Original Article

Evaluation of the osmoprotective and bioprotective effect of trehalose 3%

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Abstract

Purpose: The purpose of this study was to study the osmoprotective and bioprotective effect of trehalose 3% + carboxymethylcellulose (CMC) 0.5% during desiccation conditions.

Materials and Methods: Normal human conjunctival epithelial cells (IOBA) were exposed to (1) culture media (control); (2) CMC 0.5% + glycerin 0. 9% + polyethylene glycol 400 0.25% (vehicle); and (3) vehicle + trehalose 3% (trehalose). Cells were treated for 1 h and then exposed to desiccating conditions. Metabolic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and cell viability by trypan blue. Trehalose water retention capability was assessed by a gravimetric analysis.

Results: Metabolic activity was maintained during the first 30 min under desiccating conditions with no differences found between groups. After 45 min, metabolic activity decreased both in the control group and the vehicle group, while the trehalose group maintained activity values. Cell viability of the trehalose group was maintained throughout time and was statistically superior to those of the control and the vehicle groups at all evaluated times ($P < 0.01$). The retention time 20-RT20 - (20% of water retention) was reached at 8 min in the control group, 10 min in the vehicle group, and 15 min in the trehalose group.

Conclusions: The addition of 3% trehalose prolonged cell viability and extended water retention time in high-dryness environmental conditions.

Keywords: Bioprotection, cell viability, dry eye, osmoprotectants, osmoprotection, trehalose

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INTRODUCTION

Dry eye disease (DED) is "*a multifactorial disease characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles.*"[1] These symptoms (ocular discomfort, dryness, pain, and poor visual quality) affect negatively the patient's quality of life limiting their ability to work and challenge them when reading, watching TV, and doing other daily tasks, affecting their mental health.[2,3] Tear hyperosmolarity is one of the central events in the vicious circle of DED and refers to a state in which the osmolarity of the tear film exceeds that of the epithelial cells, leading to an outflow of water from cells, thus

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reducing their volume, stimulating epithelial cells death and inflammatory events that lead to tear film instability, and contributing circular events that perpetuate DED.[4] Tear film and ocular surface society's dry eye workshop II (TFOS DEWS II) Management report[5] recommends the addition of osmoprotectants to lubricant eye drops to protect epithelial cells from extreme osmotic stress conditions due to their property of balancing the osmotic pressure without disturbing cell metabolism. Trehalose is a natural nonreducing disaccharide of glucose synthesized by lower organisms as a stress-response factor when cells are exposed to environmental conditions such as heat, cold, desiccation, and oxidation.[6] While some authors have evaluated trehalose's protective effects when administered topically into the eye alone or with sodium hyaluronate on the corneal epithelium, no study has yet been carried out to evaluate its protective effect on conjunctival cells and its effect when combined with carboxymethylcellulose (CMC).

The objective of this work was to study the protective effect of the addition of 3% trehalose to a vehicle with 0.5% CMC and glycerin during desiccation conditions on conjunctival cells.

MATERIALS AND METHODS

Design, settings, and medications

This experimental study was performed in the Laboratory of Ocular Research, Pathology Department, Medicine School, University of Buenos Aires, Argentina. Ophthalmic preparations under study were: CMC 0.5% + glycerin 0.9% + polyethylene glycol 200 0.25% (vehicle) and vehicle + 3% trehalose (Aucic® Plus, Poen Laboratories, Argentina). Both ophthalmic preparations were used within their labeled expiration dates. The control group was culture media, Dulbecco's modified Eagle's medium–Nutrient Mixture F-12 (DMEM-F12).

Cell culture

The normal human conjunctival epithelial cell (IOBA-NHC) line was kindly provided by Yolanda Diebold, Ph.D. from the University of Valladolid, Spain.[7] Cells were subcultured once a week to maintain the cell line. Medium consisted of DMEM-F12 supplemented with 10% fetal bovine serum (Internegocios, Mercedes, Buenos Aires, Argentina), 2 ng/mL epidermal growth factor, 5 μg/mL hydrocortisone (Sigma, St Louis, MO, USA), 1 μg/mL bovine pancreas insulin (Sigma), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 mg/mL amphotericin B. Cells were incubated at 37°C in a humidified 5% CO_2 incubator.

Cell treatment

Ophthalmic preparations studied (control, vehicle, and trehalose) were added to cell culture after 1 h, then the medium was completely aspirated, and cell cultures were exposed to desiccating conditions (25°C and 36%–38% relative humidity).[8]

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cytotoxicity of treatments on IOBA-NHC cells was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which measures viability and cell proliferation.[9] MTT is a yellow aqueous solution, which is reduced by dehydrogenases and reducing agents present in metabolically active cells, to insoluble violet-blue formazan crystals. IOBA-NHC cells were plated at 3×10^4 cells per well in a 24-well culture plate for 24 h before treatment. The culture medium was removed, and eye drop treatment was performed. After that, test solutions and control medium were removed, and each well was washed twice with phosphate-buffered saline, pH = 7.4. A culture medium with 0.4 mg/mL MTT (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to each well (final volume = 500 μL). Plates were incubated for 3 h in a humid atmosphere of 37°C with 5% CO_2. After the incubation time, supernatants were discarded, and a solution of isopropyl alcohol was added to dissolve formazan crystals. Incubation was carried out for 30 min in the dark, and the absorbance of each supernatant was recorded at 570 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). Results are expressed as a percentage of cell viability and cell proliferation, where 100% represents the mean value of the absorbance obtained for the control group.

Trypan blue assay

Cell viability was assessed with a trypan blue (Sigma-Aldrich Corp, St. Louis, MO, USA) dye exclusion assay. It is based on the principle that live cells possess intact cell membranes that exclude this dye. Nonviable cells will have a blue cytoplasm. Cell cultures were treated with the three treatments before mentioned (control, vehicle, and trehalose) as explained in the cell treatment section. At 10, 15, 20, and 30 min of desiccating conditions, cells were treated with 0.05% trypsin to obtain a cell suspension. Then, trypan blue solution was added following the manufacturer's protocol. Then, cells were counted using a Neubauer chamber. Viability was expressed as the percentage of viable unstained cells in suspension related to the total of cells counted in a hemocytometer counting chamber. Counts were done in triplicate.

Water retention capability

Water retention capability was assessed by gravimetric

filter method where Whatman filter Grade 1 (1 cm^2) was impregnated with 5 mL of each solution under analysis. The weight of each filter paper was evaluated over time until the weight remained unchanged. The initial weight (time = 0 min) represents 100%. All samples were kept exposed to room temperature (25°C) and constant humidity (36%–38%) in an open container throughout the study.

Statistical analysis

Data are available at the laboratory of ocular research. The presented results correspond to three independent experiments performed in triplicate. Data are presented as mean ± standard deviation. Statistical analysis was performed using SPSS 17 (IBM, Chicago, Illinois, United States of America ;WinWrap Basic, Copyright 1993-2007 Polar Engineering and Consulting); the outcome variables evaluated were the percentage of absorbance and viable cells. The Student's *t*-test for two independent samples was used to calculate the difference between groups. Statistical significance was set at $P < 0.05$.

RESULTS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

As shown in Table 1, there were no differences found between groups during the first 30 min under desiccating conditions. After 45 min of exposure, metabolic activity sharply decreased in the control group (0.111 ± 0.007) and the vehicle group (0.107 ± 0.027), while the trehalose group maintained metabolic activity values [0.560 ± 0.008; $P < 0.001$; Figure 1]. The data show that the addition of 3% trehalose maintains cell metabolism between 15 and 30 min compared to the vehicle or control, representing approximately a 50% greater extension in conjunctival epithelial cell survival.

Trypan blue assay

Cell viability evaluated by trypan blue dye exclusion assay [Table 2 and Figure 2] was maintained throughout the time in the trehalose group (10 min: 62.7 ± 2.5, 15 min: 58.7 ± 8.1; 20 min: 62.3 ± 10.8; 30 min: 41.7 ± 8.5%) and was statistically superior to the control and vehicle groups at all evaluated times ($P < 0.01$).

Table 1: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

MTT assay absorbance media ± SD			
Time (min)	Control	Vehicle	Trehalose
15	0.586±0.012	0.575±0.028	0.523±0.027
30	0.538±0.078	0.426±0.115	0.569±0.075
45	0.111±0.007	0.107±0.027	0.560±0.008
60	0.102±0.003	0.099±0.004	0.106±0.007

Absorbance media ± SD by group. SD: Standard deviation, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Water retention capability

Filter papers soaked with DMEM medium or vehicle showed similar water retention times, with almost complete evaporation after 10 min; however, the solution with 3% trehalose showed a significant delay in evaporation with a 20% time retention (TR20) of 5 min (~50%) compared to the evaporation time of the vehicle group and 8 min (~80%) compared to the evaporation time of the control group [Figure 3]. These results show that 3% trehalose prevents evaporation by increasing the percentage of water retention between 50% and 80% compared to the vehicle without trehalose and control medium.

DISCUSSION

Dry eye is a multifactorial disease of tears and ocular surface that results in tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film which stimulates epithelial cell death and triggers inflammation events.[1,10]

In response to osmotic stress, epithelial cells lose water. Water loss brings about serious consequences, protein aggregation, oxidative stress, changes in membrane, and damaged DNA.[11] Dry eye can be temporally corrected using hypotonic eye drops; however, due to the short time of efficacy of this approach, the use of osmolytes, like trehalose, is mandatory to protect epithelial cells from death.[12] Organic osmolytes are small solutes used by numerous organisms under osmotic stress to maintain cell volume.[13] Osmolytes reduce osmotic stress by counterbalancing external osmotic stress, limiting the loss of water.[12]

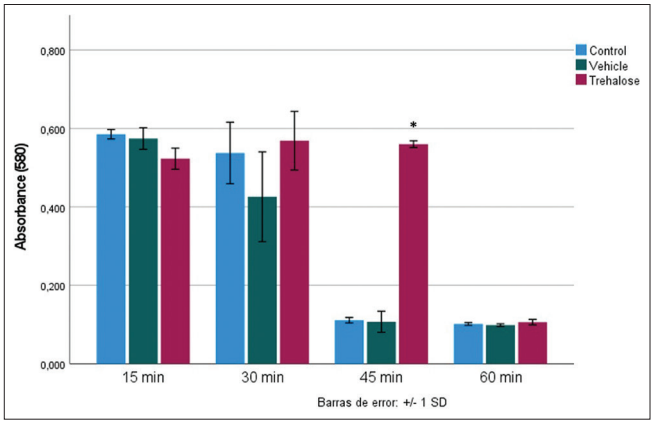

Figure 1: Metabolic activity of human conjunctival epithelial cells under desiccation conditions determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay over time. The spectrophotometry lecture was performed at 580 nm. *$P < 0.001$. SD: Standard deviation

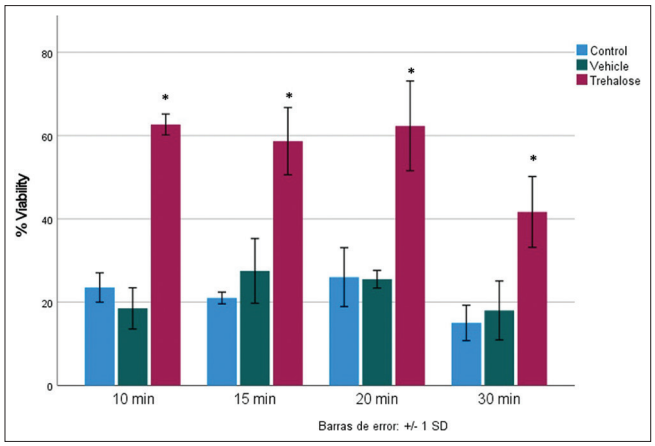

Figure 2: Viability of human conjunctival epithelial cell culture under desiccation conditions by trypan blue assay over time. The spectrophotometry lecture was performed at 580 nm. **P* < 0.01. SD: Standard deviation

Table 2: Trypan blue assay

Trypan blue assay percentage viability ± SD			
Time (min)	Control	Vehicle	Trehalose
10	23.5±3.5	21.0±5.6	62.7±2.5
15	21.0±1.4	27.5±7.8	58.7±8.1
20	26.0±7.1	23.7±3.5	62.3±10.8
30	15.0±4.2	18.7±5.1	41.7±8.5

Percentage of viability ± SD by group. SD: Standard deviation

In addition to its osmoprotective function, trehalose exerts a protective effect by stabilizing biological membranes, probably by interacting with the lipid polar head groups; this results in retention in both cell function and membrane integrity.[13,14] In situations of extreme desiccation, osmolytes can replace the lost shell of water around macromolecules, interacting directly with the dehydrated protein through hydrogen bonding between its hydroxyl groups and polar residues of the protein, alleviating damaging effects caused by drying (the water replacement hypothesis).[13,14] Scientific data suggest that trehalose cooperates by promoting proteostasis in eukaryotic cells like a "chemical chaperone," preserving the tertiary structure of proteins and preventing the formation of aggregates.[11] The formation of a trehalose–water cytoplasmic glassy matrix with the mechanical properties of a plastic solid is another mechanism by which trehalose might protect cells during severe desiccation.[13]

The protective effect of trehalose on corneal human epithelium cultures was previously investigated.[15] Matsuo studied different concentrations of trehalose and its effect on preventing death by desiccation. Compared to sodium hyaluronate 0.1% and hydroxyethylcellulose eye drops, trehalose presented significantly less cell death when exposed to ambiental dryness.[8] Hill-Bator *et al.* evaluated

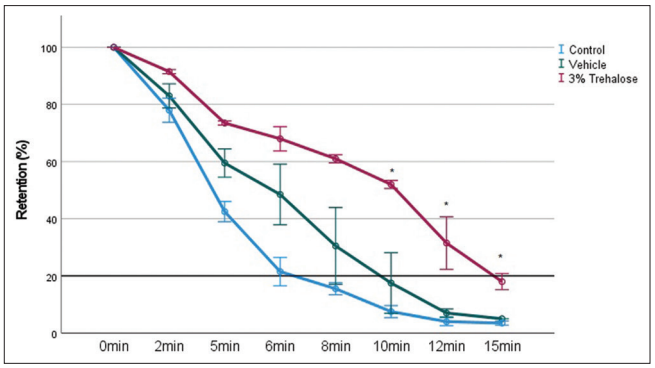

Figure 3: Percentage of water retention over time under desiccation conditions. **P* < 0.01

the effect of commercially available eye lubricants, including 3% trehalose eye drops on viability and functionality when exposed under airstream to the fume hood without any liquid. Trehalose eye drops presented the highest effectiveness in preventing cell death from desiccation and keeping cell membranes function.[16] Chen *et al.* recently evaluated the effect of trehalose in an experimental murine dry eye model. Trehalose reduced apoptosis in the cornea and conjunctiva during desiccation *in vivo*; an increase in the number of goblet cells was also found.[17] However, up to now, there is no evidence of the protective effect of trehalose on conjunctival human epithelial cells.

In our trial, human conjunctival epithelial cells were exposed to a vehicle containing CMC + glycerin and to a combination of trehalose + CMC + glycerin. We demonstrated that trehalose protects the viability and metabolic activity of human conjunctival epithelial cells of high-dryness condition compared to the vehicle and control; this could be due to its osmoprotective and bioprotective characteristics, but also to its ability to induce autophagy, a vital cellular process that involves the recycling of misfolded proteins and damaged organelles facilitating cell and tissue homeostasis.[18]

The cell line used is a representative tool of ocular surface cell biology.[7,19]

There is no evidence of the protective effect against dryness of the combination of trehalose and CMC. The last one (CMC) is a high-molecular-weight polysaccharide that increases tear viscosity, favoring lubrication of the ocular surface.[5] This polymer binds epithelial cells by interaction with glucopyranose subunits of glucose transporters and prolongs the residence time of artificial tears on the ocular surface.[20] Besides being highly viscous and mucoadhesive, CMC may have some additional properties. It has been demonstrated that CMC may have a modulatory effect on corneal epithelial wound healing.[20]

Water retention ability was also tested through the weighting of filter papers dampened with trehalose and CMC formulations. Our results showed that the weight of the trehalose-soaked filter paper was significantly higher than that of the vehicle-soaked one. This indicates that there should have been less evaporation of water from the filter paper soaked with the trehalose solution. Trehalose's water-binding ability is attributed to the strong bonds of these molecules.[21] Experimental findings demonstrate that the interaction between trehalose molecules and water is stronger than the interaction between water molecules (H_2O–H_2O).[21] This property could slow water evaporation and maintain the stability of the tear film.

Since trehalose preserves the viability of human corneal and conjunctival epithelial cells under drying conditions and delays water evaporation, the use of lubricants containing this osmolyte could be an interesting therapeutic strategy for the treatment of dry eye.

CONCLUSIONS

The addition of 3% trehalose prolonged cell viability and extended water retention time in high environmental dryness conditions. This study confirms the protective effects of trehalose on conjunctival epithelial cells, which represents an additional benefit for patients suffering from DED.

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Conflicts of interest

Giselle Rodriguez, María Silvia Passerini, and Melina Sol del Papa are Poen Laboratories employees.

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