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Histopathological and Immunohistochemical Study of the Toxicity of UVB-Irradiation on Rabbit's Cornea: Possible Antioxidant Role of Trehalose

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Authors' contributions

Author RMH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ISE and RMH managed the analyses of the study. Both authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This Study aimed to evaluate toxic changes that might occur in rabbit cornea after UVB exposure and possible protective role of Trehalose.
Study Design: Eighteen adult white female rabbits were divided into three groups, six rabbits for each one. Group I received buffered saline (negative control), Group II irradiated by UVB (positive control) and Group III irradiated by UVB with concomitant application of Trehalose eye drops.
Place and Duration of Study: Department of Pharmacology, and Department of Histopathology, Umm AL Qura University (UQU), KSA, between April 2012 and May 2012.
Methodology: Eighteen rabbits were divided into three equal groups. Group I received buffered saline (0.9%), Group II Only the cornea was irradiated by UVB, and Group III were treated (by dropping trehalose three drops in each eye, six times daily) after irradiated with UVB. Two weeks after treatment, the excised corneas were employed for histopathological and immunohistochemical examinations.

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Results: UVB exposure induced cell death and thinning of the corneal epithelium, while the irradiated epithelium was preserved well morphologically with concomitant application of Trehalose eye drops. The irradiated corneal epithelium was significantly (*P* =0.026) thicker than eyes treated with Ultraviolet and Trehalose eye drops. Apoptotic cells were significantly (*P* =0.001) reduced in negative control and Trehalose -treated eyes than positive control eyes. Trehalose was showed to prevent oxidative stress, accelerated corneal healing, restored corneal transparency and prevent corneal neovascularization in irradiated corneas. Caspase-3, was strong significantly expressed (P<0.5) in the corneal epithelium two weeks after irradiation and corneal neovascularization was evident.
Conclusion: Trehalose prevents apoptosis after the UVB irradiated cornea, so topical Trehalose administration may be a candidate treatment to prevent the damages by UVB irradiation with wide applications in clinical toxicology.

Keywords: Trehalose; UVB rays; corneal healing.

1. INTRODUCTION

Ultraviolet B (UVB) irradiation represents a significant environmental hazard that can cause acute and chronic inflammatory changes in the cornea, lens, and retina of the eye. The sources of UVB radiation are not merely room electric welding and tanning lamps, but also from sunny days on the sea when eyes are left unprotected. In recent decades, the risk of acute photochemically-induced ocular damage has increased [1,2].

One of the causes of ocular damage induced by UVB irradiation is the generation of reactive oxygen species. Reactive oxygen species (hydrogen peroxide, singlet oxygen and oxygen free radicals such as superoxide anions and hydroxyl radicals) are a danger for biological systems [3,4]. They might cause cellular damage by reacting with lipids, proteins and DNA. A number of pathologies have been attributed to the action of reactive oxygen species, and one of the dominant theories of aging suggests that senescent changes are a consequence of the accumulated action of these toxic products [5].

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 (e.g., due to the thinned ozone layer and the more pronounced penetration of solar UVB radiation), a series of harmful disturbances appear, such as changes in corneal optics [2], morphological disorders of the corneal epithelium [3,4].

Trehalose, a-D-glucopyranosyl-a-D-glucopyranoside, is a disaccharide isomer of sucrose with numerous interesting properties [6]. Trehalose is used in a variety of processed foods such as bread, animal-derived deli foods, pouch-packed foods, frozen foods, and beverages, as well as foods for eating out, or prepared at home. This use in such a wide range of products is due to the multifaceted effects of trehalose's properties, such as its inherently mild, sweet flavor; its preservative properties, which maintain the quality of the three main nutrients (carbohydrates, proteins, fats); its powerful water-retention properties, which preserve the texture of foods by protecting them from drying out or freezing and its ability to suppress bitterness, stringency, harsh flavors and the odor of raw foods, meats and packaged foods [7]. This substance is widely present in animals, plants, insects and microorganisms and plays an important role in preserving cells from different oxidant injuries [8]. Trehalose reportedly has an inhibitory effect on the denaturation of protein and

membranes in bacterial [9] and human cells [10] , protective effects on cryo-preserved cells under freeze-dried condition [11] and corneal epithelial cells under dry eye conditions [12-14] and possible therapeutic effects against the progression of Huntington's disease [15,16]. In ophthalmology, trehalose has been used to protect cells of the anterior eye surface against desiccation stress in dry eye disease [17]. The safety and efficacy of topical instillation of trehalose eye drops have been already confirmed [18]. Thus, the aim of the study was to evaluate toxic changes that might occur in rabbit cornea after UVB exposure and possible protective role of Trehalose.

2. MATERIAL AND METHODS

A) Animals and Experimental Groups

Eighteen Adult White New Zealand female rabbits (2.5-3.0 kg) were obtained from the animal house, faculty of Medicine, Umm Al-Qurra University. All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper care and use of laboratory animals. During the two weeks acclimatization and the experimental exposure periods, Rabbits were maintained in an experimental room under controlled conditions of temperature (22-25ºC), no humidity, and a 12-hour light/dark cycle with normal diet and bottles water.

Rabbits were divided into three groups, six rabbits for each Group:

Group I: received buffered saline and served as negative control.
Group II: irradiated by UVB and served as positive control.
Group III: irradiated by UVB and received Trehalose concomitantly.

B) Drugs: *ophthalmic solutions*

Trehalose (87.6mM) preservative-free eye drops (Thealoz®) were supplied by Laboratoires Thea, Clermont-Ferrand, France. Trehalose (anhydrous) was dissolved in an aqueous vehicle containing sodium chloride in order to adjust the tonicity (315 mosml/kg) and Tris buffer (pH 7.4). Phosphate-buffered saline (0.01 M) was used as the negative control.

C) Methodology

(1) UVB irradiation of the animals

The investigation was conducted according to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Six Rabbits were anesthetized by an intramuscular injection of Rometar (Xylazinum hydrochloricum, Spofa, Prague, CR, 2%, 0.2 ml/kg body weight) and Narkamon (Ketaminum hydro-chloricum, Spofa, 5%, 1 ml/kg body weight). The open eyes of anesthetized rabbits were irradiated (both eyes of each rabbit) with a UV lamp (Bioblock Scientific, Illkirch Cedex, France; 312 nm wavelength, 6W) belonging to the National Center for Radiation Research and Technology (NCRRT) with a dose of 0.5 J/cm_2 per day for 14 days days. The plane of the lamp was parallel to the tangential plane of the eye (perpendicular to the optical axis of the eye).

During irradiation, the eyes of anesthetized animals were held open. Only the cornea was irradiated, the rest of the ocular surface was protected by means of a device made of sterile

gauze (slightly soaked with aqua pro injection for softening) with the central hole of the same diameter as the rabbit cornea. Another Six animals were treated (by dropping three drops in each eye, six times daily) with trehalose and irradiated with UVB; the rest of the animals (6 rabbits) received buffered saline (0.9%) for two weeks. Two weeks after the end of treatment, animals were sacrificed under IV injection of thiopental anesthesia (Thiopental, Spofa, 30 mg/kg) and the eyes were enucleated and the excised corneas were employed for histopathological and immunohistochemical examinations.

(2) Histopathological Examination cryostat sections

After sacrificing the animals the eyes were enucleated and the anterior eye segments 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. Subsequently, the cryostat sections were fixed in acetone at 4°C for 5 min. Some sections were counter stained with Mayer's hematoxylin.

(3) Immunohistochemical parameters

For the immunohistochemical localization of active caspase-3, the following primary antibodies were used: monoclonal mouse antihuman caspase 3 (Abcam, Cambridge, UK). The binding of the primary antibodies was demonstrated using the HRP/DAB Ultra Vision Detection System (Thermo Scientific, Fremont, CA, USA) following the manufacturer's Protocol: hydrogen peroxide blocking (15 min), ultra V blocking (5 min), primary antibody incubation (60 min) (mouse anti-human caspase 2 µg/ml), secondary antibody incubation (10 min) and peroxidase labeled streptavidin incubation (10 min). Visualization was performed using freshly prepared DAB substratechromogen solution.

(4) Morphometric study

The number of caspase-3 positive cells in the corneal epithelium, as well as the number of corneal epithelial cells positively stained for caspase -3 were counted using the Olympus BX40, DOT med,s Shipping Quote Service, image analyzer computer system at the Histopathology Department, Faculty of Medicine, Umm AL-Qurra University. Measurements were performed within 10 non overlapping fields for each animal at ×1000 magnification.

(5) Corneal Cell Apoptosis

Apoptosis was scored by counting the number of apoptotic cells, as defined by chromatin condensation or nuclear fragmentation (apoptotic bodies), on PAS-stained sections.

D) Statistics

All quantitative data were presented as mean (X) ±Standard Deviation (SD) and were compared using the unpaired Student's test. P value <0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Trehalose was also found to be effective against oxidative stress of the cornea after UVB irradiation [16-18]. Trehalose has the potential for use as a new agent to control fibrosis and

is thus promising for use in oxidative oxidant stress conditions [19]. Trehalose application restored ocular surface integrity, suppressed inflammatory and proteolytic expression and keratinization in mice with dry eye damaged by a desiccative model [20]. This is important because oxidative stress accompanies a number of human corneal diseases [21]. Trehalose dropped on the ocular surface during UVB irradiation reduced pro-inflammatory cytokine induction, decreased metalloproteinase and xanthine oxidase expression and reduced the antioxidant pro-oxidant imbalance in the corneal epithelium [17].

In the UVB irradiated cornea, trehalose decreased the antioxidant/ pro-oxidant imbalance and apoptotic cell death, reduced proinflammatory cytokine and matrix metalloproteinase induction and greatly suppressed corneal neovascularization. Trehalose might act as a scavenger of oxyradicals. Toxic oxygen products have a damaging effect on amino acids in cellular proteins and the presence of trehalose in cells prevents this damage [22]. There was no change in number of animals among each group either at beginning or end of the study. Active caspase-3 immunostaining, in UVB-irradiated corneal epithelium damage was significantly higher in comparison with treated rabbits with UVB and trehalose or normal (untreated) corneal epithelium as shown in Table 1 and Fig. 1.

Table 1. Active caspase-3 immunostaining, in UVB-irradiated corneal epithelium treated with trehalose vs. untreated (normal or irradiated) corneal epithelium.

	Normal corneal epithelium (negative control) N=6	UVB irradiated corneal epithelium (positive control) N=6	Concomitant UVB irradiated corneal epithelium treated with trehalose (treated) N=6
Mean± SD	0.988±0.669	8.184±1.779	1.482±0.801
P value	0.2438	0.026	0.1674
Significance		*	

Values are expressed as mean ± SD, n=number per each group.
** P < 0.5: Significant statistically difference; while N.S. P> 0.5: non significant value*

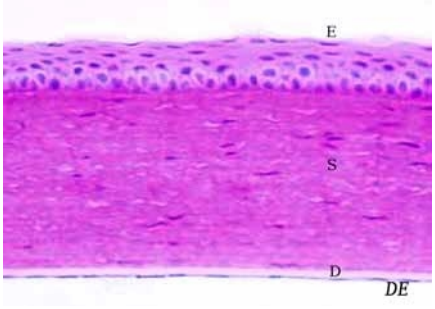
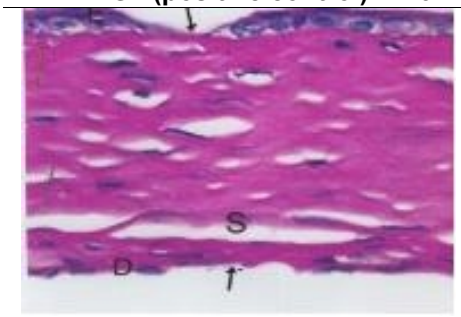
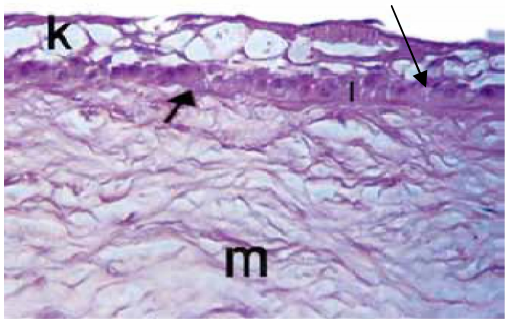
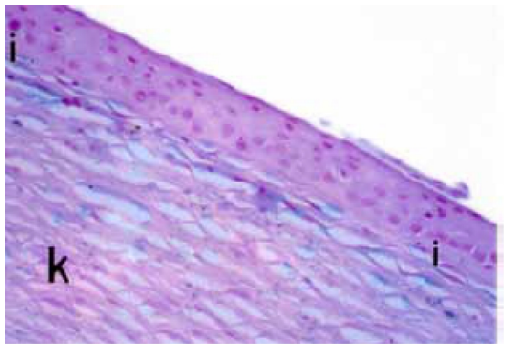


Fig. 1. A photomicrograph of a section of rabbit cornea from the control group (group 1) showing stratified squamous nonkeratinized epithelium (E), substantia propria (S) with regular parallel collagen fibers and flattened keratocytes in between. D, Descement's membrane; DE, Descement's endothelium (b).(H & E x 400).

After two weeks, severe epithelial damage and many apoptotic cells were found in the UVB-irradiated non treated corneal epithelium. The corneal epithelial cells of concomitant UVB irradiated corneal epithelium treated with trehalose revealed the staining for active caspase which was in contrast to the trehalose-treated UVB-irradiated corneas, where following cornea of the histopathological changes and the immunehistochemical staining of corneal

epithelial cells for active caspase-3, it was similar to the normal untreated cornea as shown in Table 2 that contain Figs. 2-9 expressing the histopathological and immunohistochemical changes.

Table 2. Comparison of Microscopic Results of the Studied Groups after the Two Weeks of the Study

UVB irradiated corneal epithelium G2 (positive control) N=6	UVB irradiated corneal epithelium treated with trehalose (G3 i.e. treated) N=6
 **Fig. 2. A photomicrograph of a section in rabbit cornea from the irradiated group showing a loss of corneal epithelium (E) in some areas and apparent decrease in thickness of the epithelium (arrow); corneal stroma fibers were widely separated (S). Descement's endothelium cells are irregular and flat (D). Note loss of Descement's membrane and some endothelial cells (↑↑). (H & E x 400).**	 **Fig. 3. A photomicrograph of a section in rabbit cornea from group 3 irradiated with UVB and treated with trehalose showing normal epithelium. Neither pyknotic nuclei nor congested nuclei were present. (H & E x 200).**
 **Fig. 4. A photomicrograph of a section in rabbit cornea from the irradiated group with UVB showing irregular epithelial cells containing pyknotic nuclei (k) with vacuolation of cells of the middle layer of the corneal epithelium (arrow). The stroma shows irregular keratocytes with separation of collagen fibers (m). (H & x 400).**	 **Fig. 5. A photomicrograph of a section in rabbit cornea from concomitant UVB irradiation and trehalose treatment group after two week showing regenerative epithelium with no blood vessels and no cytoplasmic vacuolation (i) as well as mild separation of stromal collagen lamellae (k). (H & E x 400).**

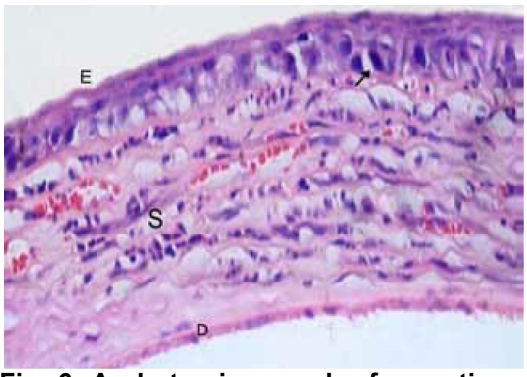


Fig. 6. A photomicrograph of a section in rabbit cornea from the UVB irradiated group showing vacuolation of cells of the middle layer of the corneal epithelium (E) (wing cells) and congested dilated blood vessels and cellular infiltration (arrow). Some cells showed hyperchromatic nuclei (↑). Noticed loss of demarcation between the epithelium and the stroma. (H & E x 400).

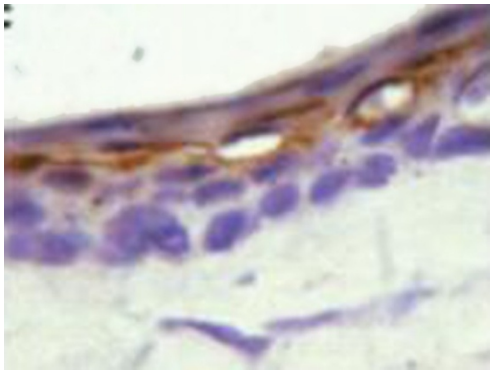


Fig. 7. A photomicrograph of a section in rabbit cornea of concomitant UVB irradiation and trehalose treatment group one day after irradiation showing MILD caspase-3 reactivity in the corneal epithelium. (Caspase-3, × 1000 magnification).

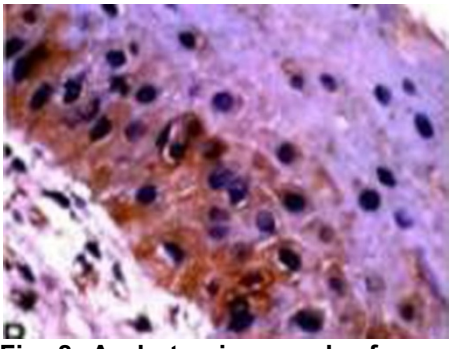


Fig. 8. A photomicrograph of a section of rabbit cornea from the irradiated group showing HIGH caspase-3 reaction in the corneal epithelium, especially in the middle layer. (Caspase-3, × 1000).

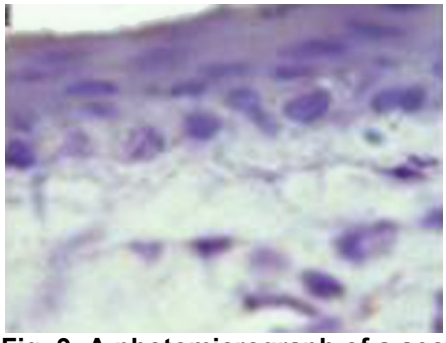


Fig. 9. A photomicrograph of a section in rabbit cornea of concomitant UVB irradiation and trehalose treatment group after two weeks, Collagen fibers of the stroma (s) show an apparently NORMAL proper histological pattern. (Caspase-3, × 1000).

UVB-irradiated corneal cell showed significantly increased apoptosis in comparison with normal or treated cells with trehalose drops, apoptosis was four times in irradiated rabbit cornea. The number of apoptotic cells was low along in the group with corneal irradiation and treatment with trehalose drops as shown in Table 3.

Table 3. Effect of Treatment by Trehalose vs. Untreated (irradiated) on Apoptosis in Corneal Epithelium of the Rabbit

Studied group	Apoptotic cells in 30×40 field Mean ±SD at day 1	Apoptotic cells in 30×40 field Mean ±SD after 2 Ws	P value and significance
Normal corneal epithelium G1(negative control)	8±2	7±3	
UVB irradiated corneal epithelium G2 (positive control)	16±7 *	60±29 *	*P* =0.001*
concomitant UVB irradiation and trehalose eye drops treatment G3 (treated)	10±9	12 ±8	

Values are expressed as mean ± SD, n=number per each group.
** P < 0.5: Significant statistically difference; while N.S. P> 0.5: non significant value*

In this experiment**,** UVB-irradiated corneas in which trehalose were dropped repeatedly for two weeks on the corneal surface during irradiation, showed that trehalose greatly prevented the apoptotic death of corneal cells. These results indicated that apoptotic cell death was significantly reduced in the irradiated corneas treated with trehalose. After two weeks of trehalose repeated treatment since irradiation, apoptopic cells were not observed in the cornea, whereas in untreated irradiated corneas, apoptotic cell death was apparent in the cornea at the end of the second week of treatment (Table 3).

The results of this study showed the efficacy of trehalose in accelerating corneal antioxidant effect and inhibiting apoptosis. According to Jain and Roy, 2009 [10], sub-solar UVB radiation has already caused irreversible damage to the corneal epithelium. Apoptosis appears to be a mechanism of corneal cell death after UVB ray exposure [15, 23,24]. The exposure of human corneal epithelial cells to UVB rays leads to the activation of caspase-3, which serves as a critical marker of apoptosis [9,15,24].

According to some other reported studies, trehalose is an example of how this disaccharide molecule can enhance protein integrity and limit protein degradation not only in oxidant injury, but also in hypoxia or anoxia [2,25].

Although after the end of irradiation, a high number of UVB-irradiated corneal epithelial cells were stained for apoptosis markers, with concomitant treatment for two weeks of trehalose, the number of positively stained corneal epithelial cells was reduced, absent, or present in a very low expression. The staining was not present similarly as in the normal untreated corneal epithelium.

In addition, it is widely accepted that trehalose can protect cells against desiccation. Trehalose was reported to be more efficient for treating dry eye syndrome than commercial eyedrops containing hyaluronan or hydroxyethylcellulose [26]. This beneficial effect is also attributable to trehalose's ability to protect cell membranes [27] and proteins from oxidative

injury by acting as a free-radical scavenger [28]. Furthermore, trehalose suppresses proinflammatory phenotype activation in macrophages in experimental septic shock [29] and in a model of peritoneal inflammation by protecting against I-kappa B-alpha dephosphorylation [30]. Trehalose supplementation also protects against apoptosis [20, 31].

Our results supported by Cejková et. al, 2012 [32] who provided the first time important evidence that trehalose applied on the surface of corneas for two weeks with repeated UVB irradiation (312 nm, daily dose 0.5 J/cm2) accelerated corneal healing, restored corneal transparency and suppressed corneal neovascularization. Compared to buffered saline treatment, following which caspase-3, nitrotyrosine, malondialdehyde and urokinase-type plasminogen activator were still strongly expressed in the corneal epithelium two weeks after irradiation and corneal neovascularization was evident, apoptotic cell death was already significantly reduced after one week of trehalose application. The expression of other markers of injury returned to normal levels during two weeks of trehalose treatment and explained his results that toxic oxygen products induced by UVB irradiation in the cornea are sufficiently removed by antioxidant effect of Trehalose.

While, De Cooman et al. [33] founded the need for caution when interpreting composite electron paramagnetic resonance (EPR) spectra and thermally induced spectral changes of radiation-induced species, even in case of single dose administration of Trehalose. It also provides further evidence that the pathways for radiation damage critically depend on the specific conformation of a molecule and its environment, but also that carbonyl group formation is a common process in the radiation chemistry of sugars (as trehalose) and related compound.

4. CONCLUSION

Trehalose had promoting healing effect on UVB corneal toxicity which is likely to be related to a decrease in the hypoxia-response injury of the cornea. We can also conclude that trehalose may inhibit apoptotic cell death, so can be used as a treatment in clinical toxicology.

CONSENT

Not applicable.

ETHICAL APPROVAL

Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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