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Efficacy of a New Ocular Surface Modulator in Restoring Epithelial Changes in an In Vitro Model of Dry Eye Syndrome

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ABSTRACT

Purpose: So far tear substitutes have demonstrated a limited role in restoring ocular surface damage in dry eye syndrome (DES). The aim of this study was to assess the efficacy of a new ocular surface modulator in an in vitro model of human corneal epithelium (HCE) damaged by severe osmotic stress mirroring the features of dry eye conditions.

Methods: A reconstructed HCE model challenged by the introduction of sorbitol in the culture medium for 16 h was used to induce an inflammatory pathway and to impair the tight junctions integrity determining a severe modification of the superficial layer ultrastructure. At the end of the overnight stress period in the treated HCE series, 30 µl of the ocular surface modulator (T-LysYal, Sildeha, Switzerland) and of hyaluronic acid (HA) in the control HCE series were applied for 24 h. The following parameters were quantified: scanning electron microscopy (SEM), trans-epithelial electrical resistance (TEER), immunofluorescence analysis of integrin β1 (ITG-β1), mRNA expression of Cyclin D-1 (CCND1), and ITG-β1.

Results: In the positive control after the osmotic stress the HCE surface damage was visible at the ultrastructural level with loss of cell–cell interconnections, intercellular matrix destruction, and TEER reduction. After 24 h of treatment with T-LysYal, HCE showed a significant improvement of the ultrastructural morphological organization and increased expression of ITG-β1 at the tissue level when compared to positive and control series. A significant increase of mRNA expression for ITG-β1 and CCND1 was shown in the HA-treated cells compared to T-LysYal. TEER measurement showed a significant reduction in all groups after 16 h without modifications after the treatment period.

Conclusions: This study has shown the possibility of a new class of agents denominated ocular surface modulators to restore corneal cells damaged by dry eye conditions. Further in vivo studies are certainly necessary to confirm these results.

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Corneal epithelium; dry eye syndrome; inflammation; ocular surface modulator; scanning electron microscopy

Introduction

Dry eye syndrome (DES) is one of the most frequent pathologies in ophthalmology, affecting millions people worldwide. On the basis of questionnaires and the diagnostic criteria, the current estimates of the prevalence of DES range from over 5% to 33% at the age of 65 years.[1] The importance of dry eye is based also on its capacity to affect the quality of life of patients by means of symptoms of pain and irritation, which in the severe forms of the disease are comparable to those reported for moderate to severe angina,[2] and to limit and degrade the performance of common vision-related daily activities, such as reading and driving.[3]

It is not presently known what triggers the pathogenic mechanism, but a growing body of evidence suggests that chronic dry eye disease is characterized by increased osmolarity and an inflammatory process affecting both the lacrimal gland and the ocular surface system.[4] The cornea becomes non-wettable in a vicious cycle, eventually leading to severe corneal damage and chronic dry eye. Corneal epithelial cells exposed to dry eye conditions have demonstrated reduced intercellular connections, loss of cell surface microplicae and desmosomes, disruption of cell membranes, epithelial desquamation, and mucous threads.[5]

Substitute tears are widely used to treat DES, but this conventional therapy focuses on tear replacement and, when used alone, cannot interrupt the cycle of inflammatory disease and restore the homeostasis of the ocular surface epithelia. This hypothesis is strongly supported by the efficacy of treatment of severe forms of DES with steroids and topical cyclosporin A in animal and human clinical trials.[4] Use of anti-inflammatory therapy on a large scale is expected, but at present the well-known side effects of steroids (intraocular hypertension and cataract) and the efficacy of cyclosporin A restricted to T-lymphocytes are important limitations. Furthermore, the direct effect of an anti-inflammatory treatment on changes of corneal epithelial cells has not been demonstrated yet.

Recently, a new molecule has been developed to improve cell conditions during tissue regeneration processes. In particular, T-LysYal (T-LYS) is a supramolecular system containing lysine hyaluronate, thimine, and sodium chloride stereochemically aimed at creating nanotubes that has

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shown important results in promoting the healing process of decubitus ulcers in hospitalized patients[6] and in restoring nasal mucosa after functional endoscopic sinus surgery.[7]

The aim of our study was to evaluate the potential effects of T-LYS on damaged corneal epithelial cells in dry eye conditions. We used an experimentally induced in vitro model based on reconstructed human corneal epithelium (HCE) that mimics changes that occur in patients with dry eye.[8] We studied the ultrastructural modifications by scanning electron microscopy (SEM), the impairment of the epithelial barrier by trans-epithelial electrical resistance (TEER), the genomic response by qRT-PCR of mRNA expression of Cyclin D-1 (CCND1), and integrin β1 (ITG-β1) and at the protein level by immunofluorescence in dry eye conditions in two HCE series treated with T-LysYal and hyaluronic acid (HA), respectively.

Materials and methods

Biological model

The reconstructed HCE model supplied by EPISKIN Laboratories (Nice, France) consisted of immortalized human corneal epithelial cells (IHCEC) cultured on an inert, permeable polycarbonate filter of 0.5 cm^2 for 5 days at the air–liquid interface in a supplemented chemically defined medium (modified MCDB 153). The resulting 3D construct showed the morphology of the stratified cellular organization of HCE and has been characterized for different relevant markers. Upon arrival, cells were removed and placed in a six-well culture plate with 1 ml of chemically defined maintenance medium, which was changed every 24 h.

Experimentally induced In vitro dry corneal epithelium in hyperosmolar condition

A dry hyperosmolar (HYP-DRY) in vitro corneal epithelium has been induced by placement of the HCE tissue under controlled environmental conditions (<40% humidity, 40°C temperature, and 5% CO_2) in the presence of Sorbitol 0.6 M in medium for 16 h as previously described.[8] At the end of the overnight stress period, product treatment was applied for 24 h on stressed HCE (Figure 1).

Products, controls, and treatments

T-LysYal (0.32%, 260 mOsm/kg) from Sildeha (Lugano, Switzerland, marketed by Farmigea, Pisa, Italy) and HA (0.32%, 270 mOsm/kg), two ophthalmological formulations, were tested for their efficacy and for their capability to prevent desiccation and to restore physiological conditions on damaged corneal cells. The two products were applied on the stressed HCE: 30 µl was directly applied to, and gently spread over, the whole epithelium surface on tissues that were already exposed for 16 h in dryness and hyperosmolar conditions, and were followed for further 24 h to test the ability to homeostasis restoring. As a negative control, HCE placed in standard condition (90% humidity, 37°C, and 5% CO_2) for the same amount of time has been used.

Tissues maintained in the controlled setting (20–25% humidity, 40°C temperature, and 5% CO_2 and Sorbitol 0.6 M in the medium) but without treatments were used as positive control of the HYP-DRY condition.

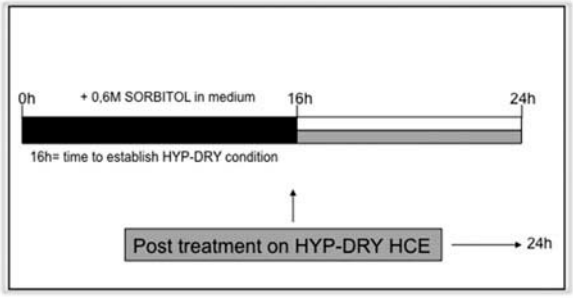

Figure 1. Schematic representation of protocol of HYP-DRY induction and products treatment. HYP-DRY: dry hyperosmolar in vitro corneal epithelium.

Scanning electron microscopy

At the beginning and at the end of the study, tissue samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 h at 4°C. Slices were washed in 0.1 M sodium cacodylate buffer, pH 7.4 and then carried out in 1% osmium tetroxide (OsO4) in the same buffer (2 h at RT).

Samples were dehydrated through ascending grades of ethanol and Hexamethyldisilazane overnight, and then critical-point-dried in a CO_2 liquid Bemar SPC 1500 apparatus. Specimens were placed on pins with carbon tabs and later they were coated with a layer of gold using Polaron Equipment limited SEM coating unit E5100 and then transferred to the SEM Zeiss Sigma Electron Microscope (Oberkochen, Germany) for viewing and photography. Magnification of 2000×, 10 000×, and 20000× was performed.

Trans-epithelial electrical resistance (TEER) measurement

A total of 0.5 ml of saline solution was directly applied on the tissue placed in a six-well plate containing 5 ml of saline solution as well. The instrument Millicell-ERS (Electrical Resistance System, range 0–20 kΩ, Merck Millipore, Darmstadt, Germany) was placed with the two electrodes in the two chambers: the measure directly appeared on the display.

Three measurements for each tissue were performed, because of variability within the tissues the measurement performed at t = 0, which was taken as the basal value and the reference of each single tissue. The blank value (insert without tissue) was subtracted from the sample value (mean three measurements). This result was then corrected considering the tissue surface (0.5 cm^2). Ω (mean three measurements) sample – Ω blank = Ω × tissue surface (0.5 cm^2). No measurements were taken in the T-LYS and HA groups after the stress period to avoid any cell modifications.

Immunofluorescence analyses on frozen sections and entire epithelia

After deparaffination and rehydration, 8 min at 99°C incubation in citrate buffer was performed as antigen retrieval, and then permeabilized with Triton X-100 for 10 min. The tissues were then blocked with 3% BSA-PBS (bovine serum albumin [BSA], phosphate buffer saline [PBS]) for 30 min and sections were subjected to immunofluorescence with antibodies against ITG-

β1 (Santa Cruz, Dallas, Texas, USA, sc-9936), diluted 1:50 for 1 h incubation at room temperature; the secondary antibody was Alexa flour 555 donkey anti-goat (Invitrogen, California, USA). After three washes in PBS, the slices were mounted with Fluoroshield mounting medium with DAPI (4′,6-diamidino-2-phenylindole) and sealed with nail varnish (Invitrogen).

The slices were examined under the Leica TCS SPE confocal laser scanning microscope (Leica, Wetzelar, Germany).

Confocal images of the sections were taken. Images from individual optical planes were analyzed by confocal software (Multicolor Package, Leica).

Transcriptional study of ITG-β1 and CCND1 mRNAusing qRT-PCR

After 24 h of treatment on stressed HCE, total RNA was extracted from HCE using the RNAqueous kit according to the manufacturer's protocol (Thermo Fisher Scientific, Rodano, Italy). The cDNAs were then synthesized in a 20 µl reaction using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). A total of 10 µl RNA was added to the master mix, then subjected to reverse transcription in a thermal cycler (Thermo Fisher Scientific ABI 7500 Real Time PCR System) under the following conditions: 25°C for 10 min, 37°C for 60 min, and 85°C for 5 s. Real-Time Polymerase Chain Reaction (RT-PCR) was then performed in triplicate in a 25 µl final reaction volume using TaqMan® assay (Thermo Fisher Scientific).The cDNA was amplified using TaqMan Universal PCR Master Mix and TaqMan gene expression assay provided as a 20X Assay mix (Human Cyclin D-1: Taqman probe CCND1 Hs00277039_m1, integrin β1: Taqman probe ITG-β1 Hs00559595_m1 and Human GAPDH, as the calibrator gene: Taqman probe GAPDH Hs99999905_m1). The PCR conditions were 95°C for 10 min (AmpliTaq Gold DNA Polymerase Activation), followed by 40 amplification cycles (95°C for 15 s; 60°C for 1 min).

The relative quantification (RQ) minimum and RQ maximum define statistical boundaries for RQ, based upon a 95%-specified RQ Min/Max confidence setting. A confidence setting of 95% means that the user can expect the true RQ value to fall within the RQ Min/Max range with a 95% confidence. The RQ Min/Max is calculated using Eq. (1):

$$2^{-(\Delta\Delta C_{T(s,t)} \pm TxVA(C_{T(s,t)}))} \quad (1)$$

where $\Delta\Delta C_{T(s,t)} = \Delta C_{T(s,t)} - \Delta C_{T(calibrator,t)}$
s = sample name, t = target detector, T = student's T value at the selected confident setting using a degree of freedom that is associated with the test sample $\Delta C_{T(s,t)}$, and VAB is the Applied Biosystems' variability function for calculating the variability of the test sample ΔC_T statistic. Calculations of relative gene expression used relative differences in the threshold cycle C_T (the cycle of PCR at which the fluorescence reaches a given value or a threshold that is in the log-linear range of amplification). Because each cycle in the PCR reaction corresponds to a twofold increase in the PCR product, a difference of one in the threshold cycle number represents a twofold difference in the expression of a particular gene or internal control target sequence and can be considered significant. Duplicate cultures were analyzed and the data were represented as RQ data ± standard deviation.

Results

Scanning electron microscopy

SEM analysis revealed an HCE standard tissue with a normal epithelium surface at different magnifications at baseline, and after 16 and 16 + 24 h of culture in standard conditions (Figure 2a). In the HYP-DRY group, significant changes from baseline were demonstrated after 16 h of stress with a progressive reduction of the cell–cell connections and with a visible dehydration (Figure 2b). After 24 h recovery post stress, the HCE surface damage was still visible at the cellular level with loss of cell–cell interconnections and intercellular matrix destruction (Figure 2c).

In the study group the treatment with T-LysYal for 24 h after the stress period produced significant changes. The HCE surface appeared restructured with an ultrastructure similar to the negative control (Figure 2d). The surface of cells was smooth and rare intercellular connection was present. The cells could grow tightly and no extracellular matrix protein structures were visible on the surface.

In the control group the treatment with HA induced a limited recovery of the tissue surface ultrastructure compared to the study group (Figure 2e).

Barrier properties evaluation by TEER measurement

After 16 h post stress, a significant TEER decrease was observed as a result of an epithelial barrier impairment in HYP-DRY positive control tissues and the damage was not recovered after 24 h. In the adopted experimental conditions none of the products (T-LYS and HA) had an influence on the physical state of the barrier compared to the positive control. In fact, no changes were recorded after 24 h of treatment. No measurements were taken in the T-LYS and HA groups after 16 h (Figure 3).

Immunofluorescence

Immunofluorescent analysis of HCE following 24 h of treatment after stressed conditions compared to HCE standard is shown in Figure 4. In standard conditions HCE revealed staining associated with the plasma membranes of cells, while in the HYP-DRY group the signal was not homogeneously present. After 24 h of treatment with T-LYS a more prominent staining (higher signal) around the perimeter of the cells was observed compared to HYP-DRY and HA groups. In fact, the images show a weak signal in the HA group.

RT-PCR of CCND1 and ITG-β1 mRNA in the HCE model

Cyclin (CCND1) mRNA expression was up-regulated for both products after 24 h of treatment compared to the positive control, and in particular HA showed a significant increase compared to T-LYS (Figure 5a).

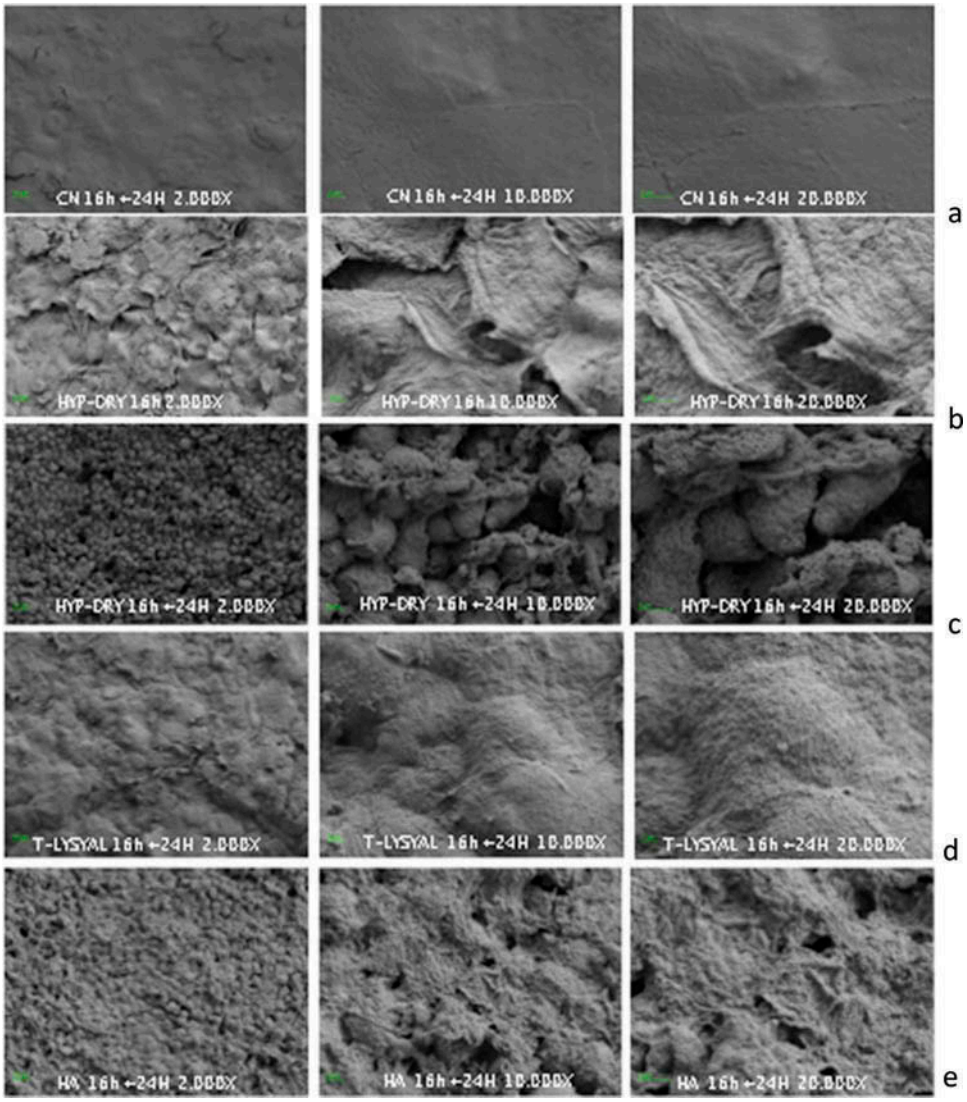

Figure 2. Scanning electron microscopy images of human corneal epithelium in standard, HYP-DRY condition and after treatment with T-LysYal and hyaluronic acid (HA). Magnification: 2000-10000-20000×. (a) Human corneal epithelium in standard conditions: the cell surface appears smooth and covered by microvilli. (b) After 16 h in HYP-DRY condition the surface of human corneal cells appears irregular with visible dehydration and reduction of cell-to-cell connections. A similar picture was visible after 24 h recovery post stress (c). (d) After 24 h of treatment with T-LysYal the cell surface appears restructured with an ultrastructure similar to cells in standard conditions. (e) Cells treated with HA for 24 h show a limited recovery compared to cells treated with T-LysYal.

ITG-β1 mRNA expression was significantly increased in the HA-treated group compared to T-LYS and control group after 24 h of treatment (Figure 5b).

Discussion

The corneal epithelium has a fundamental role in the homeostasis of the ocular surface and its damage in patients with dry eye is a key factor that mirrors the alteration of the system and is responsible for the invalidating symptoms. In this study we demonstrated for the first time that the use of T-LYS can significantly improve the conditions of the corneal epithelium when damaged in dry eye conditions. Furthermore, we showed that T-LYS can modulate tissue growth factors expression in an active manner.

The components of the ocular surface are functionally linked by the continuity of the surface epithelium. It is essential that all of the components of the ocular surface function normally in order to preserve optimal visual function. Protective mechanisms of the ocular surface epithelium include its ability to produce mucins that cover the epithelial surface and bind the tear film, its capacity to heal rapidly, and adhere to the underlying connective tissue.[9] This is critical since the ocular surface is exposed to the "outside" environment. In addition, blinking and rubbing of the eye put mechanical stress on the epithelium.

The barrier function of the corneal epithelial layer can be disrupted in patients with DES and induce symptoms of discomfort and visual disturbances.[10] Osmolarity certainly plays an important role in the pathogenesis of ocular surface damage.[11] In fact, cellular processes such as metabolism, protein folding, and intracellular transport require stable osmolarity/tonicity.[12] Responses of epithelial cells exposed to hyperosmolarity include reduced cell volume, increased concentration of solutes, oxidative stress, disruption of DNA repair systems and therefore DNA damage, and increased

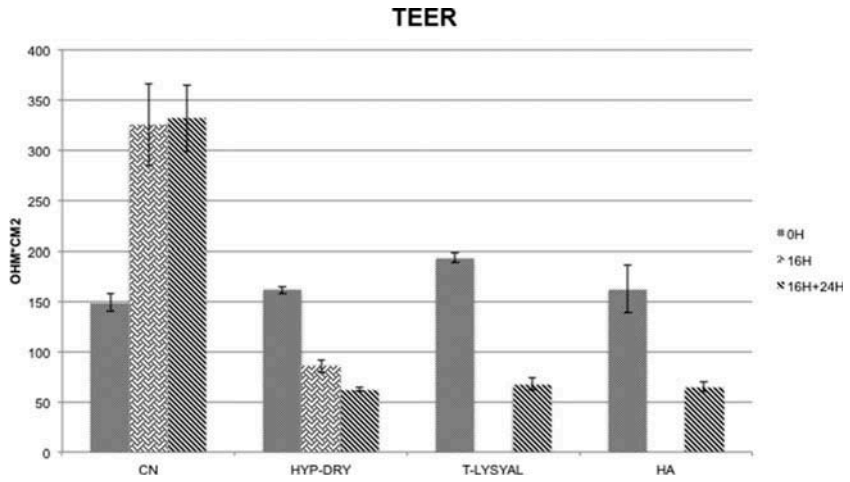

Figure 3. Trans-Epithelial Electrical Resistance (TEER) expressed in OHM*cm^2 at basal level (0 h), after dryness and hyperosmolar stress induction (16 h), after 24 h of recovery period, and after products treatment. No measurements were taken in the T-LysYal and HA groups after the stress induction (16 h). HA = hyaluronic acid.

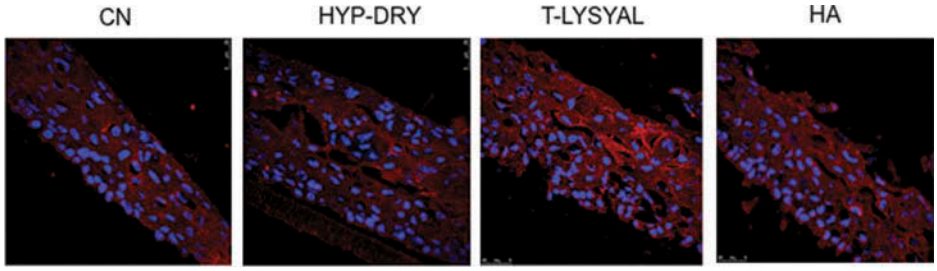

Figure 4. Immunofluorescent analysis of HCE without treatment and in HYP-DRY conditions or following 24 h treatment with ophthalmological formulations T-LysYal and hyaluronic acid (HA).

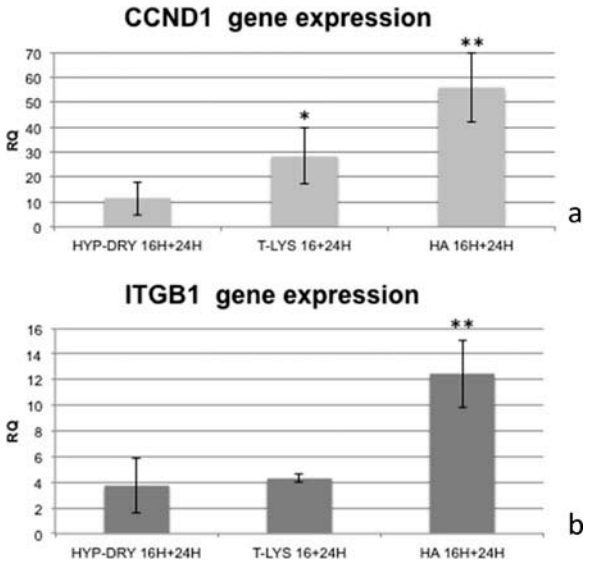

Figure 5. Quantitative analysis of cyclin (CCND1, a) and integrin (ITGB1, b) mRNA expression by qRT-PCR (Taqman assay) in the control non-treated HCE and the HCE treated with T-LysYal (T-LYS) and hyaluronic acid (HA) for 24 h on stressed HCE. The CN (untreated control) was used as a calibrator (RQ = 1). * $p < 0.05$ vs. HYP-DRY; ** $p < 0.05$ vs. all groups.

pro-apoptotic signaling. Corneal epithelial cells exposed to hyperosmotic conditions have demonstrated reduced intercellular connections, loss of cell surface microplicae and desmosomes, disruption of cell membranes, epithelial desquamation, and mucous threads.[13]

During the last 10 years in vitro 3D models of HCE have been developed.[14] These living 3D constructs are versatile for the setup of predictive experimental models allowing an objective and reproducible quantification of complementary testing parameters.[15,16] Compared to in vivo studies on animals and cell culture models, the in vitro tissue is an easy-to-handle model of human origin that more closely resembles human epithelial physiology than conventional monolayer models. In this study we used the HCE in an experimental setting developed by our group that has previously demonstrated its ability to induce changes in the corneal epithelium similar to dry eye conditions.[9] In particular, the SEM images clearly demonstrate that the epithelium exposed to dry conditions presents severe damages.

In our study we tested the hypothesis that a new molecule, T-LYS, could restore the epithelial conditions after exposure to severe dry conditions. Interestingly, the images obtained with the SEM demonstrated that the use of T-LYS for 24 h can improve epithelial conditions, and the final result is a picture similar to normal conditions, with restored integrity of the cells and a normal surface. The use of HA in the control group induced positive changes in the damaged epithelium but was not able to reproduce the changes induced by T-LYS. The effect of this molecule is probably due to its structure: it is a supramolecular system containing lysine hyaluronate,

thimine, and sodium chloride that form longer chains than HA alone and a 3D structure with nanotubes. This particular conformation of the molecule gives T-LYS some unique properties: while HA binds water but it is not able to move it, T-LYS can attract water and has the capacity to move it, and therefore is able to modify the cells environment modulating growth factors expression and possibly inflammatory mediators.[17] Furthermore, T-LYS is more resistant to the lytic enzyme hyaluronidase than HA because of the presence of lysine hyaluronate and thimine on the target sites of the enzyme. This fact accounts for a superior stability of the product that may exert a repairing activity for a longer period (Gruppo Biolab, data on file).

The evidence of the active role of T-LYS is demonstrated by the modulation of ITG-β1, which is involved in cell-to-cell contacts allowing epithelial cells to stick to each other and to repair the tissue. The images obtained after 24 h of treatment demonstrate an increased expression in the corneal tissue compared to the control group. The mRNA expression, however, showed opposite results. In our opinion the dynamic of the genomic response is a possible explanation related to the speed of action of T-LYS: after 24 h the protein is already expressed at the tissue level while HA is still inducing mRNA synthesis. Future studies with different follow-up times could be of help to clarify this point, which appears controversial. However, clinical evidence of the properties of T-LYS to induce improvement of damaged tissues has been recently reported at the level of the nasal mucosa[7] and also when compared to HA in the healing process of decubitus ulcers in hospitalized patients.[6]

In our study we evaluated also the expression of mRNA for CCND1. Both treatment and control groups showed an increased expression compared to cells exposed to dry conditions only. Similar to ITG-β1, HA induced a higher expression compared to T-LYS. The fact that CCND1 mRNA is overexpressed is certainly a positive sign, because it is the demonstration that the exposure to treatment can induce an activation of repairing mechanisms in damaged cells. Considering the whole ultrastructural and morphological investigation SEM and staining images, it seems that T-LYS acts as a modulator of the ocular surface because the final result, restoring of the normal epithelium conditions as shown by SEM images, could be considered better than the HA result. Based on this mechanism of action in our opinion, we could define T-LYS as an ocular surface modulator.

The present study has some limitations. There is a body of evidence that inflammation plays a pivotal role in dry eye pathogenesis and is responsible for corneal damage.[4] In this study we did not measure the effect of T-LYS on pro-inflammatory mediators. However, Gelardi et al.[7] have already shown that after surgery, such as functional endoscopic sinus procedure, intranasal T-LYS is able to significantly reduce the grade of eosinophilic and neutrophilic infiltrate as well as increasing the quote of well-being epithelial cells in the nasal mucosa. Moreover, T-LYS has been demonstrated to decrease the expression of ICAM-1 on human umbilical vein endothelial cells (HUVEC) induced by tumor necrosis factor (TNF-α) in a dose-dependent manner.[17] Another limitation of this study is the duration of the follow-up. Therefore, further studies are certainly necessary to evaluate the effect of T-LYS on the inflammatory cascade and after a longer exposure, but from this first study we can conclude that this new molecule is capable of significantly improved epithelial conditions in DES conditions.

Declaration of interest

The authors report no conflicts of interest, except Demetrio Manenti who is business partner of Sildeha, Switzerland. The authors alone are responsible for the content and writing of the paper.

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