Impression conjunctival cytology in sicca syndrome – correlations between clinical and histological findings related to dry eye severity

CARMEN-LUMINIȚA MOCANU1), SANDA JURJA2), ANDREA-GBRÉILA DECA3), FLORICA BÎRJOVANU4), ANDREI OLARU1), DENISSA-GRETA POPA1), ALIN-ȘTEFAN ȘTEFĂNESCU-DIMA1), MARIA-RODICA MĂNESCU5)

1)Department of Surgical Specialties II, University of Medicine and Pharmacy of Craiova, Romania
2)Department of Ophthalmology, Faculty of Medicine, “Ovidius” University, Constanța, Romania
3)Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania
4)Department of Ophthalmology, “Dr. Ștefan Odobleja” Emergency Military Hospital, Craiova, Romania
5)Department of Morphological Sciences, University of Medicine and Pharmacy of Craiova, Romania

Abstract
Keratoconjunctivitis sicca represents a progressive deterioration of ocular surface produced by a deficient secretion of lachrymal film (quantitative disorder) or excessive tear evaporation (qualitative disorder). The cytological analysis of conjunctival impression in 42 patients with dry eye syndrome established a strong correlation between the clinical grade of severity of disease and the grade of squamous metaplasia, including goblet cell loss. The cellular anomalies were represented by modifications of keratinization, epithelial cells’ anisocytosis, anisochromia, the nuclear condensation and the cytoplasmic vacuolization. Pyknotic nuclei and anucleated cells were only seen in the most severe dry eye.

Keywords: impression cytology, conjunctiva, sicca syndrome, goblet cells, ferning test.

Introduction
Sicca syndrome consists in ocular and oral signs and symptoms, determined by a decreasing of lachrymal and salivary glands. The importance of dry eye syndrome in ocular pathology has increased in the last decades, being considered a very common disorder of tear film that results by either decreasing tear production or excessive tear evaporation.

The intimate relationship between lachrymal glands and the other adjacent ocular structures – cornea, conjunctiva – explains the intricate pathological manifestations; the tear film becomes unstable with progressive deterioration of ocular surface ensues [1].

Conjunctival impression cytology using cellulose acetate filter paper is an innovative technique to study the viability of conjunctiva. Impression cytology represents a non-invasive or minimally invasive biopsy of the ocular surface epithelium with no side effects or contraindications [2]. The method was used for the first time in 1974 by Egbert et al. [3], which they called a simple conjunctival biopsy.

It has demonstrated to be a useful diagnostic support for a wide variety of processes involving the ocular surface. In addition, and mainly during the last decade, its use as a research tool has experienced an enormous growth and has greatly contributed to the understanding of ocular surface pathology [2].

Consequently, during the past decade, the method has been used increasingly for diagnose and research, improving our understanding of the pathophysiology of ocular surface disease, and provide biomarkers to be used as outcome measures in clinical trials [4].

After experimenting with a variety of methods, including cellophane tape, photographic film, and various synthetic filters (Duralon, Polivy, Mitex), Nelson et al. [5] found that the original Millipore (mixed esters of cellulose) filter seemed effective for the purpose.

In the original method of staining, the material obtained by the cellulose acetate paper is transferred on the surface of slide at 4°C [6]. In time, the method suffered several modifications, for example the procedure of transferring the impressions from the filter paper to the slide, now almost all people doing that at room temperature [7].

Filippello et al. [8] used a very creative variation of this method, applying the Millipore filter on the ophthalmodynamometer piston to maintain a constant pressure on the ocular surface.

Purpose
The purpose of this study is to evaluate the clinical and histopathological lesions in sicca syndrome, related to stage of the disease, using the technique of conjunctival impression and to establish significant correlations with other clinical tests for qualitative and quantitative production of lachrymal secretion.

Patients and Methods
Forty-two patients with sicca syndrome in different stages of evolution and 10 normal were included in this...
study. A complete general and ocular examination has been performed, to establish the etiologic diagnosis. Standard ocular examination consisted in both eyes visual acuity (VA), and intraocular pressure (IOP) measurement, biomicroscopy and ophthalmoscopy.

We used two different vital colorations for each patient to establish the diagnosis of keratitis and to evaluate the most superficial lesion of conjunctiva. The Fluorescein staining was performed initially using 1% Fluorescein; in the second staining, we used 1% Rose Bengal (imposed as diagnostic criteria by the European Study Group in Sjögren’s syndrome). We graded using van Bijsterveld scale from 0 to 9, a score ≥4 representing a diagnostic criteria for sicca syndrome.

Quantitative examination of lacrimal film consisted in Schirmer I test measure, and Jones test examination.

Qualitative examination of lacrimal film has been represented by tear crystallization test and break-up time (BUT) evaluation.

Histopathological examination was represented by impression cytology and histopathological examination of superior conjunctiva.

Conjunctival impression cytology was realized using a cellulose acetate filter paper (Millipore paper) to pick up a layer of cells from the conjunctival surface.

The procedure is extremely simple and non-invasive; the protocol requires the following steps:

- Perform surface anesthesia with 0.3% Oxybuprocaine instilled in the conjunctival sac, than remove any excess liquid from conjunctival sac;
- Prepare cellulose acetate filter paper Millipore into 3 mm square pieces and place the filter paper on the surface of the eye in the temporal bulbar part using a smooth ended forceps;
- Gently press the paper on surface of the conjunctiva, where will be kept for 3–5 seconds;
- Move the filter paper on the surface of a clean glass slide, at room temperature where the impression is transferred by gentle pressure;
- Fixed tissue with 95% ethanol and 1% formalin;
- Staining with Periodic Acid–Schiff (PAS), Hematoxylin–Eosin (HE), Giemsa, Gömöri and van Gieson.

The dental tests include one of the following exams: salivary flow, salivary scintigraphy or salivary biopsy.

Results

Based on general examination, we classified cases in primary or secondary Sjögren’s syndrome, and based on ocular examination we divided in four stages of severity:

- Stage I: sicca conjunctivitis, normal cornea;
- Stage II: superficial punctate keratitis;
- Stage III: filamentous keratitis;
- Stage IV: risk of corneal cecity (corneal ulcus).

In our series, we identified 12 cases with minor form, 11 cases with moderate form, and 19 cases with severe form of disease; 10 normal subjects were introduced in this study to compare results.

For Jones test, all values were lower than the normal values. The smallest values <4 mm were recorded in most severe cases. We did not demonstrate an absolute relationship between the test results and clinical evolution; in some cases, we considered that the poor general statement, and the association with severe general diseases, represented the most important factor in the evolution. Comparing the clinical results of Schirmer I test and Jones test, we have concluded that the results were quite similar and we did not find more clinical information, using Jones test measurements.

BUT test is mandatory in determining sicca syndrome. Values less than 10 seconds are pathological, between 10–12 seconds they were inconclusive. BUT translates the mucinic layer integrity from the lachrymal film structure, and it was correlated with the other quantitative or qualitative methods of lachrymal secretion. The study results have revealed that 33 patients have had BUT less than 10 seconds and nine patients between 10–12 seconds.

Schirmer/BUT ratio was 78%, demonstrating that a decreasing quantity of lachrymal secretion is not always associated with qualitative disorder (tear instability).

Ferning test presented four types of crystallization:

- Type I – uniform “fern leaf”-like ramifications with a rigid trunk and perpendicular branches, covering the entire microscopic field without spaces between “leaves” (Figure 1A);
- Type II – very similar with type I, but the “fern leaves” are smaller, less ramified with larger spaces between (Figure 1B);
- Type III – in this stage, the “fern leaf” crystallization is partially present; “leaves” are small and incompletely formed, with rare or absent ramifications and large gaps (Figure 1C);
- Type IV – “fern leaf” crystallization is rare or absent (Figure 1D).

Type III and IV of crystallization occurred in 87% of patients in correspondence with the intensity of the dry eye symptoms, and with a high correspondence with conjunctival impression and histopathological lesions.

Conjunctival impression, revealed in normal cases, numerous small, round epithelial cells with eosinophilic cytoplasm and large basophil nuclei (nucleus/cytoplasm ratio of 1:2). The goblet cells are identified by the PAS-positive cytoplasm, eccentric placed nuclei and large size (Figure 2, A–J).

In sicca syndrome patients, depending of severity, morphological changes of epithelial cells are recorded:

- In stage I, the epithelial cells are slightly larger and more polygonal; the nuclei are smaller with a nucleus/cytoplasm ratio of 1:3.
- In stage II, the epithelial cells appeared to be smaller with squamous metaplasia – there was a poor cytoplasm and a pyknotic nucleus;
- In stage III, squamous metaplasia was detected in 89% of temporal bulbar conjunctiva – clumped mucous aggregates on the bulbar conjunctiva.

In normal specimens, the goblet cells are abundant and plump, oval and have an intensely PAS-positive cytoplasm. With grade I change, their numbers reduce, still the morphology is unchanged. The average density of goblet cells in normal control subjects was around 200/mm²; the average density of goblet cells in dry eye...
was remarkably lower in parallel with the severity of the disease (in advanced stages of disease around 80/mm²). The difference found between normal control subjects and those with dry eye in three different stages of evolution was statistically significant ($p<0.001$).

The histopathological exam of conjunctiva in dry eye patients showed extensive, progressive hyperplasia, hypertrophy and cellular flattening of epithelial cells, with loss of goblet cell density. We have identified small epithelial cells only in the control group and incipient forms of dry eye, their size increasing with the severity of the disease. Also, depending on evolution stage, we noticed different pathological grades of squamous metaplasia.

Clear nuclear alterations were present from very early stages, but pyknotic nuclei and anucleated cells were seen only in the most severe dry eye.

Figure 1 – Ferning test type I to type IV showed the crystallization in “fern leaf” ramifications ($×40$): (A) Type I; (B) Type II; (C) Type III; (D) Type IV.

Figure 2 – Conjunctival impression of normal subjects and in different type of sicca patients. Normal cellularity of conjunctival impression (A), mild keratoconjunctivitis sicca with anisocytosis (B and C), and racket cells (D). HE staining: (A and C) $×200$. Giemsa staining: (B and D) $×100$. 
Figure 2 (continued) – Conjunctival impression of normal subjects and in different type of sicca patients. Moderate keratoconjunctivitis sicca – the modifications in epithelial cells and conjunctival goblet cells reveal cellular sufferance (E and F). Severe keratoconjunctivitis with anisocytosis, anisochromia, the nuclear condensation, and the cytoplasmic vacuolization, eccentric nucleus, and decreased nucleus/cytoplasm rapport (G–I). Keratoconjunctivitis sicca complicated with corneal ulcus (J) very rare cellularity, with altered morphology. HE staining: (E, G, I and J) ×200; (F) ×400. Giemsa staining: (H) ×100.

Depending of severity, we noticed an increasing in cellular separation with progressive stratification, and mucous layer decreasing. Blood and lymphatic vessels showed alterations only in the most severe eyes, with increase of inflammatory cells number (Figure 3, A–F).

In all stages, we proved a very high parallelism between histopathological examination and impression conjunctival cytology.

Figure 3 – Histopathological aspect of conjunctiva in keratoconjunctivitis sicca different stages of evolution: Mild form. HE staining: (A) ×100; (B) ×200.
Discussion

Keratoconjunctivitis sicca is a chronic, progressive, inflammatory disease characterized by two distinct etiopathogenic mechanisms: a deficient production of the aqueous part of lacrimal film (quantitative disorder) or excessive tear evaporation (qualitative disorder), the last one by inadequate production of lipid layer.

Both, quantitative and qualitative tear film disorders will produce corneal and conjunctival surface alteration, causing ocular symptoms that vary in severity, related to stage of diseases; on the other hand, even the diagnosis is clear in almost all cases, the symptoms never indicate the type of disorders (qualitative or quantitative).

The complete diagnostic is important, because the most of treatments accomplish only the qualitative or the quantitative side of disease.

The diagnosis becomes totally clear only performing histopathological examination, and observing the goblet cells, or determining the biochemical tear composition.

Still, they cannot be introduced in practice, the first one because of huge discomfort of invasive procedure, the second because is expensive, being utilized only for research reasons. In this moment, clinical tests give clear information in less than 50% of cases, about quantitative and qualitative disorders of tears.

In this study, we try to demonstrate the sensitivity and specificity of conjunctival impression in dry eye, making correlations with the other clinical diagnostic tests, and comparing with histopathological exam.

The most common histopathological feature observed in dry eye syndrome is squamous metaplasia, accompanied by keratinization with advanced epithelial changes (nucleus/cytoplasm ratio between 1:3 and 1:6 with pyknotic nuclei) [9, 10].

Extensive goblet cell loss (>75%) on the temporal bulbar conjunctiva, and mucous aggregates on the temporal bulbar and inferior bulbar conjunctiva were observed in a significantly greater percentage of severe dry eyes than in the other groups [11, 12].

A strong correlation was noted between the inferior tarsal inflammatory infiltrate and the extent of squamous metaplasia of the bulbar conjunctiva in dry eyes specimens, different stages [13].

The results of previous studies indicate that the diagnostic specificity of impression cytology in ocular surface disorders relies on the constellation and topographic distribution of cytological features including squamous metaplasia, goblet cell loss, keratinization, and mucous aggregate formation at three different conjunctival sites: exposed (temporal bulbar) and non-exposed (inferior bulbar) bulbar, and the inferior tarsal conjunctiva [14, 15].

Tamer et al. [16] found squamous metaplasia of the temporal and inferior bulbar in combination with mucous aggregates of the bulbar conjunctiva to be highly specific for sicca syndrome (97% specificity).

The qualitative alteration of lacrimal film is determined by decreasing of quantity of mucin produced by the conjunctival goblet cells. The detection of a significant reduction in the number of goblet cells in patients with dry eyes is parallel with the severity [17–20].

A significant reduction in the level of mucin implies a consequential rapid degeneration of the tear film that is, the creation of hydrophobic areas on the cornea and conjunctiva, which in turn can give rise to increased risk of infection [8, 21, 22].

Because the ferning test strictly depend on quantity and quality of mucin from lacrimal film, is easy to understand that the alteration of mucin layer is parallel with the alteration of ferning test [23].
The reduction of mucin layer in patients with dry eye disease will increase the evaporative process, determining a vicious circle, with progressive alteration and losing of goblet cells [24].

We used in our study this test together with conjunctival impression, and histopathological examination to obtain a complete imagine about histopathological lesions in dry eye; also, we made all necessary correlations in order to standardize conjunctival impression in function of stage of the disease, to be useful and clear in practice.

In our specimens, the conjunctival impression revealed various types of modifications in different intensities; the cellular anomalies from ocular cytology were presented in different associations and intensities in all grades (I–IV) of sicca syndrome. The lesions are similar with the previous studies, regarding the alteration of aspect and the modification of number of cells.

The cellular anomalies are not specific, representing by modifications of keratinization, epithelial cells’ anisocytosis, anisochromia, the nuclear condensation and the cytoplasmatic vacuolization. The modifications in epithelial cells and conjunctival goblet cells reveal cellular sufferance, with an evident parallelism between these anomalies and clinico-functional signs in keratoconjunctivitis sicca. In our study, the conjunctival impression offers a sensitivity >90% and a specificity >95% in sicca syndrome patients (all specimens were analyzed in double blind manner by two different examiners).

In the absence of a codification and of a gravity scale of the cellular anomalies, only few comparative studies have been performed between the clinical forms and stages of sicca syndrome and cytological aspect.

Regarding our experience, we consider that conjunctival impression provide an easy and quick identification of the lacrimal film alterations existent in the sicca syndrome and gives us valuable information about the qualitative disorders.

Impression cytology is a simple, very well tolerated, non-invasive, but unfortunately not very standardized technique to evaluate ocular surface epithelial changes. In our opinion, it can offers highly confident histopathological information, in a disease where a routine histopathological exam is inappropriate. It is fast, the entire procedure takes several minutes, and it is very cheap, the paper and the stainings are affordable in any medical unit. It can be incorporated without any important adjustment in clinical standard investigation for patients with conjunctival chronic diseases.

The method offer a high development potential, like cytological analysis of eye surface sebaceous carcinoma [25] and recently the conjunctival impression specimens have been used for indirect immunofluorescence performed with mouse immunoglobulin G1 (IgG1) anti-HLA-DR-α chain as primary monoclonal antibody [26].

Conclusions

The cytological analysis of conjunctival impression in 42 patients with dry eye syndrome established a strong correlation between the clinical grade of severity of disease and the grade of squamous metaplasia, including goblet cell loss. Having more than 90% sensitivity and specificity, the method is valuable to detect and define histopathological lesions in dry eye syndrome, where a standard histopathological exam is not indicated.

Conflict of interests

The authors declare that they have no conflict of interests.

References

Impression conjunctival cytology in sicca syndrome – correlations between clinical and histological findings...


**Corresponding author**
Andreea-Gabriela Deca, University Assistant, Pharm, PhD, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40756–099 642, e-mail: gabriela_deca@yahoo.com

Received: April 7, 2015
Accepted: February 29, 2016