DRY OR WET: MEMBRANE ASSOCIATED MUCIN AND THE WETTABILITY OF CORNEAL EPITHELIAL CELLS

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ABSTRACT

The purpose of the presented study is to evaluate the influence of membrane-associated mucins (MAM) on the wettability of human corneal epithelial cells, as a part of the pathophysiology of the Dry Eye Syndrome.

Human corneal epithelial cells (HCEC) were cultivated 7 days after they reached 100% confluence and formed stratified culture where the most upper layer of cells, i.e. the one in direct contact with the culture medium started to express membrane associated mucins. The presence of membrane associated mucins (MAM) was demonstrated via (i) immunofluorescence, using human-specific MUC-16 (OC125) targeted antibody and (ii) the resistance of the cells to rose bengal (RB) staining. The MAM shedding was achieved, by treating cells with neutrophil elastase (NE). The wettability of the cell surface was evaluated by measuring equilibrium, advancing and receding contact angles via sessile bubble method.

The MAM shedding via neutrophil elastase resulted in (i) decrease of immunofluorescence, (ii) increase of RB staining and (iii) decrease of wettability of HCEC cells manifested as an increase of equilibrium and dynamic (advancing and receding) contact angles of HCEC coated plate.

This study shows that MAM shedding might affect normal wettability of human corneal epithelium, thus leading to the certain visual discomfort. It also confirms the role of the secretory mucins in the underlying colloid interactions within tears.

Key words: *membrane-associated mucins, human corneal epithelium cells, Dry Eye Syndrome*

Introduction and aim

Dry eye Syndrome, also known as keratoconjunctivitis sicca is the most common eye disease, affecting the human population. Although the etiology of DES isn't completely clarified, it is confirmed, that the presence of mucins MUC1, MUC4 and MUC16 is important in the formation of a stable tear film [3]. There are evidences of mutual connection between the concentrations of the membrane-associated mucins, the time spent at the visual displays and the development of dry eye syndrome [6,7,8]. Our efforts were focused on the evaluation of the contribution of the membrane-associated mucins to the overall wettability of the cells forming the eye surface.

Materials and methods

1. *Cell culture.* We cultured HCEC (*Invitrogen™, Life technologies*) on a standard №1.5 cover slip glass following routine protocol and using Keratinocyte-SFM, (*Invitrogen™, Life technologies*) culture medium supplemented with EGF Recombinant Human Protein (*Invitrogen™, Life technologies*). The cells were cultured until 100% confluence and the culture medium was switched to Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM / F-12) +10% FBS for 7 days in order to achieve stratification of the cell culture and expression of MAM.

2. *Mucines shedding procedure.* HCEC cells were incubated with DMEM/Ham F-12 containing 5 μg/mL neutrophil elastase (NE) (*Enzo Biochem, Inc, NY*) for 30 minutes [4]. Cell viability was checked via MTT test, and no significant damage of HCEC population had been observed (data not shown).

3. *Rose Bengal staining.* Slides covered with stratified HCEC cells were stained with 1% Rose Bengal solution, and the results were compare between previously treated with NE and non-treated control.

4. *Fluorescent microscopy.* After stratification, HCEC cells in the culture chamber slides were fixed in 4% paraformaldehyde. Cultures were rinsed in PBS, blocked with PBS with 1% bovine serum albumin (BSA) and incubated for 1 hour at room temperature with primary antibodies clone OC125 to a peptidic epitope on MUC16 (Anti-CA 125 Mouse (OC 125) Antibody - *Merck Millipore*). Cultures were then incubated for 1 hour at room temperature with the fluorescein-conjugated secondary antibody (Alexa Fluor® 546 Goat Anti-Mouse IgG, *Thermo Scientific)*. Slides were coverslipped with fluorescence mounting medium and observed under a fluorescence microscope.

5. *Sessile bubble technique.* The wettability of samples of HCEC culture was evaluated by the method of the shape analysis of axisymmetric sessile bubble (SB-ABSA) [1,2], i.e. by measuring the contact angles between an axisymmetric air-bubble with V=0,3µl, submerged in 0.15M phosphate buffer saline solution (PBS), put in contact with the cells. Three different types of contact angles were measured:

   i. *Equilibrium contact angle* between the air-bubble and the HCEC covered solid support - the air bubble is left static over the surface and is allowed to reach equilibrium with the solution and with the cell covered support. Provides information about the overall wettability.

   ii. *Advancing contact angle* between the bubble at contraction and the cell covered surface of the solid support. Provides information about wetting capability of the exposed cell surface. Low advancing angle corresponds to better wetting abilities of the cell surface.

   iii. *Receding contact angle* between the bubble at expansion and the cell covered surface of the solid support. Receding angle provides information on the dewetting endurance of the cells, i.e. lower values correspond to higher resistance to dewetting.

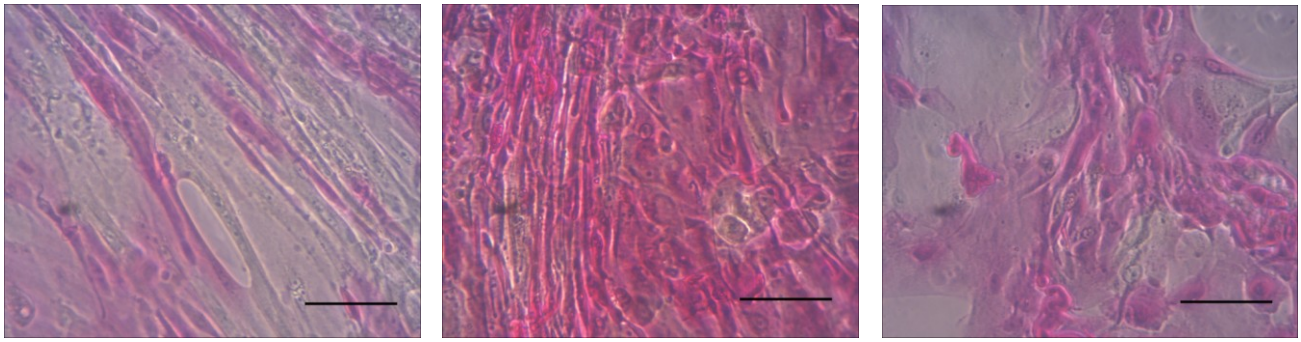
   All measurements were performed on CAM101 contact angle tension meter (KSV NIMA, Finland), with complete curve fitting based on Young - Laplace equation.

6. *Statistical analysis. All the results were compared statistically with one way ANOVA test with subsequent Tukey-Kramer analysis on the GraphPad InStat software (GraphPad Software Inc., CA, USA).*

Results and Discution

I. Rose Bengal staining

Permeability to Rose Bengal dye was compared in intact and treated with NE stratified HCEC cells (fig.1). The level of staining corresponds to the level of mucins shedding. Immediately after the treatment with NE, the cells permeability to the Rose Bengal increased and within a period of 24h it diminished, hence to the partial recovery of the mucins layer.

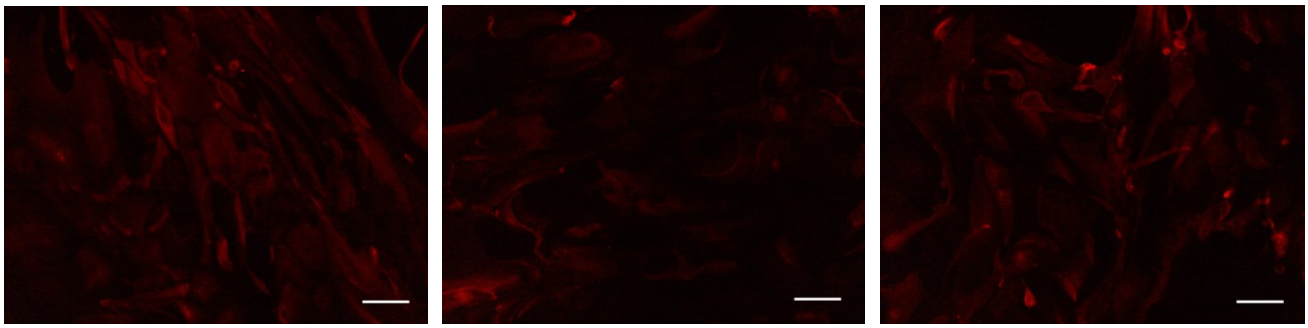


A B C

Fig.1 HCEC permeability for the Rose Bengal dye. A. Untreated control. B. HCEC after treatment with NE. C. HCEC 24h after treatment with NE. Magnification 100x, bar equals to100μм.

II. Fluorescent microscopy

For further confirmation of the presence of MUC-16 in the intact and treated with neutrophil elastase HCEC cells, we compared the levels of fluorescent emission under the same conditions of exposure and recovery times (fig.2). Significant loss of the signal was observed, after the treatment with NE, and subsequent partial recovery was observed 24 hours after the treatment.



A B C

Fig.2 Fluorescence microscopy images of the stratified HCEC cell culture A. Untreated control. B. HCEC after treatment with NE. C. HCEC 24h after treatment with NE. Magnification 200x, bar equals to50μм.

III. Sessile bubble - axisymmetrical bubble shape analysis (SB-ABSA)

Measurements of the dynamic (advancing and receding) and static contact angles (fig.3) showed significant correlation between the level of wettability and the presence of MUC-16. Static angle θ_s equals to 39.55deg, for the untreated control, while after the treatment it arose to θ_s = 44.73deg, showing diminished wettability. Partial recovery was observed 24h after the treatment with NE (θ_s=43.50deg). The data for the receding (control θ_r =41.68, θ_{r0h}=46.44, θ_{r24h}=44.70) and for the advancing angle (control θ_a =42.41, θ_{a0h}=47.31, θ_{a24h}=44.89) confirmed the connection between the wettability of the cellular surface and the presence of MAM.

As in our previous study [5], receiding contact angle was measured by increasing the volume of the sessile bubble, i.e. by increasing the hydrophobic zone over the cellular surface, while the advancing angle was measured by the decreasing the volume of the sessile bubble.

In a consecutive manner, all the presented results confirmed the role of MUC-16 in maintaining the overall wettability of the HCEC cell line. Implementation of the SB-ABSA measurement technique greatly facilitates the process and could be used in further investigations or possible *in vitro* tests of compounds for DES clinical signs amelioration.

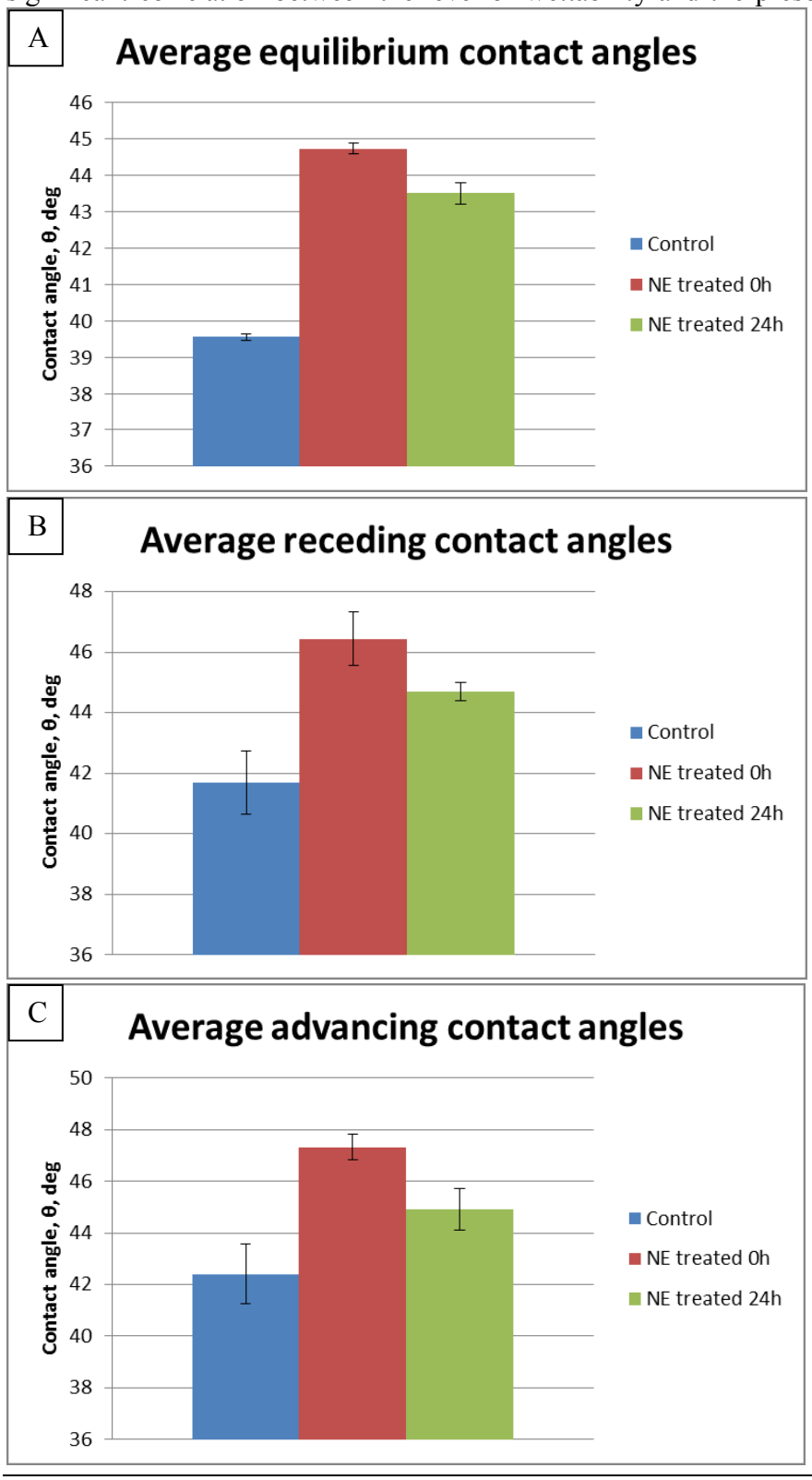


Fig. 3 Contact angle measurements of the static (panel A), receding (panel B) and advancing (panel C) angles.

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