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Correlation of impaired tear mucin production with relative expression of neurtrophil activation marker CD 15+ in patients with type 2 diabetes mellitus

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Aim: *To determine tear mucin levels in patients with type 2 diabetes mellitus (T2DM) and correlate them with the levels of the neutrophil activation marker CD15+ in epithelial cells of the bulbar conjunctiva in patients with T2DM, depending on the grades of squamous metaplasia.*

Material and Methods: *Thirty-seven patients (37 eyes) with ocular surface damage and T2DM comorbidity were enrolled in this study. Fifteen sex- and age-matched participants without T2DM comprised the control group. Based on the cytological changes in the bulbar conjunctiva and according to Nelson's classification, patients with T2DM were divided into two main groups: Study group 1 – 29 patients (29 eyes) with changes in the bulbar conjunctiva corresponding to grades II and III of squamous metaplasia according to Nelson's classification; study group 2 – 8 patients (8 eyes) with grades 0-I according to Nelson's classification.*

Results: *The mucin level was significantly lower in study group I - 0.95 ± 0.18 g/l compared to study group II - 1.28 ± 0.09 g/l and controls - 1.59 ± 0.08 g/l ($p<0.0001$). Median relative expression of CD15+ was 4 % in study group I whereas only one patient in study group II (12.5 %) and the control group (6 %) was positive for CD15+ expression. Relative expression of CD15+ strongly negatively correlated with the mucin level in the study group I ($rs = -0.87$; $p < 0.0001$). Nelson's grades also negatively correlated with mucin levels in patients with T2DM ($rs = -0.87$; $p<0.0001$).*

Conclusion: *Tear mucin levels are significantly reduced in patients with T2DM and show a strong negative correlation with the neutrophil activation marker CD15+ in the epithelial cells of the bulbar conjunctiva depending on the grade of squamous metaplasia. This indicates dysregulation of ocular surface homeostasis and the inflammatory component in the development of ocular surface damage in T2DM.*

Key words:
mucin, CD15+, ocular surface damage, DED, inflammation, T2DM, diabetes mellitus, cornea

Introduction

Despite various underlying causes, there are several common histopathological changes of the ocular surface epithelium in patients with dry eye disease (DED), including the loss of goblet cells in the conjunctiva, abnormal epithelial cell hyperplasia (presenting as squamous metaplasia), increased cellular stratification, and keratinization. The loss of goblet cells may be directly linked to chronic inflammation and apoptosis of epithelial cells, associated with cellular hyperosmolarity and chronic damage due to mucin deficiency (Baudouin et al., 2013, 2018) [1, 2]. Changes in the mucin layer can lead to increased tear evaporation, contributing to tear hyperosmolarity, and subsequently triggering the release of inflammatory mediators. These mediators may further negatively affect the differentiation, proliferation, and secretion of mucins by goblet cells causing a vicious circle of DED.

According to recent studies, chronic low-grade inflammation is associated with the development and clinical course of T2DM [3, 4]. Elevated levels of pro-inflammatory biomarkers are observed in the early stages of T2DM and its complications [5]. Moreover, high levels of IL-6 and C-reactive protein (CRP) are strongly associated with an increased risk of developing T2DM [5, 6, 7]. Significant associations with risk of T2DM have been observed for neutrophil and lymphocyte fractions in complete blood count [8]. In patients with T2DM, there is a reduction in neutrophil function, with impaired migration, and adhesion to the epithelium, which promotes the release of cytokines and chemokines, leading to subclinical and evident inflammation. This systemic immune response affects the ocular surface and the function of the tear system by activating the innate immune system and increasing the levels of pro-inflammatory cytokines (IL-6, IL-1, TNF-α). Inflammatory mediators damage goblet cells, leading to ocular surface damage [9, 10, 11]. Furthermore, hyperglycemia has a direct damaging effect on the ocular surface, specifically the epithelial and goblet cells of the conjunctiva. Tissue

dysfunction also induces an adaptive response called para-inflammation which, despite its ubiquitous nature, plays a crucial role in pathogenesis of DED [12].

Neutrophil activation marker CD15+ participates in the functions of neutrophils, particularly in intercellular interactions, adhesion to epithelial and endothelial cells, phagocytosis, and stimulation of degranulation. Given the spectrum of activity and expression sources of CD15+ (epithelial cells of the conjunctiva), determining mucin level and its correlations with CD15+ may provide valuable insights into the pathogenic mechanisms of DED development in patients with T2DM, as well as new perspectives for evaluating diagnostic biomarkers of ocular surface damage and treatment strategies.

Aim. To determine tear mucin levels in patients with T2DM and correlate them with the levels of the neutrophil activation marker CD15+ in epithelial cells of the bulbar conjunctiva in patients with T2DM, depending on the grades of squamous metaplasia.

Material and Methods

Thirty-seven patients (37 eyes) with ocular surface damage and T2DM comorbidity were enrolled in this study. Fifteen sex- and age-matched participants without T2DM comprised the control group. Patients with T2DM were divided into two main groups based on the cytological changes in the bulbar conjunctiva and according to Nelson's classification [13]: Study group 1 – 29 patients (29 eyes) with changes in the bulbar conjunctiva corresponding to grades II and III of squamous metaplasia according to Nelson's classification; study group 2 – 8 patients (8 eyes) with bulbar conjunctival changes corresponding to grades 0-I according to Nelson's classification.

Exclusion criteria of the study included history of ocular surgery, ocular trauma; use of artificial tear substitutes, topical steroids, or cyclosporine; other ocular conditions such as glaucoma, eyelid anomalies, chalazion, use of contact lenses; and systemic autoimmune diseases.

Mucin spectrometric assay

The tear mucin level was determined by placing filter paper strips (Filtrak, No. 338, Germany, size 7 × 12 mm) in the lateral part of the lower conjunctival fornix for 5 minutes. After soaking, the tear fluid was eluted from the strips into physiological saline. The obtained solution was then centrifuged for 10 minutes at 3000 rpm. The supernatant was used for biochemical analysis. Mucin content was measured in the supernatant using an SF-26 spectrophotometer (Lomo, Ukraine) and a "Spekol-21" spectrophotometer (Jena, Germany).

CD15+ immunocytochemical assay

Relative expression of CD15+ was determined in conjunctival samples collected by the standard CIC procedure followed by the immunocytochemical study.

Primary antibody CD 15+ clone Carb3 (Dako, Denmark) and the Mouse/Rabbit PolyVueTM polymer imaging system for antigen detection HRP/DAB (Diagnostic BioSystems, USA) were used according to the standard protocol.

The samples were analyzed using a light microscope (×100; ×400).

CD15+ cells were counted per 100 epithelial cells in each sample. The percent value was computed by multiplying the numeric value of the antigen-binding ephitheliocyte ratio by 100.

Ethics

All procedures performed in this study involving human participants were conducted ethically according to the ethical standards of Vinnytsia National Pirogov Memorial Medical University Committee of Bioethics (approval number 08.11.2024 № 1) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study."

Statistics

Statistical analysis of the obtained data was performed using the software package Statistica v.10.0 (StatSoft, USA). Data are presented as mean ± standard deviation (M ± SD), as well as in percentage (%). A two-tailed t-test was used to evaluate intergroup differences for continuous variables with normal distribution (the Kolmogorov–Smirnov test). Correlations between mucin levels and laboratory tests were evaluated using Spearman's rank correlation. P-value <0,05 was considered statistically significant.

Results

Patients of both study groups demonstrated good and fair glycemic control (< 8 mmol/l) (Table 1). Basic characteristics of groups are given in the table 1.

The mucin level was significantly lower in study group I (0.95 ± 0.18 g/l) compared to study group II (1.28 ± 0.09 g/l) and controls (1.59 ± 0.08 g/l) ($p < 0.0001$). Median relative expression of CD15+ was 4% in study group I whereas only one patient in study group II (12.5 %) and the control group (6 %)

was positive for CD15+ expression. Further intragroup comparative analysis between patients with Nelson's grades II (n=16) and III (n=13) has revealed significant differences in both relative expression of CD 15+ and mucin level ($p < 0.0001$) (Table 2).

Despite showing the same Nelson grades, study group II and controls also demonstrated significant differences in mucin level ($p < 0.0001$). It may be due to the possible pathogenetic impact of T2DM on mucin production and quality.

Further correlation analysis has been conducted to assess interactions between mucin levels and age, glycemic control, relative CD 15+ expression, and Nelson's grades (Table 3).

Relative expression of CD15 strongly negatively correlated with the mucin level in the study group I ($r_s = -0.87$; $p < 0.0001$) [14]. Despite moderate negative correlation, CD15+ failed to show significance in study group II, which may be due to the small size of this group ($p = 0.1282$). CD15+ level was also insignificant among controls. However, conducting correlation analysis among all T2DM pa-

Table 1 Basic characteristics of groups

Characteristic		Study group 1 (n=29 eyes)	Study group 2 (n=8 eyes)	Control group (n=15 eyes)	p
Age, years		62.07±5.94	60.00±3.92	63.93±7.00	$p_{1,2}$=0.3612 $p_{2,3}$=0.1590 $p_{1,3}$=0.3588
HbAc1, %		6.79±0.57	7.46±0.21	n/a	$p_{1,2}$=0.0029*
CD 15+		4 (range – 0-10)	0 (range – 0-3)	0 (range – 0-3)	$p_{1,2}$=1,000 $p_{1,3}$=1,000
Mucin level, g/l		n/a	1.28±0.09	1.59±0.08	$p_{1,3}$ < 0.0001* $p_{1,2}$ < 0.0001* $p_{2,3}$ < 0.0001*
Nelson's grade	0-1	n/a	8	15	
	2	16	n/a	n/a	
	3	13	n/a	n/a	

Note. * p < 0.05 is considered statistically significant; n/a – not applicable; p1 – study group 1; p2 – study group 2; p3 – control group.

Table 2 Intragroup comparison (study group I)

Laboratory test	Study group I		p-value
	Nelson's grade II	Nelson's grade III	
CD 15 + (%)	3.5±1.31	7.15±1.86	<0.0001*
Mucin (g/l)	1.08±0.11	0.79±0.10	<0.0001*

Note. * p < 0.05 is considered statistically significant.

Table 3 Correlations between mucin level, age, and laboratory indicators

Indicator \ Mucin	Study group 1 (r_s)	p	Study group 2 (r_s)	p	Control group (r_s)	p
Age	-0.09	0.6134	-0.69	0.0570	-0.34	0.2134
HbAc1	-0.24	0.1969	-0.02	0.9540	n/a	n/a
CD 15+	-0.87	<0.0001*	-0.58	0.1282	-0.43	0.1048
Nelson grade	-0.83	<0.0001*	n/a	n/a	n/a	n/a

Note. *p < 0.05 is considered statistically significant between CD15+, Nelson grade and mucin in study group 1.

tients (both study groups – 37 eyes), we obtained a very strong correlation between CD15+ and mucin level (rs = -0.9). Nelson's grades also negatively correlated with mucin levels in patients with T2DM (rs = -0.87; p < 0.0001). Analyzing mucin levels and abnormal Nelson's grades in study group I, we confirmed a very strong correlation (rs = -0.83; p < 0.0001), however, we could not evaluate the same correlations in study group II and controls. Age and glycemic control have not shown any significant correlation in all groups.

Discussion

T2DM is associated with epithelial damage to the cornea and conjunctiva which leads to a reduction in the number of goblet cells. The latter decreases mucin production and impairs the hydrophilic nature of the ocular surface, resulting in tear film instability [15]. Our study shows a significant difference in tear mucin levels between patients with T2DM and controls. Additionally, a significant reduction in mucin production was observed in patients with Nelson's grades II and III in comparison to grades 0 and I

($p<0.0001$). Baudouin C. et al. (2019) described a potential link between mucin dysfunction and inflammation as part of uncontrolled chronic inflammation, of the vicious circle in dry eye disease [16].

Recent research shows an increasing interest in the role of inflammatory processes, subclinical inflammation, and parainflammation in the development of dry eye disease (DED), with low-grade inflammation being present in T2DM [17]. The presence of polymorphonuclear neutrophil granulocytes is highly regulated in the anterior segment of the eye [18]. Neutrophil granulocytes (CD45+, CD15+) are key components of innate immunity, mainly due to their phagocytic properties and direct migration to the site of inflammation, infiltrating surrounding tissues. Furthermore, they are also involved in the adaptive immune system through interactions with T and B cells [19, 20, 21]. Increased expression of CD15+ was observed in study group 1 compared to study group 2 and controls showed similar patterns in our study. Thus, the latter may be the marker of ocular surface damage in patients with T2DM.

Also, we have demonstrated a strong negative correlation between mucin levels in the tears and the expression of the neutrophil activation marker CD15+ in the epithelial cells of the bulbar conjunctiva ($rs = -0.87$; $p <0.0001$) in the group of patients with T2DM and squamous metaplasia grades II and III according to Nelson's classification, compared to the control group. This could be related to the potentially disrupting pathophysiological effect of T2DM, specifically hyperglycemia, on mucin production and quality.

This study has several limitations. First, a relatively small number of participants and a lack of Nelson's grades variety in the study groups. Thus, larger cohort studies on this topic are required. We also did not determine mucin subtypes, which could be valuable both for diagnosis and for a better understanding of the pathogenic mechanisms of ocular surface damage in patients with T2DM. Also, we did not use qualitative analysis of goblet cell density as it is reported in modified Nelson's scales. We will address these problems in our further research.

In conclusion, tear mucin levels are significantly reduced in patients with T2DM and show a strong negative correlation with the neutrophil activation marker CD15+ in the epithelial cells of the bulbar conjunctiva depending on the grade of squamous metaplasia. This indicates dysregulation of ocular surface homeostasis and the inflammatory component in the development of ocular surface damage in T2DM.

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Data Availability Statement*. All the data obtained and analysed in this study has been reported in this study.*