TREHALOSE PROTECTIVE EFFECT ON AN IN VITRO MODEL OF DRY EYE

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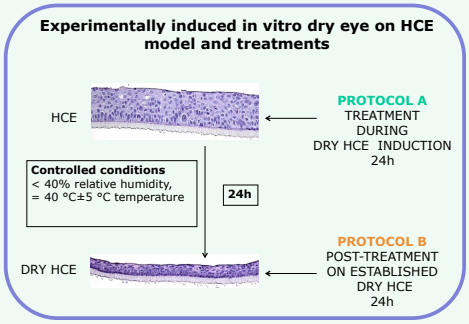
INTRODUCTION

Tear Dysfunction Syndrome (TDS) or Dry Eye Disease is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort and tear film composition modification and instability with potential damage to the ocular surface. The corneal epithelium, a non keratinized multilayered squamous epithelium of about 60 µm, has a crucial role in the barrier function and can be considered the first line of defence against many types of injury, trauma or infection: the corneal epithelium is directly involved in the establishment of TDS with discomfort and pain associated to inflammation, matrix degradation, expression of specific markers and modification of microvilli network at molecular level.

An **experimentally induced in vitro DRY EYE model on HCE** (1) mimicking dry eye ocular surface damage has been applied to assess the efficacy of a Trehalose based formulation. The in Vitro model has the characteristic features of a dry eye epithelium and could be satisfactorily used for a pre-clinical assessment of the protective activity of ophthalmic formulations.

Trehalose (2) is a naturally occurring disaccharide comprised of two molecules of glucose. The sugar is widespread in many species of plants and animals, where its function appears to be to protect cells against desiccation, but it is not found in mammals. Some physical chemistry properties of Trehalose in more concentrated solution are particularly beneficial during desiccation. In drying it has a lower tendency to form crystals that can damage cell organelles than other disaccharides, but instead it is thought to form a gel phase that might contribute to resilience to cellular desiccation. Recent studies have shown that Trehalose can also prevent damage to mammalian eyes caused by desiccation and oxidative insult. These unique properties of Trehalose have thus prompted its investigation as a component in treatment for dry eye syndrome.

EXPERIMENTAL DESIGN PROTOCOL	Dry eye Model has been induced by controlled environmental setting (reduced relative humidity and increased temperature= RH<40% and T=40°C) for 24h. The HCE tissues were transferred in the defined controlled conditions for 24h in order to induce the dry eye. A good cell viability of the tissues during this time has been previously confirmed. Negative (HCE in standard conditions) and positive (DRY HCE) controls, compared to Trehalose 3% treated HCE (30µl) have been analyzed to quantify the expression of the most relevant biomarkers, MMP-9, MUC-4 related to the dry eye symptoms by qRT-PCR with Taqman assays, histo-morphological (H&E) and immuno-histochemistry analysis.  **PREDICTION MODEL FOR THE ESTABLISHMENT OF DRY HCE** The Dry-HCE model is characterized by increase in MUC4, MMP9, TNF-α, and DEFB2 expression (1)
TEST ITEM and TREATMENT	A) THEALOZ has been topically applied once (30µl) during the induction of the DRY EYE conditions in order to assess their efficacy to counteract the effects of induced dryness: the treatment has been done for **24h.** B) THEALOZ (30µl) has been applied after having induced the DRY EYE conditions for 24h: then a **post treatment of 24h** has been done in order to assess a possible recovery after the damage.
END POINTS	inflammation, morphological modification, expression of relevant markers (the found over expression, MMP-9 and MUC-4 correlate with the dry eye symptons in Vivo) and reduction of the microvilli "network". 1) gene expression by using TaqMan gene assay technology (MUC-4, MMP-9) 2) Histology and immunohistochemistry staining (MUC4 and MMP9)



RESULTS

MMP-9 and MUC-4 GENE EXPRESSION RESULTS (qRT-PCR)

Fig. 1 A MMP-9:
THEALOZ treatment reduced at 24h (PROTOCOL A) the MMP-9 gene expression at values comparable to those of the negative control.
On established DRY HCE (PROTOCOL B), the product induced a significant down regulation of the gene

Fig. 1B MUC-4:
The treatment with THEALOZ has determined at 24h (PROTOCOL A) an increase of MUC-4 expression.
On established DRY HCE (PROTOCOL B), THEALOZ induced a significant up-regulation of MUC4. The up-regulation of MUC-4 suggested a positive protection mechanism.

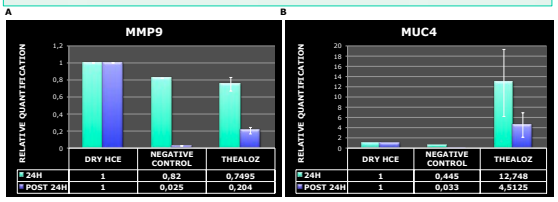


Fig. 1 MMP-9 and MUC-4 gene expression by real time PCR; CALIBRATOR DRY HCE=1

BIOLOGICAL ROLE OF METALLOPROTEINASES
MMP9(4)

MMP-9 is the most important gelatinase present on the ocular surfaceMMP-9 levels appear to be higher in the tear fluids of patients with TDS and in particular in patients with ulceration.
MMP- increased in the tear fluid of patients with dry eye and increased production and activation of pro-inflammatory cytokines (TNF-α and IL1-α) have been observed at the stressed ocular surface in dry eye.

The in vitro **DRY-HCE MODEL** stimulated the production of inflammatory factors (TNF-α and MMP-9).
The increased MMP-9 activity in TDS may be responsible in part for the impaired corneal epithelial barrier function, increased corneal epithelial desquamation, and corneal surface irregularity.

BIOLOGICAL ROLE OF MUCINS
MUC4(3)

Mucins network keeps the ocular surface wet and protected from adverse environmental conditions. Mucin function at the ocular surface has been ascribed to secreted gel-forming mucins acting as lubricating agents and clearing molecules.

The transmembrane mucin MUC-4 is expressed at low level in patient with dry eye syndrome.

In the in vitro **DRY-HCE MODEL** MUC-4 upregulation can be interpreted as an early marker that acts as a positive signal to stimulate the production of mucins: stress conditions lack mucin protein.

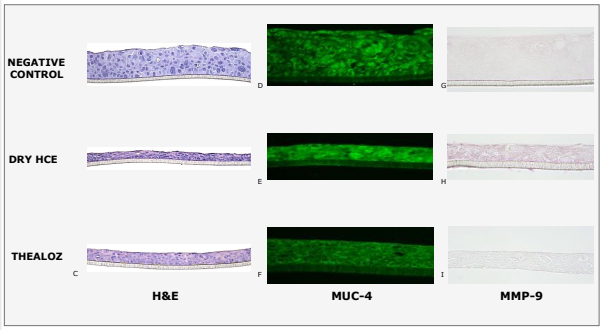


Fig. 2 Histological analysis of DRY HCE and THEALOZ on established dry HCE (A-C). Immunofluorescence staining of MUC4 (FITC) (D-F). Immunohistochemical analysis of MMP9 (G-I).

HISTOLOGICAL ANALYSIS H&E
NC: standard morphology

DRY HCE: Epithelial thickness was reduced in DRY-HCE compared to NEGATIVE CONTROL-HCE 24H post dry induction

THEALOZ: After 24 h treatment on established DRY-HCE, THEALOZ restored the initial morphology of HCE, which resembled the physiological conditions.

MUC-4

NC: clear signal on the whole section

DRY HCE: cellular structures appear severely modified both at cellular than extracellular level; the signal is clear in the overall thickness.

THEALOZ: cellular structures appear conserved and the signal is almost clear in the overall thickness
MUC-4 protein expression as defence mechanism is not required.

MMP-9

NC: very low signal in the whole tissue; the standard morphology is perfectly observed.

DRY HCE: the signal is moderate/ clear in the whole tissue. The cellular and tissue stucture appears severely modified and the thickness is dramatically reduced

THEALOZ: very low signal, even not visible signal. Strong protection offered by the treatment against the matrix degradation induced by the dryness conditions

CONCLUSION

The present study investigated the biologic response of a 3D model of Human Corneal Epithelium to dry environmental cultivation conditions by monitoring the kinetics of a biologically relevant gene signature over a 24h time period after DRY EYE induction. This dynamic approach could be predictive of corneal damage in vivo and allows to investigate the activity of a trehalose ophtalmic preparation, THEALOZ. The Dryness conditions have deeply modified the HCE's genes pathways and the treatment with THEALOZ have induce specific tissue responses.
The **THEALOZ** treatment has globally determined a significant and early down regulation of MMP-9 and a significant up-regulation of MUC-4 and for both genes these results correspond to a protective activity of the product to prevent the establishment of the dry eye conditions and to restore the epithelium when it is applied on established dry eye conditions (post treatment).
For MUC-4 immunostaining the cellular structures appeared conserved and the signal were almost clear in the overall thickness. On the contrary, the MMP-9 was almost not detectable which is in agreement with the down regulation of the gene.
In this in vitro dry eye model, TREHALOSE polysaccharide 3%, **THEALOZ,** induced down regulation of MMP-9 and up regulation of MUC-4, in favour of a protective activity of corneal surface.

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