Original article

 Conjunctival impression cytology in patients with normal and impaired OSDI scores

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 A B S T R A C T
 Purpose: To describe goblet cell density and Nelson grading in different areas of the ocular surface using conjunctival impression cytology (CIC) among patients with normal and impaired Ocular Surface Disease Index (OSDI) scores.
 Materials and methods: Patients (n = 166) under assessment for dry eye were recruited between 2011 and 2012 and classified according to the OSDI score in 4 categories (normal and impaired). Cytological study (CIC plus Papanicolaou staining) using the Nelson grading system, with modifications in staging, and goblet cell counting were performed on the nasal, temporal, inferior, and superior bulbar conjunctival surfaces.
 Results: Nelson grading was significantly higher in patients with a severely impaired OSDI score (1.41 ± 0.14) compared to normal patients (0.86 ± 0.09) (p < .01). Goblet cell density was significantly reduced in patients with a severely impaired OSDI score (310.24 ± 56.24 cells per sample) compared with normal subjects (497.31 ± 50.07 cells per sample) (p < .001). Compared with the photoexposed bulbar conjunctiva, goblet cell density on the non-photoexposed conjunctiva was significantly higher both in patients with mild (p < .01) and moderate (p < .001) OSDI scores.
 Conclusion: Patients with severely impaired OSDI scores have less goblet cells and a higher Nelson grade. Goblet cells are more abundant on the non-photoexposed conjunctiva.

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Cvitología de impresión conjuntival en pacientes con valores OSDI normales y alterados

RESUMEN

Objetivo: Describir la graduación de Nelson y la densidad de células caliciformes en distintas áreas de la superficie ocular usando citología de impresión conjuntival (CIC), en pacientes con valores Ocular Surface Disease Index (OSDI) normales y alterados.

Materiales y métodos: Los pacientes (n = 166) en evaluación por ojo seco, reclutados entre 2011 y 2012, fueron clasificados según el cuestionario OSDI en 4 categorías (normal y alterados). Se evaluó citología (CIC con tinción Papanicolaou) aplicando el sistema de graduación de Nelson, con modificaciones en la determinación de la estadificación, y recuento de células caliciformes en zonas nasal, temporal, superior e inferior de la superficie conjuntival.

Resultados: El grado de Nelson fue significativamente mayor en pacientes con valores OSDI severos, variando desde 0,86 ± 0,09 en pacientes normales a 1,41 ± 0,14 en OSDI severo (p < 0,01). La densidad de células caliciformes disminuyó desde 497,31 ± 50,07 células por muestra en pacientes normales a 310,24 ± 56,24 células por muestra en pacientes con OSDI severo (p < 0,001). La conjuntiva bulbar no fotoexpuesta presentó un número de células caliciformes significativamente mayor (p < 0,0001) que la zona fotoexpuesta en pacientes con OSDI leve (p < 0,01) y moderado (p < 0,001).

Conclusión: La densidad de células caliciformes es menor y la clasificación de Nelson es mayor en pacientes con OSDI severo. La densidad de células caliciformes es mayor en la conjuntiva bulbar no fotoexpuesta.

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Introduction

The ocular surface and lacrimal film are essential for the formation of images as they constitute the first refractive medium. Accordingly, adequate maintenance of their structure and functionalities has a direct repercussion on the visual quality of patients. Alterations in any of their parts produce an imbalance in normal trophism which can give rise to adaptive changes or tissue damage.1,2

The normal corneal and conjunctival epithelium of the ocular surface is flat, multi-stratified and non-keratinized, comprising 5–7 layers of polygonal cells. In addition, the conjunctiva comprises numerous goblet cells which contribute to the formation of the lacrimal film. In diseases such as dry eye this epithelium undergoes reversible adaptive changes which may or may not be associated to inflammation. One of these changes is a pathological transition to a keratinized non-secretory corneal and conjunctival surface, characterized by goblet cell depletion.3,4 A range of methods have been applied to support the dry eye syndrome diagnosis, including the Ocular Surface Disease Index (OSDI) survey, comprising 12 questions on ocular surface symptomatology divided into three categories (ocular discomfort, functionality and effect of environmental conditions).5,6 The survey provides an estimation of dry eye severity degree.6 At present, the clinical assessment as well as lab examinations (histological and cytopathological techniques) evidence ocular surface damage and contribute to establish a pathological clinical correlation with dry eye which has not yet been perfected.3

Conjunctival impression cytology (CIC), based on the application of cellulose acetate filters over the ocular surface, provides samples of superficial epithelium layers for subsequent cytological, immuno-cytological or molecular analysis. This methodology detects incipient changes in the process of squamous metaplasia before it becomes clinically evident and irreversible, and also can be useful for studying conjunctival goblet cells, the number of which could be diminished in patients with severe dry eye.2,3,7,8 The said method, originally described by Egbert et al. in 1977,9 has been modified and utilized for studying various ocular surface diseases.3,7,10 For analyzing CIC, several squamous metaplasia grading systems based on qualitative or quantitative cytological criteria are applied. The best-known methods include the systems by Nelson,11 Tseng,12 Blades et al.13 and Oroza.14 Nelson grading system considers the density, morphology, cytoplasmatic staining affinity and nucleus/cytoplasm ratio of conjunctival epithelial and goblet cells. According to the system, 4 stages can be identified (0–3), with 0 and 1 being normal, 2 and 3 altered.11 The present study proposes to assess CIC based on the use of the Nelson grading rendered objective by the use of semi-quantitative estimations of cytological criteria and goblet cell density in different ocular surface areas in patients with normal OSDI values, and comparing these measurements with those obtained from patients with altered OSDI values.

Materials and methods

A prospective study included 166 patients of the Los Andes Ophthalmological Foundation (Santiago, Chile), with confirmed or suspected dry eye diagnosis during the period 2011–2012. The study was carried out in accordance with the
Fig. 1 – Ocular surface samples with various Nelson gradings (400×). (A) Grade 0. (B) Grade 1. (C) Grade 2. (D) Grade 3. Reference bars = 75 μm.

principles of the 1975 Helsinki declaration and the recommendations of the Ethics Committees of participating institutions. The patients filled in the OSDI survey and were grouped according to the scores obtained in the following categories: normal (0–12 points), slight (13–22 points), moderate (23–32 points) and severe (33 points or more). Representative samples of the superior, inferior, nasal and temporal bulbar conjunctiva of both eyes were obtained from each patient. To this end, after instilling topical anesthesia (0.5% proparacaine), sterile cellulose acetate filters of approximately 5 × 5 mm² were applied (pore size 0.45 μm, Merck Millipore membrane, Darmstadt, Germany) in each area of the ocular surface during 5 s. The said membranes were fixed in 70% ethanol, stained with the Fapanicolou technique, rinsed in xylene (Merck KGaA, Darmstadt, Germany), set in Entellan (Merck KGaA, Darmstadt, Germany) and assessed under light microscope (Axio Lab.A1, Carl Zeiss, Gottingen, Germany). Goblet cell number and Nelson grade were determined for each sample. In order to assess the Nelson grade, the first step was to identify 5 cytological criteria derived from the grading system described by Nelson et al. A numerical value was assigned to each of these criteria according to magnitude (Table 1). The sum of the values supplied by each of the 5 criteria (overall score) determined the Nelson grading for each eye (Table 2). Cytological analysis and goblet cell count were performed by a single observer with an increase of 400×. The goblet cell count was made separately taking areas corresponding to a surface of 25 mm² each. Goblet cell count results (average ± standard error) on the conjunctival ocular surface are presented as cell number in 100 mm² and correspond to the total counts of the 4 areas of each eye. In the differential study based on exposure to light, the results (average ± standard error) are expressed as goblet cell number for

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Reference ranges</th>
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<tr>
<td>Cellularity</td>
<td>0: abundant</td>
</tr>
<tr>
<td></td>
<td>1: moderate</td>
</tr>
<tr>
<td></td>
<td>2: scarce</td>
</tr>
<tr>
<td>Cell-cell contact</td>
<td>0: associated cells</td>
</tr>
<tr>
<td></td>
<td>1: associated cells and isolated cells</td>
</tr>
<tr>
<td></td>
<td>2: isolated cells only</td>
</tr>
<tr>
<td>Nucleus/cytoplasm ratio</td>
<td>0: 1:2</td>
</tr>
<tr>
<td></td>
<td>1: 1.3–1.4</td>
</tr>
<tr>
<td></td>
<td>2: &gt;1.5</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>0: abundant (&gt;300)</td>
</tr>
<tr>
<td></td>
<td>1: moderate (&lt;300)</td>
</tr>
<tr>
<td></td>
<td>2: scarce (&lt;100)</td>
</tr>
<tr>
<td></td>
<td>3: absent (&lt;10)</td>
</tr>
<tr>
<td>Metaplasia</td>
<td>0: absent</td>
</tr>
<tr>
<td></td>
<td>1: incipient</td>
</tr>
<tr>
<td></td>
<td>2: moderate</td>
</tr>
<tr>
<td></td>
<td>3: advanced</td>
</tr>
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</table>
50 mm² and correspond to the sum of the counts of the 2 areas exposed to light (nasal and temporal) and not exposed to light (superior and inferior) of each eye.

A photograph was taken of each sample with a Canon EOS Rebel T3 (Canon, Miami, United States) camera and EOS Utility software version 2.10.0.0 (Canon, United States) (Fig. 1). The results were analyzed and graphed utilizing the GraphPad Prism software version 5.01 (GraphPad, San Diego, California, USA) for the application of statistical tests, T for student and ANOVA, and Bonferroni and Kruskal-Wallis post-tests. The p values p < 0.05 were considered as statistically significant.

### Results

Of the 166 patients (65.8% female and 34.2% male), 43.4% exhibited OSDI within normal ranges and 56.6% exhibited altered values. The latter were subdivided into slight, moderate and severe OSDI categories and percentage-wise were 13.8%, 15.7% and 27.1% of patients, respectively.

The population of the study exhibited Nelson grade increases together with OSDI symptomatology increases (Fig. 2). In patients with normal OSDI, Nelson grading of 0.86 ± 0.09 was obtained and in patients with altered OSDI, Nelson grade of 1.07 ± 0.18; 1.13 ± 0.16 and 1.41 ± 0.14, for the slight, moderate and severe groups, respectively, was obtained. The difference observed between the normal and severe groups was significant (p<0.01).

**Fig. 2 – Nelson grading in patients with normal or altered OSDI values.**

The analysis of the goblet cell population took into account each eye of the studied patients as a sample unit. The analysis excluded samples that were insufficient and those from elderly adults with degenerative goblet cells (hyaline bodies). In accordance with these criteria, only one eye of 3 patients was taken into account and 11 patients were excluded. Therefore, this part of the study comprised 307 eyes.

The number of goblet cells in the overall samples of each eye diminished significantly in a direct relationship with the degree of alteration of the OSDI indicator (Fig. 3). Accordingly, the overall number of goblet cells in normal patients was 497.31 ± 50.07 and in patients with altered OSDI it was 408.54 ± 89.70, 315.1 ± 40.07 and 310.24 ± 56.24 in the slight, moderate and severe groups, respectively. The differences between the normal and severe groups were significant (p<0.001).

On the other hand, a comparison of the number of goblet cells taken from the areas exposed to light (nasal and temporal) and not exposed to light (superior and inferior) of the bulbar conjunctiva, regardless of the OSDI score, revealed that the density of goblet cells is significantly higher in the area which was not exposed to light (128.2 ± 8.24 versus 73.31 ± 7.77) (p<0.0001) (Fig. 4).

In order to establish whether the observed difference in the density of goblet cells between the areas exposed and not exposed to light was modified in subjects with altered OSDI, the goblet cell count between both areas in the patients grouped per OSDI index was compared (Fig. 5). Patients with normal OSDI exhibited a high number of goblet cells in both areas with greater although not significant presence in the areas not exposed to light. In contrast, in the groups with slight and moderate OSDI indicators the differences observed in the density of goblet cells between the areas exposed and not exposed to light were statistically significant (p<0.01 and p<0.001, respectively). In patients with severe OSDI the goblet cell density was significantly diminished in both areas, without significant differences between them.

**Fig. 3 – Goblet cell density in all eye samples based on OSDI index.**

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**Table 2 – Nelson grading in relation to the semi-quantitative estimation of cytological criteria.**

<table>
<thead>
<tr>
<th>Nelson grading</th>
<th>Total score of cytological criteria*</th>
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<tbody>
<tr>
<td>0</td>
<td>0–3</td>
</tr>
<tr>
<td>1</td>
<td>4–5</td>
</tr>
<tr>
<td>2</td>
<td>6–9</td>
</tr>
<tr>
<td>3</td>
<td>≥10</td>
</tr>
</tbody>
</table>

* Total score ranges were determined based on the description of the Nelson et al. grading.¹¹
related to a higher degree of ocular discomfort, also exhibited more ocular surface alteration, advanced squamous metaplasia and even keratinization. It must be noted that not all the abnormal OSDI cases corresponded to symmetric alterations. In these cases, the symptomatology assessed with the OSDI survey would correspond to the eye with greater alteration.

In parallel with the morphological and architectural changes of squamous epithelial cells, this study also observed a significant goblet cell reduction in patients with severe OSDI vis-à-vis normal patients. This could indicate that the conjunctival epithelium has lost its secretory capacity.

In the analysis of goblet cell distribution over the ocular surface across the entire population, higher density thereof was observed in surfaces not exposed to light. This dissimilar distribution of goblet cells on the ocular surface matches the findings of other studies. In normal patients the presence of this cell population is abundant in all areas of the studied bulbar conjunctiva. In contrast, patients with slight and moderate OSDI exhibited significant goblet cell reduction in the surface exposed to light. In addition, the surface exhibited some metaplastic changes. The differential alteration associated to exposure to light was lost in patients with severe OSDI, who exhibit a reduced number of cells in all areas and metaplastic changes affect the entire bulbar conjunctival surface.

Probably, in the initial stages of ocular damage secondary to lacrimal film alteration, higher exposure to external aggressive elements such as wind, low environmental humidity, friction due to blinking and others could be responsible for the lower goblet cell count in various surface areas. However, in the more severe stages, diminished goblet cell count would become a general condition. Accordingly, for practical purposes these studies based on CIC should always consider the ocular surface sector to be studied. Plus, diseases such as dry eye would reduce goblet cell count initially in the conjunctiva exposed to light, caustic liquid aggressions would produce greater damage in the inferior conjunctiva and various self-immune diseases would produce alterations in the entire ocular surface areas.

In the present study, the goblet cell count was lower than that observed by other authors both in patients with normal as well as altered OSDI values. The heterogeneous nature of studied patients, environmental pollution in the city in which the study was carried out or intense/habitual exposure to air conditioning are some of the factors which, on their own or in combination with each other, could partially or entirely explain the observed differences. In addition, the said differences could be related to the cell count methodology applied, which in this study was not based on mathematical approximations or assumed homogeneous goblet cell distribution.

The conclusion is that patients with severe OSDI exhibit higher grades in Nelson’s classification (conjunctival damage) and lower overall goblet cell counts. Ocular surface analysis with CIC revealed greater goblet cell density in the bulbar conjunctiva which was not exposed to light, both in healthy patients and moderately altered OSDI patients. These observations evidence the importance of studying the ocular surface as a whole.
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Conflict of interests

No conflict of interests has been declared by the authors.

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