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Impression cytology on conjunctiva and cornea in dry eye patients establishes a correlation between squamous metaplasia and dry eye clinical severity

J. MURUBE[1], L. RIVAS[2]

[1] School of Medicine, Alcalá de Henares University
[2] Service of Ophthalmology, Hospital Ramón y Cajal, Madrid - Spain

PURPOSE. *To obtain deeper knowledge of the cellular transition in squamous metaplasia, and to look for a correlation between the clinical grade of severity of dry eye and the grade of squamous metaplasia of the corneal and conjunctival epithelium, studied by impression cytology.*

METHODS. *A total of 143 patients with dry eye disorders of different grades of clinical severity and 33 control subjects of matched age and sex were studied. Symptoms, clinical tests (including Schirmer test, slit-lamp examination, break-up time, rose Bengal staining, vanishing lacunar sulci, and neovascularization), and tear osmolarity were used to establish the diagnosis of dry eye. The subjects were classified into six clinical grades, grade 0 indicating normal and grades 1 to 5 progressively more severe dry eye. Impression cytology specimens were taken from the central cornea and different areas of the conjunctiva of one eye from all patients. A morphologic and morphometric study of the photographs obtained by light microscopy showed cell size, nuclear size, nuclear-cytoplasmic ratio (N:C) in nonsecretory epithelial cells, and density of goblet cells.*

RESULTS. *Morphometric and morphologic studies of the ocular surface cells indicated significant differences, mainly in cell sizes, nuclear alterations, and the N:C ratio, in nonsecretory epithelial cells of the conjunctiva and cornea, and in goblet cell densities from the conjunctiva, between the clinically normal eyes and those with the five grades of clinical severity of dry eye, with different degrees of squamous metaplasia.*

CONCLUSIONS. *A morphologic and morphometric analysis of the ocular surface from patients with dry eye obtained by impression cytology led us to draft a new grading system containing one normal level and five levels of squamous metaplasia. This new grading system is based on a significant decrease in the number of goblet cells with less periodic acid-Schiff-hematoxylin-positive staining, an increase in nonsecretory cell size, more marked cell separation, a lower N:C ratio, and an increase in nuclear alterations. The clinical severity of the dry eye correlates with these alterations. (Eur J Ophthalmol 2003; 13: 115-27)*

KEY WORDS. *Dry eye, Ocular surface, Impression cytology, Squamous metaplasia*

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INTRODUCTION

We usually evaluate patients with dry eye syndromes in daily practice on the basis of clinical data, using a clinical grading system (1). However, laboratory and histopathologic tests can be helpful in the diagnosis and grading of dry eye syndrome (2).

The epithelium of the normal ocular surface is stratified and not keratinized. In dry eye syndromes, the normal epithelium suffers squamous metaplasia, the corneal one becoming keratinized, and the conjunctiva becoming keratinized and non secretory.

Impression cytology is more sensitive than clinical and laboratory tests, and has the advantage of being able to detect squamous metaplasia before keratinization is clinically detectable (3, 4). Impression cytology is a useful noninvasive technique, in which the first or the two outermost layers of the ocular surface epithelium are removed and studied to determine the state of the conjunctival surface (5, 6) and to classify the severity of squamous metaplasia (2). It was first used by Egbert et al (7) and modified by other authors (5, 6) for use in daily practice, giving special attention to cell surface area (8), the shape of the conjunctival cells (9), and the nuclear-cytoplasmic ratio (N:C) (2, 10).

There are several grading systems of squamous metaplasia based on different criteria. Among the best known are those of Nelson (5), Tseng (11), Blades et al (8), and Oroza (2). Nelson's system (5) looks at the number and shape of goblet cells, the size and shape of nonsecretory conjunctival epithelial cells, the cytoplasmic stain, and the N:C ratio, describing four stages, where grades 0 and 1 are normal and grades 2 and 3 are abnormal. Tseng's grading system (11) also studies only the conjunctiva, describing the number of goblet cells, size of nonsecretory epithelial cells, keratinization, cytoplasmic stain, and N:C ratio, developing six stages in a system mainly based on the keratinization of nonsecretory epithelial cells, where only grade 0 is normal. Blades et al (8) do not establish a proper grading system, as they obtained only the conjunctival surface area in a group of normal subjects and a group of patients with dry eye, including any stage of dry eye without specifying the level of severity. Oroza's grading system (2) describes the number and shape of goblet cells, the area of nonsecretory conjunctival and corneal epithelial cells, cytoplasmic stain, nuclear area, nuclear alterations, and the N:C ratio, proposing a system with five grades, where grades 0 and 1 are normal. Our present study is based mainly on these parameters, but unlike the other studies, we also studied the cornea, and correlated the results with the clinical stages of dry eye.

A new classification system is necessary for two reasons. First, the three classifications described do not include the state of the cornea, although the cornea is the most important part of the lacrimal system. The lacrimal system exists in terrestrial vertebrates to serve the cornea, which is the center of dacryology, and produces the majority of symptoms and signs in patients with dry eye. The other reason is that clinicians attend patients because of their clinical symptoms and signs, and then classify and treat them accordingly. The histopathologic classifications cited were made independently of these facts; therefore, the clinician must transfer these stages to clinical situation.

No perfect correlation has ever been described between the stages of clinical classification and stages of impression cytology. We therefore decided first to classify patients according to the clinical stage of the dry eye, and then to look for changes in the ocular surface epithelium.

MATERIALS AND METHODS

A total of 143 patients attending the Dry Eye Clinic of the Hospital Ramón y Cajal were consecutively selected, and classified according to Murube's clinical progressive grading classification:

- *Grade 0*: normal subjects;
- *Grade 1*: symptoms of dryness (dry sensation, itching, sandy sensation, etc.) only on over-exposure, which would not appear in normal people;
- *Grade 2*: symptoms in normal daily life, but no clinical signs;
- *Grade 3*: symptoms of grade 2 plus reversible clinical signs (redness, corneal staining, keratitis punctata, filaments, etc.);
- *Grade 4*: grade 3 plus irreversible signs (corneal scars, corneal vascularization, retraction of the lower fornix, of the lacunar sulci, etc.);
- *Grade 5*: grade 4 plus corneal keratinization and visual deficiency.

Samples were taken from 33 patients for each of

grades 1, 2, and 3, and from 22 patients each for grades 4 and 5. The control group comprised 33 subjects without symptoms and signs of keratoconjunctivitis sicca, attending the hospital for low refractive defects. Patients and controls were individually informed about the nature of the impression cytology study and gave informed written agreement to participate.

All patients also had at least three altered clinical tear tests and tear osmolarity findings, according to the pathological values proposed by the Pisa criteria for dry eye (11): the Schirmer test (abnormal <10 mm), tear break-up time (BUT) (abnormal <10 sec), rose Bengal vital staining (abnormal >4 crosses over 9), and tear osmolarity (abnormal >310 mOsm/L).

Patients with dry eye had an age range of 24 to 84 years (mean 55 years). There were 70 women (53.0%) and 62 men. Normal subjects without ocular surface alterations had an age range of 21 to 78 years (mean 51 years). There were 18 women (54.5%)and 15 men.

Impression cytology specimens were obtained after topical anesthesia with tetracaine hydrochloride and oxibuprocaine hydrochloride (Colircusí Anestésico Doble, Alcon Cusí SA) from only the right eye of each patient. Strips of cellulose acetate filter paper (Millipore HAWP304) were applied over the ocular surface with gentle pressure for a few seconds, using the flat surface of the forceps, and then the filter paper was lifted off with a smooth peeling motion. Samples were taken from the central cornea, central upper bulbar conjunctiva, interpalpebral conjunctiva (medial and lateral trigoni), central lower bulbar conjunctiva, and central lower palpebral conjunctiva. The samples were fixed in 96% ethanol, stained with periodic acid-Schiff (PAS) and hematoxylin, dehydrated in ascending grades of ethanol, placed in xylene to clear, and permanently mounted in Entellan resin, with a cover slip.

Later, the filter papers were placed on glass slides for identification. The number of secretory goblet cells, cytoplasmic and nuclear area from nonsecretory conjunctival and corneal epithelial cells, cytoplasmic alterations and staining, nuclear changes, and the N:C ratio were studied by light microscopy.

All these parameters from cornea and conjunctiva were measured by image planimetry Pro/Media Cybernetics with a CDA system based on IPP-Plus software. The samples were examined with a Nikon Ophtiphot-2 microscope and attached to a MicroflexDX photography and an EVI-1011P color video camera. The software is designed specifically for measuring the general appearance of cells or two-dimensional features. Areas of cytoplasm or nucleus are included in the boundary. With a pointer connected to the planimeter, morphometric characteristics were determined over photographs of 10 x l5 cm, prepared at a nominal magnification of 40x. The pixels within the nuclear and cellular peripheries were transferred to square microns, and successive cycles of mathematical morphology operations gave the size and shape. The relation between the area of the cell nucleus and the surrounding cell cytoplasm (N:C ratio) was analysed.

Quantitative studies of conjunctival goblet cells were carried out using a calibrated grid at magnifications of 400x. The mean total goblet cell densities and standard deviation were obtained by counting ten random areas for each specimen. The densities were reported per square millimeter.

TABLE I - NUMBERS OF NORMAL SUBJECTS AND PATIENTS WITH ALTERED TEAR TESTS. Statistical differences were found in comparison with the preceding grade of severity of the dry eye in each test

	Schirmer test	RB staining	BUT	Osmolarity
Grade 0 (n = 33)	1	0	1	1
Grade 1 (n = 33)	2 n.s.	2 *	2 n.s.	3 *
Grade 2 (n = 33)	6 *	4 *	5 *	8 *
Grade 3 (n = 33)	10 *	7 *	10 *	12 *
Grade 4 (n = 22)	11 *	16 *	15 *	20 *
Grade 5 (n = 22)	17 *	20 *	19 *	22 n.s.

* $p < 0.05$
RB = Rose Bengal; BUT = Breakup time;
n.s. = Not significant

Statistical analysis was done with a statistical package (SPSS 7.5 for Windows). An unpaired two-tailed Student's t-test and analysis of variance (ANOVA) were used to compare independent groups, and a nonparametric Spearman rank correlation test to compare the relations between clinical and morphologic tests. Differences were considered significant with a probability higher than 95% (confidence interval, $p < 0.05$).

RESULTS

All the results from normal subjects and patients were statistically studied in six clinical groups (one normal and five pathologic), and there was perfect correlation with the morphometric and morphologic cellular findings of the ocular surface. Any non-sufficiently prevalent parameter, such as snake-like chromatin (SLC), was eliminated from our grading system. During the statistical analysis we found significant differences and discontinuous interval data in the histogram analysis of all six grades.

Clinical and laboratory tests are shown in Table I. There were significant differences within each test compared with the preceding grade of severity of dry eye. Only two exceptions were found: one, between grades 0 and 1 in the Schirmer test and BUT, and the other, between grades 4 and 5 in tear osmolarity.

In each person the area of nonsecretory cells of the ocular surface increased in the following order: lower palpebral and bulbar area, upper bulbar area, and exposed area, in all grades of dry eye. There were constant significant differences between the various grades of severity of dry eyes. In grade 0 there were no differences between all the areas. Grade 1 only showed significant differences between the interpalpebral

TABLE II - CELL AREAS IN DIFFERENT PARTS OF THE CONJUNCTIVA (IN μm^2). Statistical differences were found in comparison with the preceding grade, and with the inferior conjunctival area of the same grade. Corneal cell area was compared to the previous grade and to conjunctival interpalpebral trigoni

	Upper bulbar	**Interpalpebral trigonal**	**Lower bulbar**	**Lower palpebral**	**Cornea**
Grade 0	252.9 ± 46.1	248.3 ± 38.6	246.7 ± 35.4	240.4 ± 30.5	322.41 ± 34.08
	n.s.	n.s.	n.s.		*
Grade 1	314.5 ± 45.7	307.6 ± 41.6	247.1 ± 40.5	240.4 ± 41.6	346.36 ± 35.57
	n.s.	n.s.	n.s.	n.s.	n.s.
	n.s.	*	n.s.		*
Grade 2	512.2 ± 75.1	333.1 ± 62.7	256.2 ± 45.9	261.3 ± 40.4	752.50 ± 49.83
	*	n.s.	n.s.	n.s.	*
	*	*	n.s.		*
Grade 3	962.4 ± 92.5	678.4 ± 72.7	344.1 ± 42.0	282.0 ± 30.3	1422 ± 160.43
	*	*	*	n.s.	*
	*	*	*		*
Grade 4	1624.4 ± 242.3	1024.4 ± 187.3	550.4 ± 82.3	384.4 ± 41.0	2238.13 ± 250.18
	*	*	*	*	*
	*	*	*		*
Grade 5	3747.4 ± 321.1	2802.6 ± 261.8	1947.3 ± 217.2	993.4 ± 207.6	2911 ± 248.33
	*	*	*	*	*
	*	*	*		n.s.

*p < 0.05
n.s.= Not significant

TABLE III - MAIN NUCLEAR CHANGES (%) IN NONSECRETORY CONJUNCTIVAL AND CORNEAL CELLS

	Upper bulbar	Interpalpebral trigonal	Lower bulbar	Lower palpebral	Cornea
Grade 0	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	-	-	-	-	-
	Normal (100%)	Normal (100%)	Normal (100%)	Normal (100%)	Normal (100%)
Grade 1	-	-	-	-	-
	Snakelike (12.1%)	Snakelike (9.1%)	-	-	Snakelike (9.1%)
	-	-	-	-	-
	-	-	-	-	-
	Normal (87.9%)	Normal (90.9%)	Normal (100%)	Normal (100%)	Normal (90.9%)
Grade 2	Binucleated (21.2%)	Binucleated (21.2%)	Binucleated (9.1%)	-	Binucleated (13.6%)
	Snakelike (15.2%)	Snakelike (12.1%)	-	-	Snakelike (12.1%)
	-	-	-	-	-
	-	-	-	-	-
	Normal (63.6%)	Normal (66.7%)	Normal (90.9%)	Normal (100%)	Normal (74.3%)
Grade 3	Binucleated (15.2%)	Binucleated (18.2%)	Binucleated (18.2%)	Binucleated (9.1%)	Binucleated (18.2%)
	Snakelike (21.2%)	Snakelike (9.1%)	-	-	Snakelike (12.1%)
	Pyknotic (24.2%)	Pyknotic (12.1%)	Pyknotic (9.1%)	Pyknotic (9.1%)	Pyknotic (9.1%)
	Anucleated (12.1%)	Anucleated (18.2%)	Anucleated (9.1%)	-	-
	Normal (27.3%)	Normal (42.4%)	Normal (63.6%)	Normal (81.8%)	Normal (60.6%)
Grade 4	Binucleated (9.1%)	Binucleated (9.1%)	Binucleated (13.6%)	Binucleated (18.2%)	Binucleated (9.1%)
	Snakelike (9.1%)	Snakelike (9.1%)	-	-	Snakelike (9.1%)
	Pyknotic (36.4%)	Pyknotic (36.4%)	Pyknotic (27.3%)	Pyknotic (18.2%)	Pyknotic (27.3%)
	Anucleated (31.8%)	Anucleated (22.7%)	Anucleated (27.3%)	Anucleated (13.6%)	Anucleated (18.2%)
	Normal (13.6%)	Normal (22.7%)	Normal (31.8%)	Normal (50.0%)	Normal (36.3%)
Grade 5	Binucleated (9.1%)	Binucleated (9.1%)	-	-	Binucleated (9.1%)
	Snakelike (9.1%)	Snakelike (9.1%)	-	-	-
	Pyknotic (9.1%)	Pyknotic (9.1%)	Pyknotic (27.3%)	Pyknotic (27.3%)	Pyknotic (9.1%)
	Anucleated (63.6%)	Anucleated (63.6%)	Anucleated (63.6%)	Anucleated (63.6%)	Anucleated (72.7%)
	Normal (9.1%)	Normal (9.1%)	Normal (9.1%)	Normal (9.1%)	Normal (9.1%)

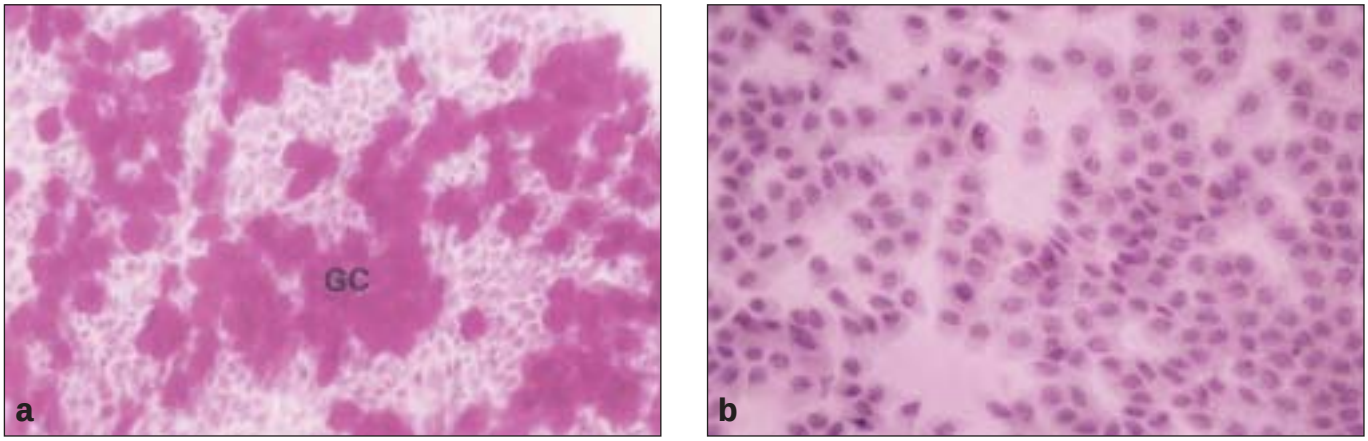


Fig. 1 - a) *Impression cytology in conjunctiva from normal subjects* (grade 0). *Sheet of small, round, nonsecretory epithelial cells with no nuclear alterations, and numerous oval goblet cells (GC) with an intensely periodic acid-Schiff (PAS)-positive cytoplasm. PAS-hematoxylin staining. Magnification x40.*
b) *Impression cytology in cornea from normal subjects* (grade 0). *Sheet of small, round, nonsecretory epithelial cells with normal nuclei and a nuclear-cytoplasmic ratio of 1:3. PAS-hematoxylin staining. Magnification x40.*

TABLE IV - NUCLEAR AREA IN CONJUNCTIVAL AND CORNEAL EPITHELIAL CELLS (IN μm^2) WITH A MORPHOLOGICALLY NORMAL ASPECT. Statistical differences were found in comparison with the preceding severity group, and with the inferior conjunctival area of the same grade. The corneal nuclear area was compared to the previous grade and to the interpalpebral conjunctival trigoni

	Upper bulbar		Interpalpebral trigonal		Lower bulbar		Lower palpebral	Cornea
Grade 0	92.7 ± 11.3	n.s.	91.3 ± 9.6	n.s.	94.1 ± 9.2	n.s.	93.9 ± 10.5	106.4 ± 10.5 *
Grade 1	83.7 ± 9.5 n.s.	n.s.	81.7 ± 8.6 n.s.	n.s.	82.8 ± 7.3 n.s.	n.s.	84.7 ± 7.3 n.s.	96.7 ± 11.0 n.s. *
Grade 2	77.7 ± 8.8 n.s.	n.s.	77.1 ± 9.2 n.s.	n.s.	76.8 ± 7.9 n.s.	n.s.	78.4 ± 8.6 n.s.	93.4 ± 9.1 n.s. *
Grade 3	72.3 ± 5.2 n.s.	n.s.	69.8 ± 10.5 n.s.	n.s.	72.3 ± 6.1 n.s.	n.s.	70.8 ± 7.3 n.s.	88.7 ± 7.8 n.s. *
Grade 4	54.1 ± 6.3 *	n.s.	54.7 ± 5.6 *	n.s.	55.0 ± 6.8 *	n.s.	56.1 ± 6.9 *	84.0 ± 7.6 n.s. *
Grade 5	51.5 ± 5.0 n.s.	n.s.	52.3 ± 5.2 n.s.	n.s.	53.5 ± 6.4 n.s.	n.s.	55.0 ± 7.7 n.s.	81.3 ± 10.7 n.s. *

*p < 0.05
n.s.= Not significant

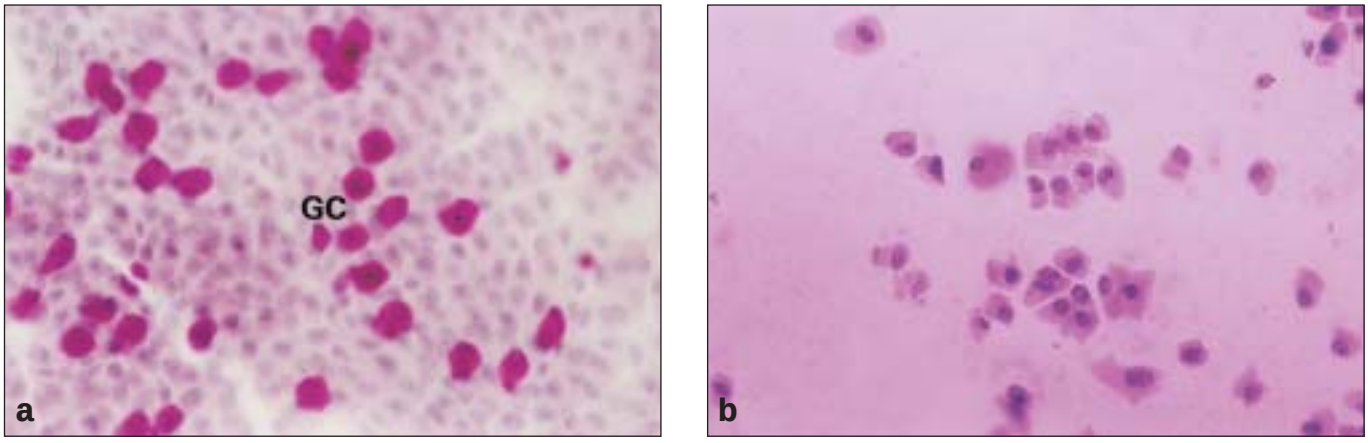


Fig. 2 - a) *Impression cytology in conjunctiva from patients with a mild grade of dry eye* (grade 1). *Nonsecretory epithelial cells were slightly separated. There is a moderate number of goblet cells (GC) with a periodic acid-Schiff (PAS)-positive cytoplasm. PAS-hematoxylin staining. Magnification x40.*
b) *Impression cytology in cornea from patients with a mild grade of dry eye* (grade 1). *Epithelial cells are slightly separated with a nuclear-cytoplasmic ratio around 1:4. PAS-hematoxylin staining. Magnification x40.*

TABLE V - NUCLEAR-CYTOPLASMIC RATIO IN DIFFERENT CONJUNCTIVAL AREAS AND CORNEA. Statistical differences were found in comparison with the preceding grade, and with the inferior conjunctival area of the same grade. The corneal cellular area was compared to the previous grade and to the interpalpebral conjunctival trigoni

	Upper bulbar		Interpalpebral trigonal		Lower bulbar		Lower palpebral	Cornea
Grade 0	1:3	n.s.	1:3	n.s.	1:2.5	n.s.	1:2.5	1:3 n.s.
Grade 1	1:4 n.s.	n.s.	1:4 n.s.	n.s.	1:3 n.s.	n.s.	1:3 n.s.	1:4 n.s. n.s.
Grade 2	1:7 *	n.s.	1:6 n.s.	*	1:3 n.s.	n.s.	1:3 n.s.	1:8 * n.s.
Grade 3	1:15 *	*	1:13 *	*	1:5 n.s.	n.s.	1:4 n.s.	1:15 * n.s.
Grade 4	1:30 *	*	1:20 *	*	1:10 *	*	1:7 *	1:20 * n.s.
Grade 5	1:50 *	*	1:30 *	*	1:20 *	*	1:14 *	1:30 * n.s.

*p < 0.05
n.s.= Not significant

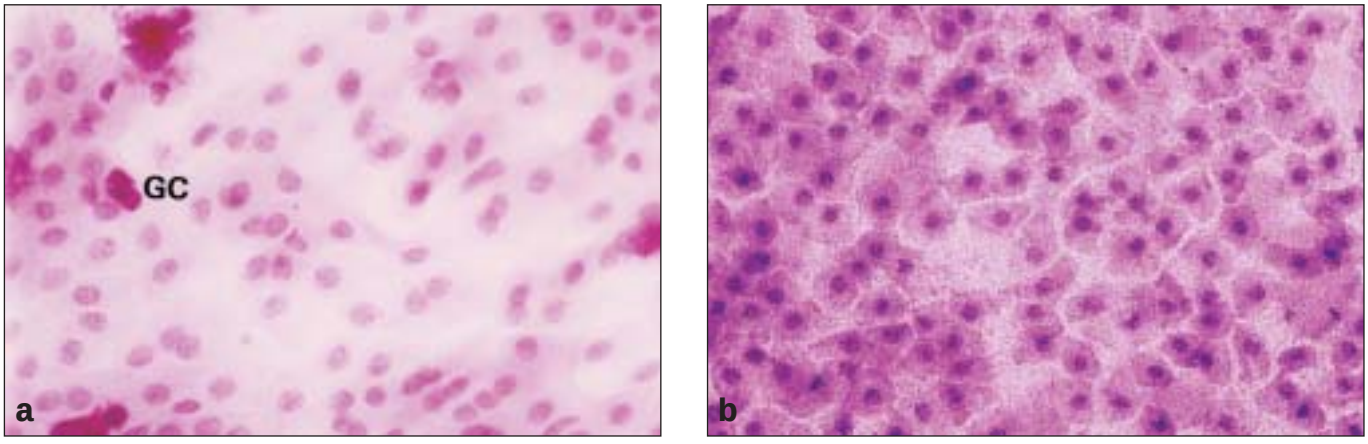


Fig. 3 - a) *Impression cytology in conjunctiva from patients with a moderate grade of dry eye* (grade 2). *Nonsecretory epithelial cells were moderately separated, and there are few goblet cells (GC) with a periodic acid-Schiff (PAS)-positive cytoplasm. PAS-hematoxylin staining. Magnification x60.*
b) *Impression cytology in cornea from patients with a moderate grade of dry eye* (grade 2). *Epithelial cells were moderately separated with nuclear-cytoplasmic ratio of 1:7. PAS-hematoxylin staining. Magnification x40.*

TABLE VI - GOBLET CELL DENSITIES IN DIFFERENT AREAS OF THE CONJUNCTIVA (cells/mm^2 ± SD). Statistical differences were found in comparison with the preceding grade, and with the inferior conjunctival area of the same grade

	Upper bulbar		Interpalpebral trigonal		Lower bulbar		Lower palpebral
Grade 0	508.2 ± 48.5	n.s.	507.0 ± 72.1	*	596.7 ± 67.6	*	973.8 ± 63.3
Grade 1	373.3 ± 53.5 *	n.s.	371.9 ± 65.4 *	*	527.8 ± 83.1 n.s.	*	927.4 ± 81.5 n.s.
Grade 2	264.6 ± 27.9 *	n.s.	230.1 ± 30.0 *	*	447.4 ± 86.5 *	*	608.3 ± 93.1 *
Grade 3	41.1 ± 24.2 *	n.s.	64.1 ± 17.3 *	*	237.8 ± 69.6 *	*	419.8 ± 71.5 *
Grade 4	9.3 ± 4.7 *	n.s.	15.0 ± 3.4 *	*	26.6 ± 4.1 *	*	40.8 ± 6.8 *
Grade 5	0.4 ± 3.7 *	n.s.	1.2 ± 3.9 *	*	6.6 ± 3.8 *	*	10.2 ± 12.3 *

*p < 0.05
n.s.= Not significant

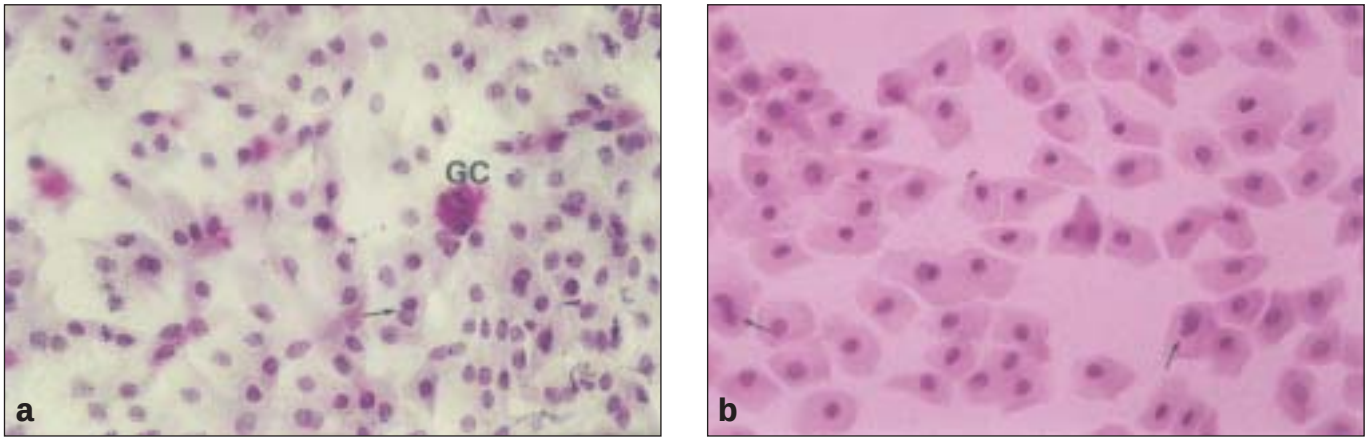


Fig. 4 - a) *Impression cytology in conjunctiva from patients with* grade 3 *dry eye. Nonsecretory epithelial cells were more separated, sometimes binucleated (↑), and there is a very small number of goblet cells (GC). Periodic acid-Schiff (PAS)-hematoxylin staining. Magnification x40.*
b) *Impression cytology in cornea from patients with* grade 3 *dry eye. Epithelial cells were more separated, sometimes binucleated (↑), with a nuclear-cytoplasmic ratio around 1:15. PAS-hematoxylin staining. Magnification x40.*

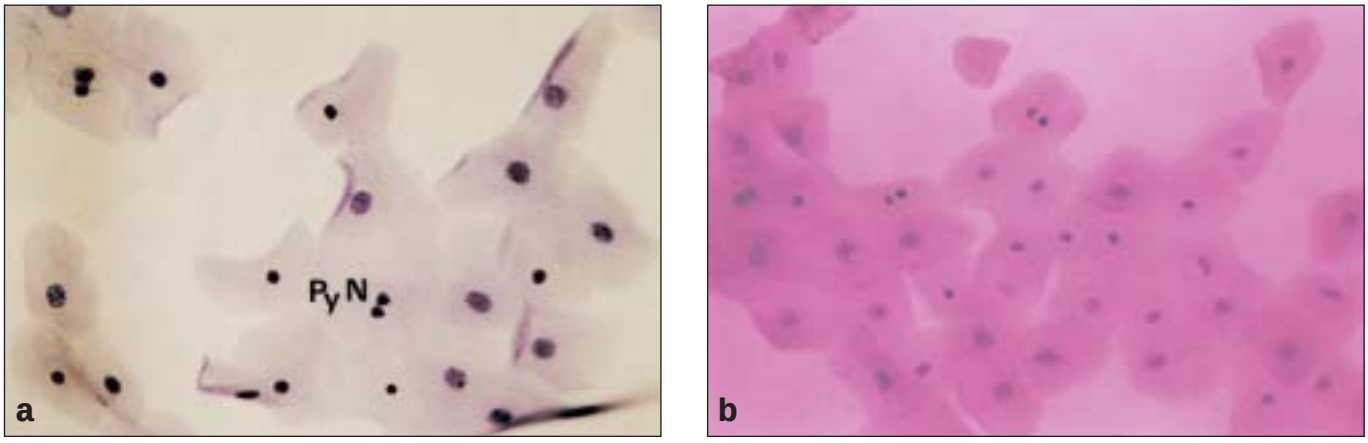


Fig. 5 - a) *Impression cytology in conjunctiva from patients with severe dry eye* (grade 4)*. Nonsecretory epithelial cells were isolated, with many pyknotic nuclei (PyN). Goblet cells have disappeared. Periodic acid-Schiff (PAS)-hematoxylin staining. Magnification x40.*
b) *Impression cytology in cornea from patients with severe dry eye* (grade 4). *Epithelial cells were isolated, frequently with pyknotic nuclei (*). PAS-hematoxylin staining. Magnification x40.*

trigoni and lower bulbar areas. Grade 2 had significant differences in all areas except the lower bulbar and lower palpebral areas. In grades 3, 4, and 5 there were significant differences in the whole conjunctiva. The size of normal corneal cells (grade 0) and grade 1 did not differ significantly, but the other grades had significant differences. When comparing corneal cell area with interpalpebral trigonal cells we found significant differences in all grades except the two most severe (4 and 5) (Tab. II).

Nuclear morphology changed in each grade of clinical severity of dry eye. Each grade had a predominant type of nuclear change, which was similar in nonsecretory cells of the cornea and conjunctiva. Clinical grades 0 and 1 showed normal cells. In clinical grade 2 there were many binucleated cells. In clinical grade 3 there was a decrease in the number of binucleated cells and prevalence of pyknotic cells. Clinical grade 4 had similar percentages of pyknotic and anucleated cells. Clinical grade 5 showed a de-

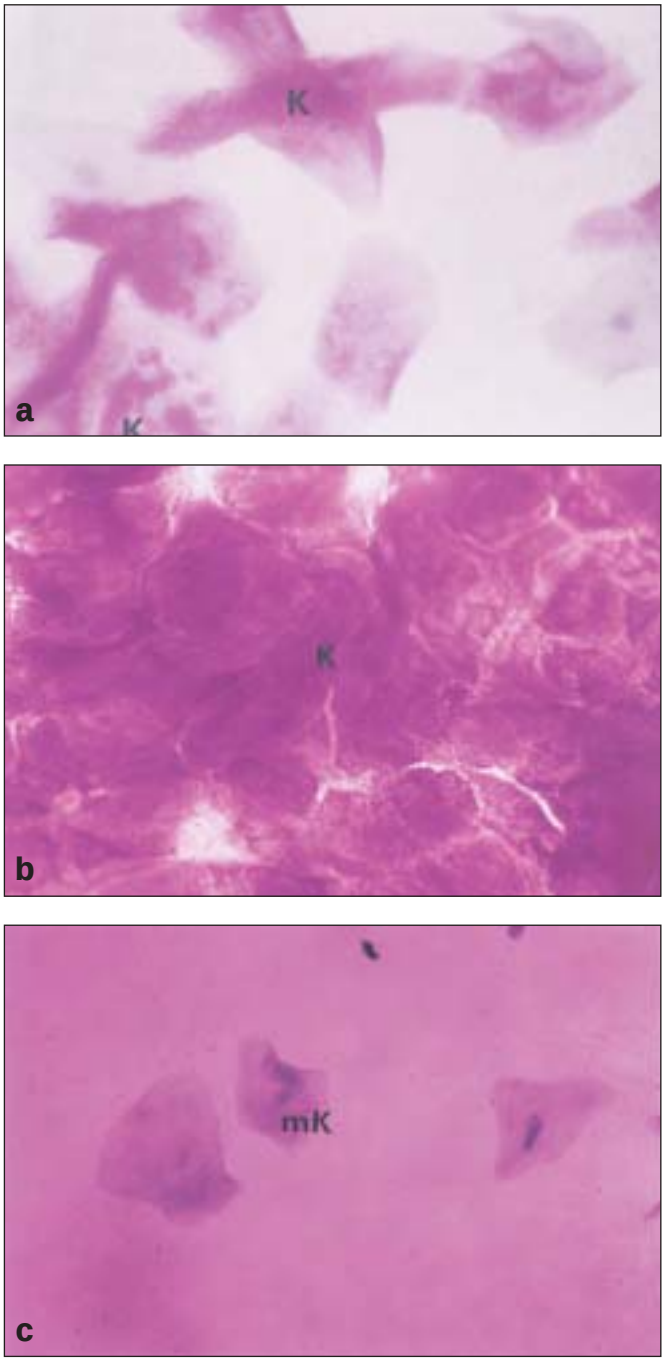


Fig. 6 - a) *Impression cytology in conjunctiva from patients with severe-plus dry eye* (grade 5). *Nonsecretory epithelial cells were isolated and their cytoplasm usually keratinized (K). Periodic acid-Schiff (PAS)-hematoxylin staining. Magnification x40.*
b) *Impression cytology in cornea from patients with grade 5 dry eye. Epithelial cells were isolated and their cytoplasm usually keratinized (K). PAS-hematoxylin staining. Magnification x40.*
c) *Impression cytology in cornea from patients with grade 5 dry eye. Some epithelial cells were mildly (mK) or not keratinized, but showed marked nuclear alterations. PAS-hematoxylin staining. Magnification x40.*

crease in the number of pyknotic cells, and had mainly anucleated cells (Tab. III).

Nuclear size did not vary significantly in conjunctival cells, except in grades 4 and 5. In corneal cells, nuclei were no different in all the grades. Compared with interpalpebral trigonal cells, the nuclei of the corneal cells were significantly bigger than conjunctival ones (Tab. IV).

The N:C ratio rose progressively from clinical grades 0 to 5. As the cellular size increased continuously with the severity of the squamous metaplasia, the nuclear area also increased slowly. The differences were significant between all six grades, except for controls (clinical grade 0) and clinical grade 1 of dry eye in all areas. Clinical grade 2 only showed significant differences in the upper bulbar conjunctiva when compared with grade 1. There were significant differences in clinical grades 2 and 3 in the upper bulbar and interpalpebral conjunctiva. Clinical grades 4 and 5 differed significantly and from the lower grades of squamous metaplasia. In corneal cells, the N:C ratio did not differ between grades 0 and 1, but the difference was significant between higher squamous metaplasia grades. On comparison with the interpalpebral conjunctiva, we found similar N:C ratios (Tab. V). The goblet cell count was significantly different between all grades when comparing the same area, except for the lower bulbar and lower palpebral conjunctiva in clinical grades 0 and 1. As the severity of squamous metaplasia increased, the goblet cell densities dropped significantly (Tab. VI).

We have described six different grades of squamous metaplasia, corresponding to the clinical grading system (1). Each grade showed significant differences from the others in clinical tests, morphologic changes, and morphometric analysis. After studying the mean values for the whole ocular surface and establishing statistical differences, we propose the following grades:

- *Grade 0*: Abundant goblet cells (more than 400 cells/mm^2) with PAS-positive cytoplasm. The nonsecretory epithelial cells are small (mean size 200-300 µm^2) with a N:C ratio of 1:2 to 1:3. Corneal cells are small and joined (mean size 300-350 µm^2) with a N:C ratio of 1:3. Conjunctival and corneal cells are perfectly joined, with an eosinophilic cytoplasm. The nucleus is round and shows no alterations (Figs. 1a, 1b).
- *Grade 1*: The goblet cell density is moderately de-

creased (400-300 cells/mm^2) with PAS-positive cytoplasm. The nonsecretory epithelial cells show slight enlargement (mean size 200-350 µm^2) and a N:C ratio of 1:3 to 1:4. Corneal cells are slightly larger (mean size 300-400 µm^2) with a N:C ratio of 1:4. Conjunctival and corneal cells are usually joined but some are slightly separated, with an eosinophilic cytoplasm, and the nucleus shows no alterations (Figs. 2a, 2b).

- *Grade 2*: The goblet cells are markedly decreased in number (300-200 cells/mm^2) with PAS-positive cytoplasm. The nonsecretory epithelial cells are moderately enlarged (mean size 350-500 µm^2) with a mean N:C ratio of 1:5. Corneal cells are larger (mean size 500-1,000 µm^2) and have a N:C ratio of 1 to 8. Conjunctival and corneal cells are moderately separated, with an eosinophilic cytoplasm (slightly light in cornea), and an occasionally binucleated nucleus (Figs. 3a, 3b).
- *Grade 3*: Goblet cells are very few (200-50 cells/mm^2) with slightly PAS-positive cytoplasm. The nonsecretory epithelial cells are large (mean size 500-900 µm^2) and the N:C ratio is around 1:10. Corneal cells are large (mean size 1000-1800 µm^2) with a N:C ratio of 1:15. Conjunctival and corneal cells are frequently isolated, with a metachromatic cytoplasm, and usually nucleus shows alterations (binucleated, pyknotic, or anucleated) (Figs. 4a, 4b).
- *Grade 4*: Goblet cells are very scarce (50-10 cells/mm^2) and usually have PAS-negative cytoplasm. The nonsecretory epithelial cells are very large (mean size 900-1600 µm^2), and the N:C ratio is around 1:20. Corneal cells are very large (mean size 1,800-2,500 µm^2) with a N:C ratio of 1:20. Conjunctival and corneal cells are always isolated, with a basophilic cytoplasm, sometimes with mild keratinization, and an altered nucleus, frequently pyknotic or anucleated (Figs. 5a, 5b).
- *Grade 5*: Goblet cells have usually disappeared. The rare ones present are completely altered (less than 10 cells/mm^2). The nonsecretory conjunctival epithelial cells are very large (more than 1600 µm^2), and the N:C ratio is around 1:30. Corneal cells are very large (more than 2500 µm^2), also with a N:C ratio of 1:30. Both conjunctival and cornea cells are isolated, have a basophilic cytoplasm, are moderately or severely keratinized, and have a very altered nucleus, which is frequently lytic or anucleated (Figs. 6a, 6b).

DISCUSSION

Each clinical severity group rated using our clinical grading system (1) is closely related to the grades of squamous metaplasia described in this article and vice versa. The morphological grading systems described by other authors have different cellular patterns. Most of them do not involve morphometric analysis, and corneal cells are not studied. One describes the severity mainly by grading cellular keratinization, but without histochemical techniques (12), and another describes few histologic grades, thus grouping several clinical grades in each histologic grade (5).Our patients with grade 0 squamous metaplasia had a clinical grade 0; morphologic grade 1 had clinical grade 1 and so on for grades 2 to 5.

We agree to a certain extent with the clinical system proposed by Oroza (2) for the three first groups of squamous metaplasia, finding continuous-interval data and histogram studies showing that all grades are homogeneous, with significant differences from the previous grades. Only the last grade of Oroza (grade 4) has heterogenous values with a discontinuous interval, and therefore we have divided this grade in grades 4 and 5 of our grading system.

When grading the squamous metaplasia, it is necessary to be careful when comparing impression cytologies from different areas of the ocular surface, and one must know where the impression cytology was obtained before comparing results between different areas or studies (13). Corneal and conjunctival cells have similar numbers of alterations in the same grade of clinical severity, particularly between the cornea and the two interpalpebral trigonal exposed areas of the conjunctiva, meaning that the ocular surface from the same patient shows close correspondence between the degree of clinical severity and the level of squamous metaplasia. In general, if morphologic and morphometric studies indicate a certain grade of squamous metaplasia in the trigonal conjunctiva, the cornea has the same grade of severity, confirming that the whole exposed ocular surface is an important morphologic and functional unit (14).

One morphologic nuclear change seen in many patients but not included in this grading system, is SLC cells, because although they only appeared in the upper bulbar and interpalpebral areas and cornea of pathological cases, the proportions are similar for different

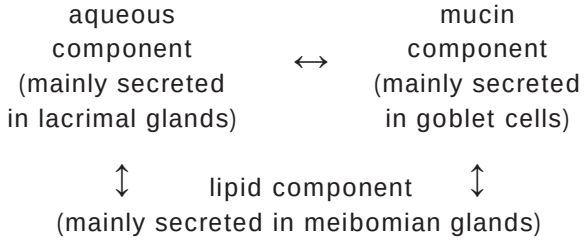
grades of squamous metaplasia. The presence of SLC has been interpreted as a result of the chronic mechanical friction of the upper eyelid over areas with inadequate tearing (15).

The goblet cell density might indicate the severity of the ocular surface damage in dry eye syndromes (16). We found significant differences in all grades. This finding, combined with the morphological changes and morphometric parameters in nonsecretory epithelial cells, is directly related to the overall health of the ocular surface (17).

For practical purposes, we must consider whether to study the impression cytology of all areas of the ocular surface or only of the exposed surface (conjunctival trigoni and cornea). The severity of the damage to the ocular surface gradually increases, in most cases proceeding in the following steps: lower palpebral and lower bulbar, upper bulbar, and interpalpebral surface (conjunctival trigoni and cornea).

The reason for this may be that when the damage of the ocular surface epithelium is secondary to tear deficiency, the exposed area is the most damaged because of all the sources of external aggression (wind, evaporation, pollution, blinking friction, etc.). However, the upper palpebral areas only add the blinking friction, and the low palpebral areas are the most protected.

The whole ocular surface, but mainly – and earlier – the exposed surface, presents secondary damage regardless of the type of dry eye, because any alterations in the lacrimal glands, meibomian glands, or goblet cells alter the tear of the conjunctival surface and damage the function of the other glands in a vicious circle in two directions, each influencing the other:



Considering that the exposed interpalpebral ocular surface is the most affected area, that the exposed area is the one that gives most of the clinical symptoms and signs, and that morphologic and morphometric techniques must be simplified as much as possible, we propose that impression cytology should only be used in the interpalpebral areas for the histologic classification of severity when trying to relate it to the clinical grade of dry eye.

Nevertheless, studying the lower bulbar/palpebral areas may also be of interest because a specific etiology of dry eye can then be considered when the severity is graded differently from that described. For instance, the maximum damage will be in the lower bulbar/palpebral areas in cases of caustication with liquids, or we may observe a similar grade of squamous metaplasia in whole areas of the ocular surface in some autoimmune syndromes, such as cicatricial ocular pemphigoid or Stevens-Johnson syndromes.

In conclusion, the diagnosis of squamous metaplasia can be confirmed by impression cytology. We believe that this approach provides the best and quickest morphologic information on ocular surface health, often without any correlation with tear film tests, and can be used when assessing the ocular surface in patients with dry eye syndromes. Squamous metaplasia appears to be a continuous process of severity from the most protected subpalpebral lower area through the subpalpebral areas to the interpalpebral areas. Consequently, we propose a new grading system of squamous metaplasia of the ocular surface based on morphologic and morphometric parameters, and statistically correlated with grades of clinical severity.

Reprint requests to:
Prof. Juan Murube
Service of Ophthalmology
Hospital Ramón y Cajal
Ctra. Colmenar, Km 9.100
E-28034 Madrid, Spain
murubejuan@terra.es

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