefficient of absorption, and d [mm] is the thickness of the light absorbing sample layer.

Morphological and immunohistochemical examination

The anterior eye segments were dissected out and quenched in light petroleum chilled with an acetone-dry ice mixture. Sections were cut on a cryostat and transferred to glass slides. Afterwards cryostat sections were fixed in acetone at 4°C for 5 min.

For the immunohistochemical localization of nitric oxide synthases, nitrotyrosine, aldehyde dehydrogenase3A1 and activated caspase-3, the following primary antibodies were used: monoclonal mouse anti-human endothelial nitric oxide synthase, monoclonal mouse anti-human inducible nitric oxide synthase (BD Biosciences, San Jose, CA, USA), monoclonal mouse anti-nitrotyrosine (Abcam, Cambridge, UK), mouse polyclonal anti-human aldehyde dehydrogenase (ALDH3A1) and mouse monoclonal (3CSPO5) anti-human caspase (Abcam, Cambridge, UK). Subsequently, an anti-mouse HRP/DAB Ultravision Detection System (Lab Vision, Fremont, CA, USA) was employed as recommended by the manufacturer: hydrogen peroxide block (20 min), ultra V block (5 min), primary antibody incubation (60 min), biotinylated goat, anti-mouse antibody incubation (10 min), and streptavidin peroxidase incubation (10 min). Visualization was performed using a freshly prepared DAB substrate/chromogen solution. Some sections were counterstained with Mayer’s hematoxylin (Sigma). Negative controls included the omission of the primary antibody. To confirm the specificity of the staining for nitrotyrosine, the antibody was incubated with 10 mM nitrotyrosine (Kooy et al., 1997). Caspase-3 positive cells and the number of inflammatory cells, as well as their positive staining for nitric oxide synthases and nitrotyrosine were counted (central as well as limbal corneal regions) over an area 100 µm long and 100 µm wide in sagittal cryostat sections.

For morphological examination, post-fixed cryostat sections were stained with haematoxylin-eosin.

Statistics

The repeated measures ANOVA with Bonferroni’s post-test calculated with GraphPad Prizm (GraphPad Software, Inc., San Diego, CA) were used for statistical evaluation of corneal thickness measurements and absorption coefficient data. The ANOVA with post hoc analysis and unpaired t-test were used to compare apoptosis in the cornea and the number of inflammatory cells (corneal morphology), as well as their positive immunohistochemical staining for nitric oxide synthases and nitrotyrosine in the corneal stroma between groups.

Results

Corneal spectrophotometry and corneal thickness (hydration)

UVB irradiated corneas treated with either solutions (buffered saline or trehalose) revealed increased hydration and corneal light absorption. However, as compared to buffered saline, after trehalose treatment corneal light absorption was significantly less increased than after buffered saline application (Fig. 1). Also, the increase in corneal thickness (hydration) after trehalose treatment was significantly less than following buffered saline drops (Fig. 2). Significant differences between transmittance and absorbance after trehalose and buffered saline treatment in UV region were found (Figs. 10, 11).

Macroscopic evaluation of corneal transparency and corneal neovascularization

Corneas irradiated with UVB rays and treated with buffered saline were highly vascularized. Corneal transparency was lost and the corneas turned white (Fig. 3a, arrows). In contrast, corneas treated with trehalose during UVB irradiation revealed lower changes of corneal transparency, as well as suppressed corneal neovascularization (Fig. 3b,c, arrows).

Corneal morphology

Corneas irradiated with UVB rays and treated with buffered saline were vascularized and many inflammatory cells were present in the corneal stroma.
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(Fig. 4a, arrows). In trehalose treated eyes the corneal vascularization was suppressed or even absent, and the number of inflammatory cells in the corneas was much lower than after buffered saline drops (Fig. 4b, arrow). Normal cornea (Fig. 4c). (Significant difference in the amount of inflammatory cells between groups P<0.01, n=6 eyes).

**Immunohistochemical staining to active caspase-3**

Apoptosis of normal corneal cells was minimal or completely absent (Fig. 5e). In UVB irradiated corneas after buffered saline treatment the number of apoptotic corneal epithelial cells and apoptotic keratocytes was highly expressed in the corneal stroma (a, b, arrows). After trehalose treatment, a low number of apoptotic cells was found in the corneal epithelium (Fig. 5c, arrows). In the corneal stroma caspase-3 staining was minimal or even absent (Fig. 5d). Differences between groups were significant at P<0.01, n=6 eyes. Positive staining was not found in control where primary antibody was omitted from the incubation medium; only the nuclei were stained with counterstaining (haematoxylin) in the corneal epithelium (Fig. 5f).

**Immunohistochemical staining for aldehyde dehydrogenase3A1**

In the normal corneal epithelium aldehyde dehydrogenase 3A1 was highly pronounced (Fig. 6c,E, epithelium). Following UVB irradiation and trehalose treatment only slightly decreased enzyme expression in the corneal epithelium was observed (Fig. 6b,E, epithelium). In contrast, after buffered saline treatment during UVB irradiation the expression of aldehyde dehydrogenase 3A1 was greatly decreased in corneal epithelial cells (Fig. 6a). In controls, stainings where the primary antibody was omitted from the incubation medium, no positive staining appeared; only the nuclei were stained with haematoxylin in the corneal epithelium (E, epithelium, Fig. 6d).

**Immunohistochemical staining for nitric oxide synthases and nitrotyrosine**

In the normal cornea endothelial nitric oxide synthase was expressed in the corneal epithelium (Fig. 7c, E, epithelium). The staining of inducible nitric oxide synthase (Fig. 8e) and nitrotyrosine (Fig. 9c) was

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**Fig. 1.** Averaged absorption spectra of corneas, expressed as the transmittance $T = T(\lambda)$ (a) or absorbance $A = A(\lambda)$ (b), of rabbits sacrificed one day after 4 days of trehalose or buffered saline treatment (N=6). The spectrum of normal rabbit corneas (mean from 16 measurements) without any treatment is also included for comparison. Note that for wavelengths shorter than about 300 nm, the spectra show the instrumental stray light error rather than the corneal optical properties. The differences between buffered saline and trehalose treated corneas were significant in UV region (Fig. 10, Fig. 11).

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**Fig. 2.** Thickness of rabbit corneas measured by an ultrasonic Pachymeter before and one day after 4 days of irradiation with UVB rays (daily dose 0.5 J/cm$^2$). During irradiation, trehalose or buffered saline (N=6) was applied. The difference between trehalose- and buffered saline-treated corneas is statistically significant (***: p <0.001)
negligible in the normal cornea. In the cornea irradiated with UVB rays and treated with buffered saline, endothelial nitric oxide synthase (Fig. 7a), inducible nitric oxide synthase (Fig. 8a) as well as nitrotyrosine (Fig. 9a, E, epithelium) were highly expressed in the corneal epithelium. Also, some inflammatory cells revealed staining for endothelial nitric oxide synthase expression (Fig. 7a, arrows). Many inflammatory cells were stained for inducible nitric oxide synthase (Fig. 8a,b, arrows) and nitrotyrosine (Fig. 9a, arrows). As compared to buffered saline, after trehalose treatment nitric oxide synthase stainings were supressed in the corneal epithelium (E) (Figs. 7b, 8c). After trehalose treatment inflammatory cells did not reveal endothelial nitric oxide synthase staining (Fig. 7b), and a lower number of inflammatory cells was stained for inducible nitric oxide synthase.

Fig. 3. Macroscopic images of the eyes of rabbits sacrificed one day after the end of irradiation with UVB rays and treatment with buffered saline or trehalose (corneas were irradiated with a daily dose of 0.5 J/cm² for four days). a. Corneas irradiated with UVB rays and treated with buffered saline are highly vascularized (arrows). Corneal transparency is lost, corneas turned white. b, c. Two typical examples of corneas irradiated with UVB rays and treated with trehalose eyedrops. Changes of corneal transparency as well as corneal neovascularization are suppressed (arrows). d. normal cornea.
nitric oxide synthase (Fig. 8c,d, arrows). For inducible nitric oxide synthase, some keratocytes also showed positive staining (Fig. 8b,d, white arrows). (Significant differences in the number of inflammatory cells and their positive staining between groups, \( P<0.01, n=6 \) eyes). In control staining where the primary antibody was omitted, no positive staining was found; only nuclei were stained with counterstaining (haematoxylin) (Fig. 7d, control for endothelial nitric oxide synthase, d, inflammatory cells in the corneal stroma; Fig. 8f, control for inducible nitric oxide synthase staining, corneal epithelium; Fig. 9d, control for nitrotyrosine staining, corneal epithelium with superficial part of the corneal stroma).

**Discussion**

The role of trehalose in protecting against various stress conditions, including oxidative stress has been well documented in lower organisms as well as in mammals. Herdeiro et al. (2006) described the role of trehalose in protecting cell membranes from oxidative injuries. According to Benaroudj et al. (2001) exposure of Saccharomyces cerevisiae to a mild heat shock evoked an accumulation of trehalose and markedly increased the viability of cells upon exposure to a free UVB-induced corneal damage decreased by trehalose

Fig. 4. Corneal morphology (haematoxylin-eosin staining) of corneas treated with trehalose or buffered saline during UVB irradiation. a. Corneas treated with buffered saline. Corneas are vascularized. Many inflammatory cells are present in the corneal stroma (arrows). b. Corneas treated with trehalose. Corneal neovascularization is strongly suppressed, the number of inflammatory cells is substantially reduced in the corneal stroma (arrows point to inflammatory cells). c. Normal cornea. Scale bar: 10 \( \mu \)M.
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Fig. 5. Apoptotic cell death detected immunohistochemically (active caspase-3) (counterstained with haematoxylin). a, b. Corneas treated with buffered saline during UVB irradiation. Apoptotic cell death is highly pronounced in the corneal epithelium (E) (a). Also, apoptotic keratocytes are present in the corneal stroma (b). Arrows point to positive staining. c, d. Corneas treated with trehalose during UVB irradiation. In the corneal epithelium (E) a low number of apoptotic cells is present (arrows). In the corneal stroma the number of apoptotic keratocytes is minimal or completely absent. e. Normal cornea. In the normal cornea apoptotic cells were not found. f. Control section (negative control, corneal epithelium) for the immunohistochemistry of active caspase-3. Sections were incubated in the absence of the primary antibody. Only the nuclei are stained with haematoxylin. Scale bar: 10 μM.
radical generating system (hydrogen peroxide/iron). Trehalose accumulation decreased the initial appearance of damaged proteins, presumably by acting as a free radical scavenger. According to these authors trehalose accumulation in stressed cells plays a major role in protecting the cellular constituents from oxidative damage. In mammals, Tanaka et al. (2005) found that oral administration of trehalose decreased polyglutamine aggregates in the cerebrum and liver and extended life span in a transgenic mouse model of polyglutamine diseases, such as Huntington’s disease. Minutoli et al. (2007) described the inhibitory effect of trehalose on UVB-induced corneal damage decreased by trehalose.

Fig. 6. Aldehyde dehydrogenase 3A1 staining (counterstained with haematoxylin). a. A cornea treated with buffered saline. In UVB irradiated corneas treated with buffered saline, the staining of aldehyde dehydrogenase3A1 is decreased in the corneal epithelium (E) (compare with the staining in the normal corneal epithelium (c), or after trehalose treatment (b). b. A cornea treated with trehalose. As compared to buffered saline (a) after trehalose treatment during UVB irradiation the enzyme expression is less reduced in the corneal epithelium (E). c. Normal cornea. Aldehyde dehydrogenase3A1 is highly expressed in the normal corneal epithelium (E). d. Control section. Using incubation medium from which the primary antibody was omitted, no positive staining appears; only the nuclei are stained with counterstained with haematoxylin in the corneal epithelium. Scale bar: 10 µm.
In this paper and also in previous studies, the effect of trehalose in a UVB irradiation model has not been investigated. Therefore, we decided to study this problem. To examine whether trehalose might protect the rabbit cornea against UVB-induced damage and changes in corneal optics. In our experimental model, the daily dose of 0.5 J/cm² corresponded to five hours exposure of the human cornea to UVB rays from sunlight (Zigman, 1995).

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Fig. 7. Endothelial nitric oxide synthase expression (counterstained with haematoxylin). a. Cornea treated with buffered saline during UVB irradiation. Endothelial nitric oxide synthase is highly expressed in the corneal epithelium (E) and also in some inflammatory cells in the corneal stroma (arrows). b. Cornea treated with trehalose during UVB irradiation. As compared to buffered saline treatment (a), endothelial nitric oxide synthase is less expressed in the corneal epithelium (E). In the corneal stroma no positive staining in inflammatory cells is seen. c. Normal cornea. In the normal cornea the enzyme is slightly expressed in the corneal epithelium. d. Negative control. Using incubation medium from which the primary antibody was omitted; only the nuclei are stained with haematoxylin in the corneal epithelium. Scale bar: 10 µm.
Fig. 8. Inducible nitric oxide synthase staining (counterstained with haematoxylin). a, b. A cornea treated with buffered saline. In a cornea treated with buffered saline during UVB irradiation the enzyme is highly expressed in the corneal epithelium (E). Many inflammatory cells reveal enzyme expression (a, b, black arrows) in the corneal stroma. Also, some keratocytes are stained (b, white arrows). c, d. Cornea treated with trehalose during UVB irradiation. As compared to buffered saline treatment (a) the staining of inducible nitric oxide synthase is less pronounced in the epithelium (E) (c). The number of positive inflammatory cells is reduced in the corneal stroma (c, d, black arrows). White arrows point to keratocytes, which show positive staining (d). e. Normal cornea. The expression of the enzyme is low in the normal cornea. f. Control section (negative control) (primary antibody was omitted in the incubation medium). No positive staining appears. Only nuclei are stained with haematoxylin in the corneal epithelium. Scale bar: 10 µm.
(Koliopoulos et al., 1979; Haaskjold et al., 1993; Čejková et al., 2000, 2005; Čejka et al., 2007), it has been described that UVB rays damage the corneal epithelium. According to Rogers et al. (2004) sub-solar UVB radiation already causes irreversible damage to the corneal epithelium. Apoptosis appears to be a mechanism of corneal cell death after UVB ray exposure (Podskochy et al., 2000; Newkirk et al., 2007; Pauloin et al., 2009). Pauloin et al. (2009) showed that the exposure of human corneal epithelial cells to UVB rays leads to the activation of caspase-3, which serves as a critical marker for apoptosis. The results of our study show that the apoptosis (detected by immunostaining for active caspase-3) of corneal cells was highly pronounced after UVB irradiation combined with buffered saline treatment. In eyes treated with trehalose during UVB irradiation, corneal cell apoptosis was significantly reduced.

Among other corneal disturbances, Kennedy et al. (1997), Pauloin et al. (2009) described that acute UVB

![Fig. 9. Nitrotyrosine staining (counterstained with haematoxylin). a. A cornea treated with buffered saline. The enzyme expression is highly pronounced in the corneal epithelium (E) and in some inflammatory cells in the corneal stroma (arrows). b. A cornea treated with trehalose. Nitrotyrosine staining is less apparent in the corneal epithelium (E). Some inflammatory cells are positive in the corneal stroma (arrow). c. Normal cornea. The enzyme staining is negligible in the normal cornea (corneal epithelium). d. Negative control (corneal epithelium with the superficial part of the corneal stroma). The antibody was incubated with 10 mM nitrotyrosine in the incubation medium. The cornea is stained only by haematoxylin. Scale bar: 10 µm.](image)
irradiation exposure resulted in the induction of cornea-derived proinflammatory cytokines. The local release of proinflammatory cytokines by cells in the irradiated cornea might be responsible for UV-mediated corneal inflammation and cell death (Caricchio et al., 2003; Yoshizumi et al., 2008). Our results show that intracorneal inflammation was reduced after trehalose treatment (the number of inflammatory cells was significantly lower in the corneal stroma as compared to the number of inflammatory cells in the corneal stroma after buffered saline treatment). Also, corneal neovascularization appearing at the limbus in UVB irradiated corneas after buffered saline treatment, was suppressed after trehalose administration. Corneal neovascularization appears in the response to angiogenic mediators released by leukocytes and damaged corneal cells (Kenyon et al., 1996; Amano et al., 1998).

In UVB irradiated corneas, morphological disturbances are accompanied by a decrease in antioxidants. Corneal antioxidants detoxify reactive oxygen species and thus protect the inner eye against oxidative injury. After repeated UVB exposure, a significant decrease in ascorbic acid in the cornea and aqueous humor was demonstrated (Tessem et al., 2005, 2006). Of enzymes that cleave reactive oxygen species, after UVB irradiation, a decrease of glutathione peroxidase and superoxide dismutase (Čejková et al., 2000; Lodovici et al., 2003), as well as aldehyde dehydrogenase (Downes et al., 1993; Manzer et al., 2003) was described in corneal cells. Aldehyde dehydrogenase3A1 is thought to have a protective effect on the cornea by absorbing UV rays, removing cytotoxic aldehydes produced by UV-induced lipid peroxidation, and generating NADPH (Buddi et al., 2002; Manzer et al., 2003). According to Manzer et al. (2003), a UVB-induced decrease of aldehyde dehydrogenase3A1 may predispose corneal stromal proteins to aggregation and degeneration. In our study, compared to buffered saline treatment where the expression of aldehyde dehydrogenase 3A1 was highly reduced, after trehalose application during UVB irradiation the decrease of aldehyde dehydrogenase3A1 staining was only slight in the corneal epithelium.

Nitric oxide synthases are enzymes that generate nitric oxide. Whereas endothelial nitric oxide synthase is expressed to some extent in the corneal epithelium and endothelium, inducible nitric oxide synthase and brain nitric oxide synthase expression is very low in the normal cornea (Čejková et al., 2005). However, during various ocular diseases or experimental injuries, nitric oxide synthase isomers are expressed in all corneal layers and also in inflammatory cells (mainly inducible nitric oxide synthase) (Buddi et al., 2002; Kim et al., 2002; Čejková et al., 2005). Nitric oxide is an important mediator of homeostatic processes in the eye. However, changes in nitric oxide generation or action have been found to be involved in various pathological states, such as ocular inflammatory or degenerative diseases (Kim et al., 2002; Tsai et al., 2002). Also, in corneas irradiated with UVB rays the expression of nitric oxide synthases (mainly inducible nitric oxide synthase) is increased. Moreover, elevated levels of nitric oxide have been found in the aqueous humor (Čejková et al., 2005). In our experiments we found that compared to buffered saline treatment during UVB irradiation, trehalose application decreased the expression of endothelial nitric oxide synthase and, in particular, inducible nitric oxide synthase in the cornea.

The staining of nitrotyrosine (demonstrating peroxynitrite, a toxic reaction product of nitric oxide and
superoxide) serves as an important marker of free radical damage of ocular tissues. Allen et al. (1996, 1998) described that peroxynitrite caused oxidative damage of cell membranes in the eye and formation of cytotoxic aldehydes. Elevated nitrotyrosine staining was also found in UVB irradiated corneas (Čejková et al., 2005) and after buffered saline treatment in our study. Trehalose application decreased nitrotyrosine expression in UVB irradiated corneas.

After UVB irradiation of the cornea, corneal hydration increases and corneal transparency is changed (the cornea turns grayish) (Doughty and Cullen, 1989; Čejka et al., 2007; Cullen, 2009). We report similar findings after the irradiation of corneas with UVB rays and treatment with either solutions. However, after trehalose application during UVB irradiation the increase of corneal hydration and the changes in corneal transparency were less pronounced than after buffered saline treatment. Altered corneal transparency due to increased corneal hydration has been previously described by Zucker (1966). The transparency is a consequence of the detailed ultrastructure of the tissue and has been attributed to the narrow, uniform diameter collagen fibrils, and to the regularity of their lateral packing (Maurice 1957; Twersky, 1974). The transparency of the corneal stroma is critically dependent on the hydration of the tissue; if the cornea swells, light scattering appears. This scattering has been ascribed to the disruption caused to the arrangement of collagen fibres; changes in the refractive index of the extracellular material make only a small contribution to the increase in light scattering when the cornea swells (Meek et al., 2003). Following UVB irradiation of the cornea, increased corneal hydration appears after several hours (a latent period) due to the damage of the corneal epithelium or endothelium. The damage of corneal cells disturbs the corneal mechanisms by which the cornea maintains normal hydration and transparency, and the cornea becomes opaque (Cullen, 2009).

Using spectrophotometry, Čejka et al. (2007) found that irradiation of the cornea with UVB rays led to increased corneal light absorption, particularly in UVB region. The increased light absorption after UVB irradiation was caused by increased corneal hydration, changes of corneal transparency and also changes to the chemical properties of the cornea (increased amount of proteins). The results of our current study show that compared to buffered saline application, trehalose treatment decreased the changes in corneal light absorption in UVB irradiated cornea.

Summarizing our results with trehalose vs. buffered saline in UVB irradiated corneas, we have shown that after trehalose treatment changes in the corneal optical properties were decreased and corneal inflammation, as well as corneal neovascularization, was inhibited. Immunohistochemically, apoptotic corneal cell death was strongly reduced after trehalose eyedrop treatment, and the expression of the antioxidant aldehyde dehydrogenase3A1 was less suppressed in the corneal epithelium. After trehalose treatment the expression of nitric oxide-generating nitric oxide synthases was decreased and the formation of toxic peroxynitrite was much less pronounced in the cornea.

In conclusion, trehalose inhibited free radical damage to the cornea evoked by UVB rays and decreased changes in corneal optics.

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