

Review

Trehalose in ophthalmology

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Summary. Trehalose, a disaccharide of glucose, is a naturally occurring nontoxic and nonreducing bioactive sugar. Trehalose is synthesized by many organisms when cells are exposed to stressful conditions, including dehydration, heat, oxidation, hypoxia or even anoxia. Although trehalose is not synthesized by mammalian cells, it has recently been demonstrated to have a number of important properties that indicate its utility in humans. Trehalose enables wound healing by protecting cells, especially cell membranes, from oxidative injury and desiccation. When the injured cornea is treated with trehalose, corneal inflammation, scar formation and corneal neovascularization are suppressed. In dry eye disease, trehalose decreased cell apoptosis and reduced oxidative, inflammatory and proteolytic activity at the ocular surface. In UVB irradiated cornea, trehalose suppressed photodamage evoked by UVB rays. It decreased the intracorneal inflammation and reduced corneal neovascularization. Trehalose prevented postoperative fibrous scar formation after ocular surgery, such as glaucoma filtration surgery. The non-toxicity of trehalose allows its administration in humans for extended periods and enables its use in various disease states.

Key words: Trehalose, Antioxidative effects, Antiinflammatory effects, Antiapoptotic effects, Antidehydration effects, Stress responsive factor

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, invertebrates and plants (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, most likely to handle ingested trehalose (Elbein et al., 2003). Trehalose is a stress responsive factor. It is synthesized in lower organisms as a stress responsive factor when cells are exposed to environmental stress conditions such as heat, cold, desiccation and oxidation (Hill-Bator et al., 2014). 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 the denaturation of proteins by trehalose, which would otherwise degrade under stress (Jain and Roy, 2009, 2010). Trehalose is an important protector of protein integrity, by limiting protein denaturation under heat stress, oxidant injury, and anoxia (Chen and Haddad, 2004), and is the most effective disaccharide for stabilizing proteins (Kaushik and Bhat, 2003). Recently, our understanding of the role of trehalose has expanded, and it has been implicated in various situations in mammals (Ohtake and Wang, 2011).

The unique properties of trehalose

Particular attention should be devoted to the fact that trehalose can protect against dehydration, both in lower organisms and also in mammalian cells. According to

the “water hypothesis”, trehalose replaces water in the desiccated organism by hydrogen bonding interactions with polar groups on membrane lipids and proteins (Elbein et al., 2003). According to the alternative hypothesis, trehalose modifies the tetrahedral hydrogen-bond structure of water and rearranges the remaining water molecules around the biological structures. This reduces the amount of water at the interface of biomacromolecules and membranes, in turn avoiding structural fluctuations and damage to cellular organelles during dehydration (Luyckx and Baudouin, 2011). A further unique property of trehalose is to maintain the integrity of phospholipid bilayers, which are essential components of cells. A degree of hydration is essential for the integrity of phospholipid bilayers. The stabilization of phospholipids with trehalose enables the cell membranes to retain their functional properties (Kapla et al., 2015).

In addition, evidence is accumulating that trehalose has a role in stabilizing proteins under stress conditions. The role of osmolytes, especially trehalose, is known to stabilize proteins under stress conditions. The physical and chemical properties of trehalose, i.e. low chemical reactivity, a nonreducing nature and high affinity to water molecules, make trehalose suitable for stabilizing partially unfolded protein molecules and inhibiting protein aggregation (Jain and Roy, 2010).

A further and unique property of trehalose involves protecting cells against oxidative stress. According to Benaroudj et al. (2001), exposure of *Saccharomyces cerevisiae* to a mild heat shock (38°C) or to a proteasome inhibitor (MG132) induced trehalose accumulation and markedly increased the viability of the cells upon exposure to a free radical-generating system (H₂O₂/iron). When the cells were returned to a normal growth temperature (28°C), or when MG132 was removed from the medium, the trehalose content and resistance to oxygen radicals decreased rapidly. The exposure of cells to H₂O₂ caused oxidative damage to amino acids in cellular proteins, and trehalose accumulation was found to reduce such damage. Trehalose accumulation decreased the initial appearance of damaged proteins, presumably by acting as a free radical scavenger. Da Costa Morato Nery (2008) described how during menadione stress, trehalose was necessary intracellularly but under H₂O₂ stress, trehalose was required on the outside of the plasma membrane. The mechanism of protection involves minimizing the oxidative damage caused to both proteins and lipids, which would require the presence of trehalose on both sides of the lipid bilayer.

Trehalose has also proved to be useful for freezing amniotic membrane and stem cells for therapeutic and investigational use. Buchanan et al. (2004) found that a low concentration of trehalose (0.2 M) possessed excellent protective characteristics, as opposed to current methods which use high concentrations (1-2 M) of dimethylsulfoxide. Trehalose was loaded into cells using a genetically engineered mutant of the pore-forming

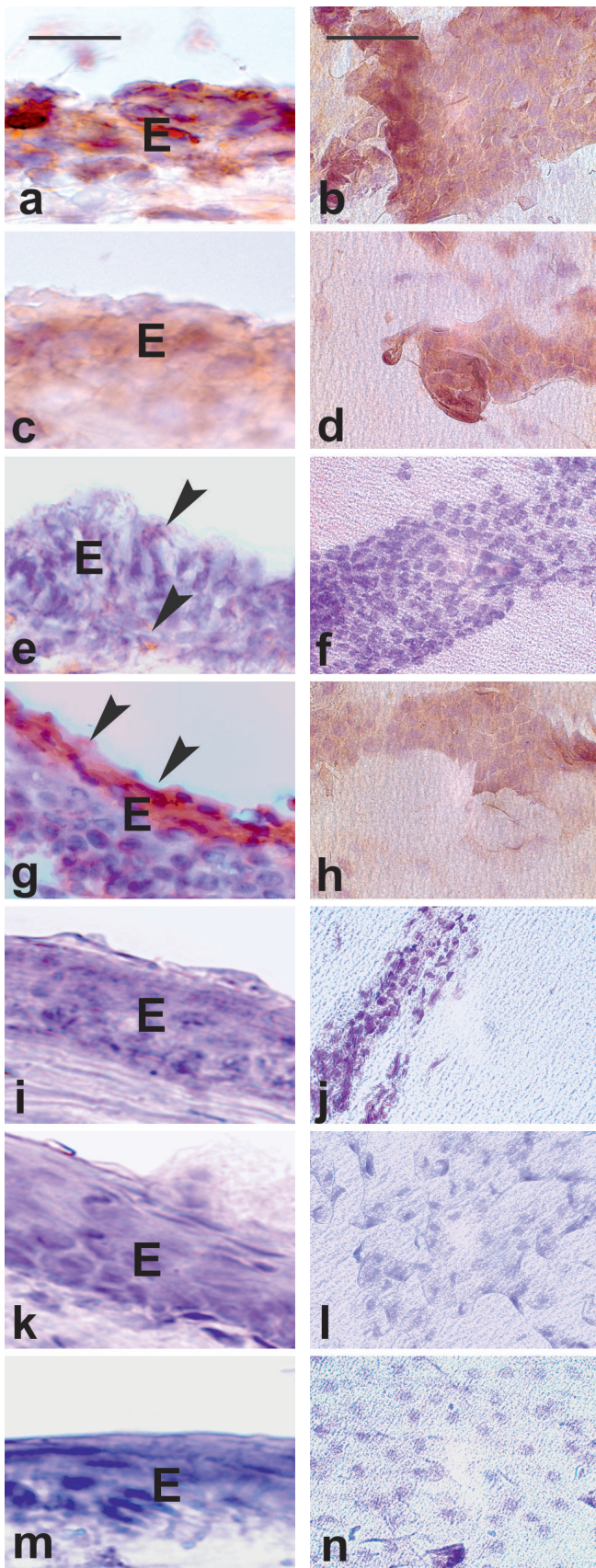
protein alpha-hemolysin from *Staphylococcus aureus*. The method of the above mentioned authors resulted in a nonselective pore equipped with a metal-actuated switch, which is sensitive to extracellular zinc concentrations, thus permitting a controlled loading of trehalose. Buchanan et al. (2004) drew attention to their preliminary experiments which characterized the effects of poration on TF-1 cells (erythroleukemic cell line from blood) and established optimal conditions for trehalose loading and cell survival. TF-1 cells were frozen at 1 degree C/min to -80 degrees C with and without intra- and extracellular trehalose. Following storage at -80 degrees C for 1 week, cells were thawed and evaluated for viability, differentiation capacity, and clonogenic activity in comparison to cells frozen with DMSO. Predictably, the cells that were frozen without any protective agent did not survive freezing. Dovgan et al. (2017) described a new cryopreservation approach using trehalose for a medically applicable stem cell employed in clinical medicine. In this study, the authors assessed the efficiency of combining reversible electroporation and trehalose for the cryopreservation of human adipose-derived stem cells. Firstly, they determined the reversible electroporation threshold by the evaluation of cell viability, using propidium iodide loading into cells. Secondly, the cells were incubated in 250 or 400 mM trehalose and electroporated before cryopreservation. The results show that electroporation seems to be an efficient method for loading nonpermeable trehalose into human adipose-derived stem cells, allowing long-term cryopreservation in DMSO-free and xeno-free conditions.

Nakamura et al. (2008) used trehalose for a freeze-dried amniotic membrane, which showed excellent biocompatibility with ocular surface tissue. Trehalose caused freeze-dried amniotic membrane to retain most of the physical, biological, and morphological characteristics of native amniotic membrane. Consequently, it is a useful biomaterial for ocular surface reconstruction.

Trehalose treatment of ocular diseases and injuries

Trehalose in dry eye disease

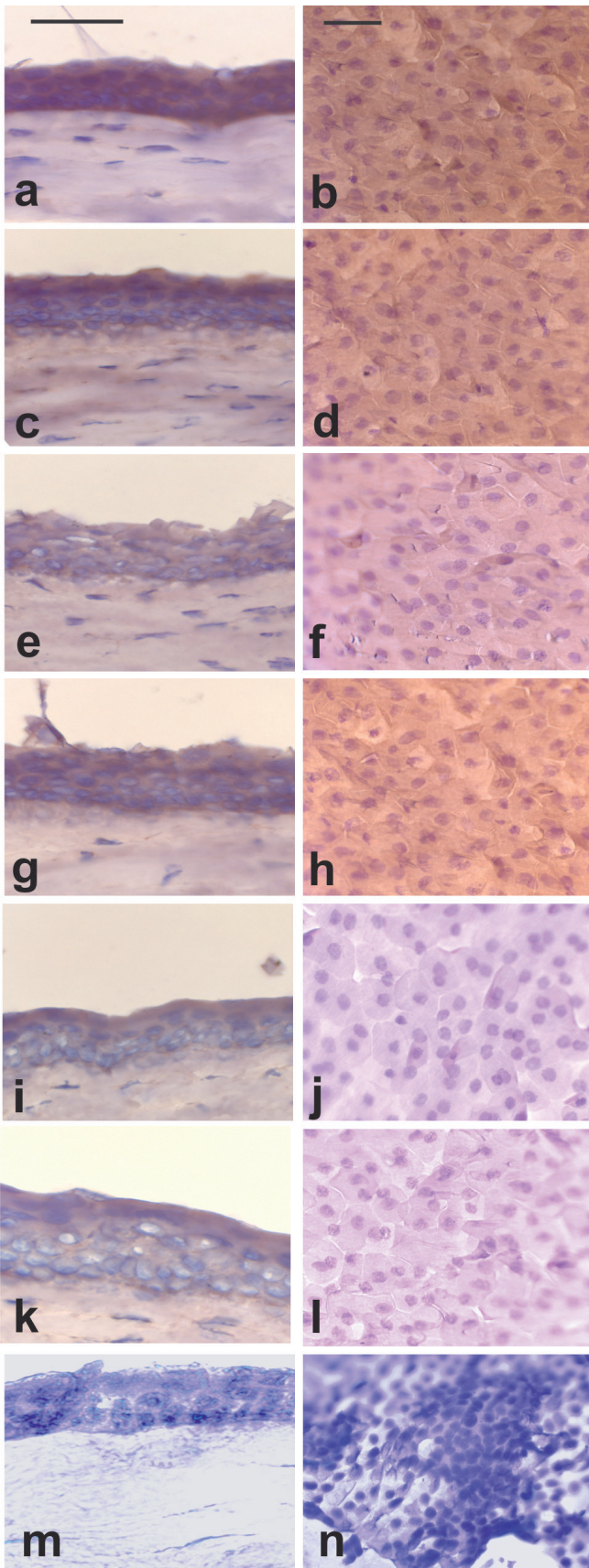
Trehalose has been used to protect the cells of the anterior eye surface against desiccation, particularly in dry eye disease (Matsuo, 2001; Matsuo et al., 2002; Chen et al., 2009; Luyckx and Baudouin, 2011; Hovakimyan et al., 2012; Li et al., 2012; Chiambaretta et al., 2017; McDonald and Fumuso, 2018). Trehalose suppressed pro-inflammatory cytokine induction and matrix metalloproteinase expression in corneal and conjunctival epithelial cells. The effect of trehalose was potentiated by the addition of hyaluronate, an anionic glycosaminoglycan polysaccharide with lubricative and water-retaining properties (Pinto-Bonilla et al., 2015; Schmidt et al., 2015). Matsuo (2004) compared the trehalose solution with hyaluronan or hydroxyethyl-



cellulose in the treatment of moderate to severe dry eye. The results showed that the trehalose solution was a better treatment for dry eye disease than the two commercially available eyedrops referred to. Acer et al. (2018) developed a novel in situ gelling artificial tear formulation, containing both lipid and aqueous components including trehalose, which resembled natural tears. The liposome-based and in situ gelling artificial tear formulation, presented good tolerance and suitable properties for topical ophthalmic administration. The authors suggested that it may be beneficial in the treatment of dry eye disease. In patients with mild to moderate dry eye disease, the effect of a single drop of different lubricant eye gels on tear thickness was measured using ultrahigh-resolution optical coherence tomography (Wozniak et al., 2017). According to these authors, ten minutes after instillation a pronounced increase in the tear film thickness was observed in all groups. However, when compared to other products, trehalose 3% + hyaluronic acid 0.15% offered a significantly longer increase in tear film thickness indicating a longer residence time. Baudouin et al. (2013) employed osmoprotectants, such as erythriol, taurine, trehalose or L-carnitine for cell protection against hyperosmolarity caused by a reduced aqueous tear flow in dry eye disease, and/or increased evaporation of the aqueous tear phase. Osmoprotectants may directly protect cells of the anterior eye surface against hyperosmolarity and thereby promote the exit from the vicious circle of dry eye disease physiopathology.

In a phase III, randomized, active-controlled, investigator-masked, multicenter study in France and Tunisia, Chiambaretta et al. (2017) compared the efficacy and safety of a new eyedrop containing trehalose and hyaluronic acid, to an established formulation containing only hyaluronic acid in patients with moderate to severe dry eye disease. Adult patients (≥ 18 years) with moderate to severe dry eye disease (DED) received either hyaluronic acid-trehalose (n=52)

Fig. 1. Staining for active caspase-3 (counterstained with haematoxylin). Cryostat sections (a, c, e, g, i, k, m), E - corneal epithelium; corneal impression cytologies (b, d, f, h, j, l, n). a, b. Cornea irradiated UVB radiation (312 nm, daily dose 0.5 J/cm² for four subsequent days). Strong caspase-3 expression is seen in the corneal epithelium. c, d. Irradiated cornea treated with buffered saline for one week after irradiation. Caspase-3 expression is prominent in the corneal epithelium. e, f. Irradiated cornea treated with trehalose for one week after irradiation. Only rare apoptotic cells are present in the corneal epithelium (arrows). g, h. Irradiated cornea treated with buffered saline for two weeks after irradiation. Active caspase-3 is present in superficial parts of the corneal epithelium. (arrows). i, j. Irradiated cornea treated with trehalose for two weeks after irradiation. No positive staining for active caspase-3 is seen in the corneal epithelium. k, l. Normal cornea. Positive staining for active caspase-3 is absent in the corneal epithelium. m, n. Negative control. No positive staining appears (primary antibody was omitted from the incubation medium). Čejková et al. 2012, *Histol. Histopathol.* 27, 1029-1040. Scale bar: 10 μ m.



or hyaluronic acid (n=53) 3-6 times per day for 84 days. The primary efficacy variable was the Oxford grading score on day 35. A questionnaire on dry eye and symptoms, Schirmer test, tear break-up time, conjunctival hyperemia, and global performance were assessed as secondary efficacy criteria at baseline, day 35, and day 84. Noninferiority of hyaluronic acid-trehalose to hyaluronic acid for keratoconjunctivitis sicca assessed by the Oxford grading score was demonstrated on day 35. For the secondary efficacy parameters, reductions in dry eye questionnaire classes of none or mild on day 84, dry eye symptoms of stinging, itching, and blurred vision on day 35, and investigator (days 35 and 84) and patient assessments (day 35) of global performance, were significantly better for hyaluronic acid-trehalose. There were no clinically meaningful differences between groups for the other secondary criteria. Both treatments were well-tolerated, and there were fewer ocular symptoms upon instillation and fewer adverse events for hyaluronic acid-trehalose than for hyaluronic acid alone. Similarly, very good results with trehalose/hyaluronate eye-drops in patients with dry eye syndrome were described earlier by Pinto-Bonilla et al. (2015).

Trehalose effects against UVB-evoked ocular photodamage

Trehalose was found to be very effective against photodamage evoked by UVB radiation (Čejková et al., 2010, 2011, 2012). The cornea absorbs and detoxifies the majority of UVB rays reaching the eye, however, under circumstances when a threshold amount of UVB rays is exceeded, a series of harmful disturbances appear, such as changes in corneal optics (Cejka et al., 2007), morphological disorders of the corneal epithelium (Koliopoulus and Margaritis, 1979; Haaskjold et al., 1993; Podskochoy et al., 2000; Rogers et al., 2004), activation of the plasminogen activator/plasmin system (Čejková and Lojda, 1995), the antioxidant/prooxidant

Fig. 2. Staining for urokinase-type plasminogen activator (counterstained with haematoxylin). Corneal cryostat sections (**a, c, e, g, i, k, m**), E-corneal epithelium; corneal impression cytologies (**b, d, f, h, j, l, n**). **a, b.** Cornea irradiated four times with UVB rays. Pronounced staining for the enzyme is apparent in the corneal epithelium. **c, d.** Cornea treated with buffer saline. Staining for the enzyme is strong in the corneal epithelium for one week after irradiation. **e, f.** Cornea treated with trehalose for one week after irradiation. The enzyme expression is reduced in the corneal epithelium. **g, h.** Cornea treated with buffered saline for two weeks after irradiation. The staining remains high in the corneal epithelium. **i, j.** Cornea treated with trehalose for two weeks after irradiation. The enzyme expression is low in the corneal epithelium, very similar to that seen in the normal corneal epithelium (more staining in the superficial layers). **k, l.** Normal cornea. The staining is low in the corneal epithelium (more staining in the superficial layers). **m, n.** Negative control. The primary antibody was omitted from the incubation medium. No positive staining appears. Čejková et al. 2012, *Histol. Histopathol.* 27, 1029-1040. Scale bar: 10 μ m.

Trehalose treatment of ocular diseases

imbalance in the corneal epithelium (Čejková et al., 2004) and alterations in corneal metabolites (Tessem et al., 2005; Fris et al., 2006). Moreover, the induction of reactive oxygen and nitrogen species (Čejková et al., 2005), proinflammatory cytokine expression and corneal neovascularization (Pauloin et al., 2009; Čejková et al., 2011) were found. Trehalose dropped on the ocular surface during UVB irradiation reduced all the above mentioned corneal disturbances (Čejková et al., 2010,

2011, 2012). Since trehalose effectively protected the rabbit cornea against the damaging effect of UVB radiation when applied during the irradiation procedure, the effect of trehalose on the healing properties of the already damaged cornea by repeated UVB radiation was examined (Čejková et al., 2012). The results of the study showed that trehalose eye drops accelerated corneal healing, restored corneal transparency and suppressed corneal neovascularization. Immunohistochemical

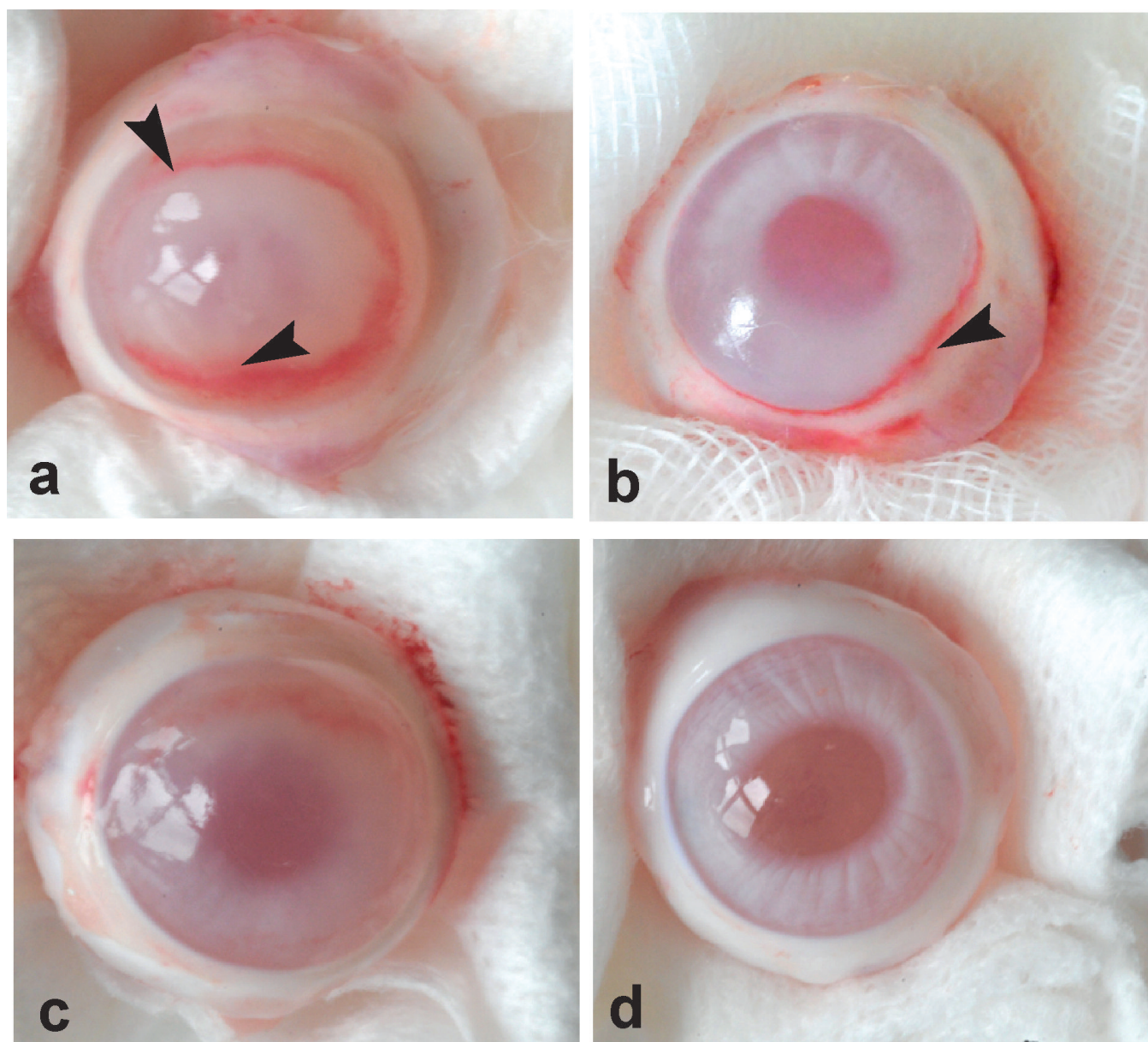


Fig. 3. Macroscopic images of the rabbit eyes one day after the end of UVB irradiation, with a daily dose of 0.5 J/cm^2 for four days, and subsequent treatment with buffered saline or trehalose. **a.** Cornea treated with buffered saline are highly vascularized (arrows). Corneal transparency is lost, and 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. Čejková et al. 2010, *Histol. Histopathol.* 25, 1403-1416.

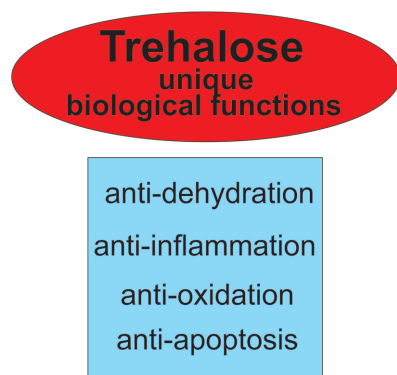


Fig. 4. Graphical scheme of the most important properties of trehalose which verify it for substantial therapeutic use.

results on corneal cryostat sections corresponded with those obtained using corneal impression cytologies, thus confirming that corneal impression cytologies are useful for diagnostic purposes (Figs. 1, 2).

Trehalose protection against ocular hypoxic or anoxic injury

Chen and Haddad (2004) described the efficacy of trehalose against hypoxic or anoxic injury. According to these authors trehalose enhances the protein integrity and reduces protein degradation not only in oxidant injury, but also in hypoxia or anoxia.

The injured cornea is not only damaged by reactive oxygen species induced by the injury but also by hypoxic-response injury, as damaged corneal cells cannot utilize oxygen normally, although the oxygen supply to the cornea is normal (Čejková et al., 2012). This hypoxia is called histotoxic hypoxia. Histotoxic hypoxia greatly contributes to delayed re-epithelialization, the ingrowth of vessels into the cornea and apoptotic cell death. Trehalose effectively suppressed these disturbances (Čejková et al., 2012).

Trehalose in controlling corneal fibrosis and in LASIK (laser-assisted in situ keratomileusis)

Takeuchi et al. (2010, 2011) suggested that trehalose has the potential for use as a new agent to control fibrosis and is thus promising for use in glaucoma surgery. These authors found that in *in vitro* experiments, the growth activities of cultured fibroblasts and keratinocytes were inhibited by trehalose in a dose-dependent manner. Fibroblasts were strongly inhibited by trehalose concentrations $\geq 5\%$, whereas keratinocytes were less inhibited compared to fibroblasts. Expressions of vimentin and α -smooth muscle actin were reduced by trehalose. With *in vivo* experiments, the postoperative application of trehalose resulted in a less firm adhesion between conjunctiva and sclera compared to the controls. Immunohistochemical studies showed a

reduced staining of isolectin B4, vimentin and α -smooth muscle actin in conjunctival wounds treated by topical trehalose. Additionally, after trabeculectomy, intraocular pressure remained in a low range during the instillation of the topical trehalose solution. Takeuchi et al. (2010, 2011) concluded that trehalose has inhibitory effects on the proliferation of fibroblasts and vascular tissues, partially due to the inhibition of the transformation of fibroblasts into myofibroblasts in wound tissues. Mateo Orobía et al. (2017) evaluated the effect of 3% trehalose as an adjuvant in the standard treatment after laser-assisted in situ keratomileusis. The results in the study indicated that the adjuvant treatment with 3% trehalose could be superior with respect to the standard treatment, with improvements in the objective and subjective parameters of tear quality.

Conclusion

Trehalose is a naturally occurring non-toxic bioactive sugar with unique properties (Fig. 4), which verifies it for substantial therapeutic use in various human disease states.

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