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## Cytological conjunctival changes in patients with type 2 diabetes-associated dry eye in the presence of treatment with a combination of trehalose plus sodium hyaluronate

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**Purpose:** To assess cytological conjunctival changes in patients with type 2 diabetes mellitus (T2DM)-associated dry eye disease in the presence of treatment with a combination of trehalose and sodium hyaluronate.

**Material and Methods:** This study was conducted at the Pirogov Vinnytsia Regional Clinical Hospital, the clinical site of the National Pirogov Memorial Medical University, from April to December 2023. We used prospective data of 46 patients (92 eyes; mean age,  $62.47 \pm 6.24$  years).

**Results:** Most patients (67%) showed abnormal conjunctival impression cytology (CIC) changes (Nelson's grade 2 and 3) before treatment. Among these, grade 3 squamous metaplasia was twice as common as grade 2 squamous metaplasia; this demonstrated apparent CIC changes in the group of patients, and a substantial prevalence of squamous metaplasia in type 2 diabetics. After treatment with a combination of trehalose 3% plus sodium hyaluronate 0.15%, neutrophil infiltration was observed only in 5 (11%) patients ( $p = 0.0375$ ), which may indicate an anti-inflammatory effect of the combination.

**Conclusion:** We found a significant effect of a combination of trehalose 3% plus sodium hyaluronate 0.15% on the state of the ocular surface, with the resolution of inflammatory conjunctival infiltration, in patients with T2DM ( $p = 0.0375$ ). Because stabilization or slowing of damage to epithelial cells is important for patients with T2DM and was observed in the presence of treatment with the combination eye drop, these patients require long-term treatment with the medication.

### Keywords:

trehalose, T2DM, dry eye disease, impression cytology, inflammatory infiltration, ocular surface, cornea

### Introduction

The mucosal epithelium of the ocular surface performs a vitally important protective barrier function against the external environment. Mucins not only contribute to the preservation of tear film integrity to maintain tear film moisture and stability, but also act as an antimicrobial and anti-inflammatory agent [1, 2].

Dry eye disease (DED) is the most common ocular surface disorder. Elucidating the pathogenesis of DED requires a multidimensional understanding of several contributing factors. The initiation of DED often starts with tear film instability, leading to tear hyperosmolarity. This hyperosmolarity, in turn, activates a cascade of inflammatory pathways involving various signaling molecules and immune cells, with an increase in the levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNF- $\alpha$  [3].

The persistent inflammation further damages the ocular epithelium and exacerbates tear film stability, creating a vicious cycle that culminates in chronic DED. Additionally, the involvement of both the innate and adaptive immune systems in DED creates a complex immunological landscape, with T-cell activation and infiltration of the conjunctiva playing a key role. This, in turn, results in impaired goblet cell function, thereby worsening the state of the ocular surface. A deeper understanding of these mechanisms is critical for developing targeted therapies [3, 4].

Studies have reported on decreased levels of the goblet mucin MUC5AC in tears of patients with DED, which may be associated with damage to as well as a reduced number of goblet cells [5, 6].

Ocular surface damage in patients with type 2 diabetes mellitus (T2DM) is caused not only by factors of the external environment, but also by loss of homeostasis of the structural and functional components of the ocular surface [7].

It had been suggested that one or more of the following initial events may lead to the alterations described in the tear film and ocular surface of diabetic patients: chronic hyperglycemia, corneal nerve damage and impairment on insulin action [8]. This results in reduced regenerative capacity and causes pathological production of humoral factors and inflammatory markers in the presence of progressive neuropathy, which affects the state of the conjunctiva in diabetic patients [9]. A mismatch between subjective patient complaints and objective data on ocular surface damage highlights the need for adequate assessment of the selection and efficacy of treatment strategies for T2DM.

We have previously found that the magnitude of changes in the bulbar conjunctival epithelium in patients with T2DM depended on diabetes duration and blood glucose level [10]. Our impression cytology study of the bulbar conjunctival epithelium found that most patients (94.1%) with T2DM had squamous metaplasia grade (Nelson) of 2 or 3, and 80% of those with squamous metaplasia grade (Nelson) of 3 had T2DM duration of more than 5 years. In addition, squamous metaplasia of grade 3 was fourfold more common among patients with T2DM duration of more than 5 years than among those with T2DM duration of less than 5 years [10].

Trehalose is a natural disaccharide which is recognized for its regenerative, anti-oxidative, bio- and osmoprotective and moisturizing effects [11]; given the pathogenetic components of T2DM, trehalose-based eye drops might be considered for the treatment of ocular surface damage in T2DM. Recent studies have demonstrated that trehalose/hyaluronate eye drop statistically significantly improved conjunctival epithelium metaplasia and reduced the number of goblet cells in the conjunctiva in patients with DED only [12]. However, no such data is available with regard to patients with T2DM-associated DED.

**The purpose** of this study was to assess cytological conjunctival changes in patients with T2DM-associated DED in the presence of treatment with a combination of trehalose and sodium hyaluronate.

#### Material and Methods

This study was conducted at the Pirogov Vinnytsia Regional Clinical Hospital, the clinical site of the National Pirogov Memorial Medical University, from April to December 2023. We used prospective data of 46 patients (92 eyes; mean age,  $62.47 \pm 6.24$  years). Patient data at baseline and after a two-month treatment with a combination of trehalose and sodium hyaluronate (group 1a and group 1b) were analyzed.

The sample was representative in terms of gender. Twenty (43%) patients were males, and 26 (57%) were females. All patients had compensated T2DM with a

mean Hb1Ac of  $6.9 \pm 0.54\%$  and disease duration of at least 3 years. They were prescribed with a trehalose 3%/sodium hyaluronate 0.15% eye drop thrice a day for 2 months. Impression cytology of the conjunctiva was used in a routine manner [13] to assess cytological changes. Color images of portions of the cells were graded by the Nelson scale [10], and inflammatory neutrophil infiltration in conjunctival samples was assessed before and after the treatment. A total of 15 patients had Nelson's grade 0 to 1, 10 patients had Nelson's grade 2, and 30 patients had Nelson's grade 3 squamous metaplasia.

Inclusion criteria included compensated T2DM; an Ocular Surface Disease Index (OSDI) of 13-32 [14]; a noninvasive tear break-up time (NIBUT)  $< 10$  s (Ophthalmic slit lamp, SLM-6E; Kanghua Inc, Chongqing, China); a Schirmer test score less than 10 mm/min and a corneal fluorescein staining score (Oxford scheme) of 1 or 2 [15].

Exclusion criteria included history of eye surgery or trauma; DED treatment, particularly that with artificial tears, topical steroids, or cyclosporine; ocular comorbidities such as glaucoma, eyelid abnormalities, and chalazion; contact lens wearers; or systemic lesions related to hyperthyreose to rheumatoid diseases.

The study followed the ethical standards stated in the Declaration of Helsinki. Informed consent was obtained from all patients included in the study.

Statistical analyses were conducted using Statistica 10.0 software (Statsoft, Tulsa, OK, USA). Data are presented as mean plus or minus standard deviation (SD). The Kolmogorov-Smirnov test was applied to test for normality. Parametric analysis of normally distributed data was performed by the Student two sample t test for paired samples or Student two-tail t test for independent samples. Non-parametric data were analyzed with the Fisher exact test. The level of significance  $p < 0.05$  was assumed.

#### Results

Before treatment, 15 (33%) patients in group 1a had normal (Nelson's grade 0 to 1) conjunctival surface epithelium, with sheets of mostly round and, less frequently, polygonal epithelial cells, with well-preserved intercellular bonds, a normal nucleocytoplasmic ratio, and a sufficient number (more than 6-10) plump goblet cells in the field of vision (Fig. 1).

Most patients (67%) showed abnormal conjunctival impression cytology (CIC) changes (Nelson's grade 2 and 3) before treatment. Among these, grade 3 squamous metaplasia was twice as common as grade 2 squamous metaplasia; this demonstrated apparent changes in conjunctival cytology in this group of patients as well as a substantial prevalence of squamous metaplasia in type 2 diabetics (Table 1).

The cytological picture of patients with grade 2 (Nelson) changes in the conjunctival mucosa was characterized by epithelial sheets with widened intercellular spaces; these sheets were composed mostly of polygonal cells with a nucleocytoplasmic ratio of 1 : 2 or 1 : 3. Round cells were

rarely seen, and sometimes showed vacuolated cytoplasm and clusters. Some loss of intercellular connections was observed. Goblet cells were small, round, and seen in reduced numbers (0-2-5 in the field of vision).

The absence of goblet cells, loss of intercellular bonds, and presence of separate somewhat polymorphic polygonal epithelial cells with a cytoplasmic ratio > 1 : 4, sometimes two piknotic nuclei with vacuolated cytoplasm containing keratohyalin granules (Fig. 2) were characteristic for grade 3 changes.

After treatment, there was a 2-percent increase in the number of patients with Nelson’s grade 0 to 1 squamous metaplasia, but this was not significant ( $p = 0.8270$ ). The change from Nelson’s grade 2 or 3 to grade 0 or 1 squamous metaplasia in group 1b was also not significant (Table 1).

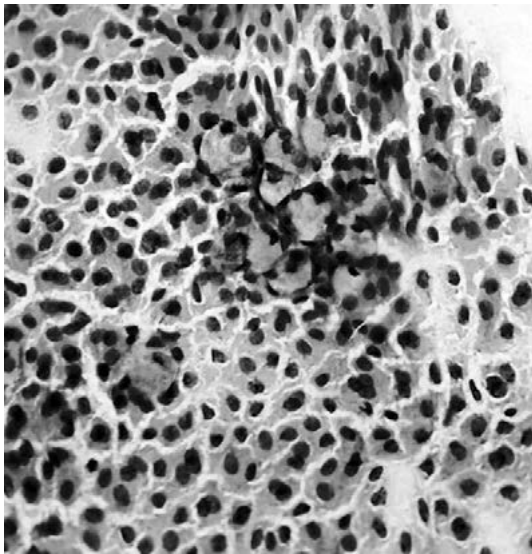
In the presence of treatment with trehalose-based eye drops, there was a tendency to improvement in some characteristics; this was indicated by some increase in the number of goblet cells, transition to plump, rounded goblet cells, restoration of intercellular bonds in sheets

of polygonal cells, and a decrease in the number and severity of signs of degeneration, which is important for the functional competence of goblet cells (Fig. 3).

Patients of group 1b were 1.2 times more likely to exhibit Nelson’s grade 0 or 1 squamous metaplasia than patients of group 1a (OR, 1.2; 95 % confidence interval [CI], 0.513-2.861;  $p = 0.06617$ ). This was likely to be associated with the relatively short period of the study and a reduced regenerative capacity of the conjunctiva in the presence of T2DM as well as the impact of chronic hyperglycemia on the process of conjunctival epithelial regeneration.

Low-grade inflammatory infiltration into the conjunctiva was noted in 14 (30%) patients of group 1a, indicating some autonomy of inflammatory processes in patients with T2DM, and the transition from compensatory mechanisms of regulation of conjunctival homeostasis (parainflammation) to the active process (Fig. 4).

After treatment with a combination of trehalose 3% plus sodium hyaluronate 0.15%, neutrophil infiltration was observed only in 5 (11%) patients ( $p = 0.0375$ ), which may indicate an anti-inflammatory effect of the combination.



**Fig. 1.** Impression cytology of the conjunctival epithelial cells showing Nelson’s grade 0 squamous metaplasia. Hematoxylin and eosin staining. Microscopic magnification, x400.

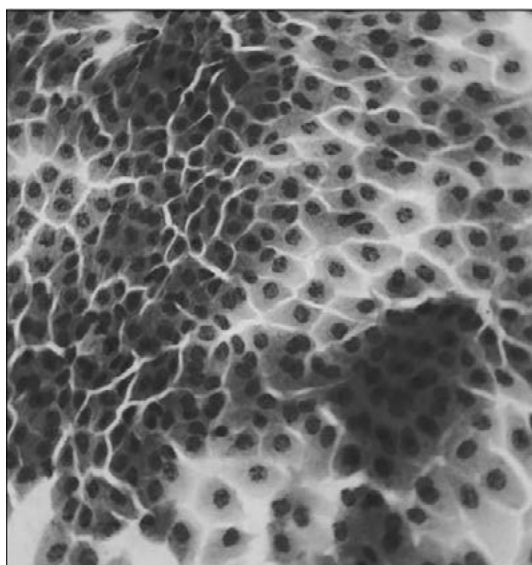


**Fig. 2.** Impression cytology of the conjunctival epithelial cells showing Nelson’s grade 3 squamous metaplasia. Polygonal cytolytic cells arranged separately are seen. Hematoxylin and eosin staining. Microscopic magnification, x400.

**Table 1.** Conjunctival cytological changes as assessed by impression cytology

Squamous metaplasia grade (Nelson)	Before treatment (group 1a)		After treatment (group 1b)		p
	Absolute count	Percentage	Absolute count	Percentage	
0-I	15	33	17	37	0,8270
II	10	22	14	30	0,4768
III	21	45	15	33	0,2854

Note: The level of significance  $p < 0.05$  was assumed



**Fig. 2.** Impression cytology of the conjunctival epithelial cells showing Nelson's grade 3 squamous metaplasia. Polygonal cytolytic cells arranged separately are seen. Hematoxylin and eosin staining. Microscopic magnification, x400.

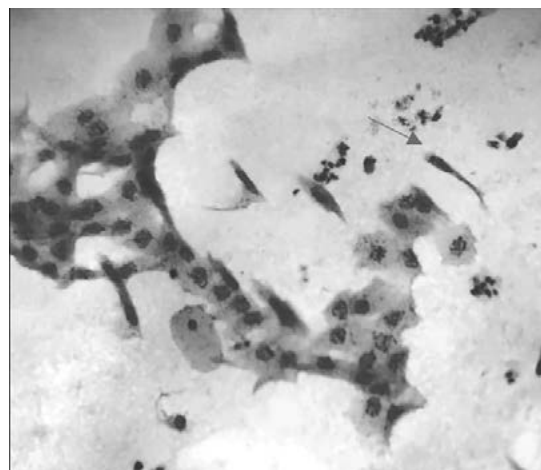
### Discussion

Trehalose is a natural disaccharide which is resistant to acid hydrolysis and dehydration and can protect a broad array of biological materials (e.g., DNA, proteins and conjunctival and corneal cells) [12, 16], which gives ground for its use as an effective agent for symptomatic treatment of DED and ocular surface damage. Cejková and colleagues [17] reported that ultraviolet B-radiation-induced apoptotic death of corneal epithelial cells was significantly reduced after one week of trehalose application, and the expression of injury returned to normal levels during two weeks of trehalose treatment.

In the current study, there were no statistically significant changes in the scores of objective tests (e.g., NIBUT and Schirmer test) on completion of the treatment period of 60 days compared to baseline.

Doan and colleagues [18] compared the effect of a hyaluronic acid (HA)-trehalose solution to an established eyedrop solution containing HA alone using OSDI score < 19 as a threshold for moderate to severe DED. They evaluated the efficacy and safety of HA-trehalose and HA administered for 84 days. Significantly more patients had OSDI < 19 at day 84 in the HA-trehalose group than in the HA group (78.8% versus 58.5%;  $p = 0.025$ ). At day 35, more patients had OSDI < 19 in the former group than in the latter group, but this difference was not statistically significant. Because these data indicate that the effect from trehalose develops gradually, and given an improvement in the Nelson grade on conjunctival impression cytology in our group 1b after treatment, we suppose the need for a long-duration administration of the combination eye drop to achieve a significant improvement.

In a study by Fariselli and colleagues [12], two-month treatment with a trihalose-HA tear substitute resulted in



**Fig. 4.** Impression cytology of the conjunctival epithelial cells showing Nelson's grade 3 squamous metaplasia. Inflammatory infiltration is observed, with neutrophils seen on the right side of the image. Hematoxylin and eosin staining. Microscopic magnification, x400.

a significant goblet cell density recovery, decrease in the grade of squamous metaplasia, and improvement in the fluorescein tear break-up test in patients with DED. The treatment regimen used in that study was the same that in the current study. Although in the current study, the percentage of patients with both DED and T2DM having a squamous metaplasia grade (Nelson) of 3 decreased from 45% to 33%, and the percentage of patients having a squamous metaplasia grade (Nelson) of 0 to 1 increased from 15% to 17% after two months of treatment compared to baseline, these changes were not statistically significant.

Cagini and colleagues [11] evaluated efficacy of trehalose/sodium hyaluronate eye drops for postoperative discomfort and tear film stability in patients with healthy ocular surface, subclinical or mild dry eye undergoing cataract surgery. They found a significant improvement in the OSDI, foreign body and puncture sensation and blinking discomfort as early as four weeks after surgery.

Limitations of the current study included small sample size and a fixed dose combination that includes two active pharmaceutical ingredients. Another limitation was that treatment outcome assessments were exclusively intragroup. These limitations will be overcome in the future with the involvement of a control group of patients without T2DM and assessment of the impact of the combination eye drops on the conjunctiva in practically healthy individuals versus patients with T2DM. In addition, a study will be conducted to assess the impact of the combination eye drops on conjunctival cytological changes in patients with T2DM.

### Conclusion

We found a significant effect of a combination of trehalose 3% plus sodium hyaluronate 0.15% on the state of the ocular surface, with the resolution of inflammatory conjunctival infiltration, in patients with T2DM ( $p = 0.0375$ ). Because stabilization or slowing of damage

to epithelial cells is important for patients with T2DM and was observed in the presence of treatment with the combination eye drop, these patients require long-term treatment with a medication containing both trehalose 3% and sodium hyaluronate 0.15%.

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### Disclosures

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**Abbreviations:** DED, dry eye disease; T2DM, type 2 diabetes mellitus