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Normal optical densitometric parameters in exfoliative cytology from different zones of the oral mucosa free of pathology

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SUMMARY

Exfoliative cytology is a rapid, inexpensive and non-invasive diagnostic tool, and is therefore very useful in clinical practice. The purpose of this study was to examine the behaviour of the optical densitometric parameters of normality in five different zones of the oral mucosa free of pathology. Thirty three patients, ranging between five and seventy five years of age, free of general and oral pathology were studied. Smears were collected with the Cytobrush® and fixed with Labofix®. The smears were stained with Hematoxylin Eosin and analysis was carried out with a semiautomatic image analyser (Microimage 3.0). The following parameters were studied: luminic density of the cytoplasm, luminic density of the nucleus, red density of the cytoplasm, blue density of the cytoplasm and green density of the cytoplasm. SPSS 7.5 was used in the statistical analysis. Variance analysis was preformed with the Bonferroni correction for multiple comparison of means, which considered $p<0.01$ as being statistically significant. Significant differences were found in the parameters studied (optical density) among the different zones of the oral mucosa.

Key Words: Exfoliative cytology - Mouth mucosa cytology - Optical densitometry - Image processing - Computer assisted - Image cytometry.

ABBREVIATIONS:

LDC: LUMINIC DENSITY OF THE CYTOPLASM.
RDC: RED DENSITY OF THE CYTOPLASM.
BDC: BLUE DENSITY OF THE CYTOPLASM.
GDC: GREEN DENSITY OF THE CYTOPLASM.
LDN: LUMINIC DENSITY OF THE NUCLEUS.

INTRODUCTION

Exfoliative cytology is a rapid, inexpensive and non-invasive method of diagnosis (Cowpe et al., 1988). It can be used on herpes lesions (Barret et al., 1986; Bagg et al., 1989), and on fungal lesions (Jones et al., 1995). Exfoliative cytology can be used also on primary oral precancer and superficial oral cancer as an effective tool for the early diagnosis of suspect lesions in high-risk patients like smokers, drinkers, patients with a family history of cancer and those who have undergone radiation treatment (Jones et al., 1994). Other use is the follow-up of malignant lesions that have been treated. As shown in table 1, it is an effective tool in determining an appropriate biopsy site in extensive lesions and in the study of patients who choose not to have a biopsy or who are considered to be high risk for surgery. It also has a very important role in quantitative analysis (cellular morphometry and densitometry) (Cowpe et al., 1988). The morphofunctional alterations of cell atypia (table 2) or any other cell dysfunction can be quantified with a quantitative analysis using exfoliative cytology. Morphological alterations (nuclear and cytoplasmatic areas and nuclear pleomorphism) are quantified using morphometric analysis. Functional alterations (increased keratinization, nuclear hyperchromatism, chromatin clumping) can be quantified using densitometric analysis.

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The prognostic accuracy of flow cytometric DNA analysis is less than that of static densitometry, in which the morphological selection of malignant cells permits quantitative measurements (Cusick et al., 1990). The image analysis method has been used to study the optical density of cell nuclei by Tucker (1979), Aggarwal and Bacus (1977), Dairkee and Glaser (1984), Buromskii (1980), Yoshii et al. (1995), François et al. (1997) and Tucker et al. (1994) to determine nuclear DNA contents, and by Dairkee and Glaser (1984) to determine the heterogeneity of the integrated optical density of mammalian cell colonies. Optical densitometry offers a reproducible and objective series of parameters that can provide information about the functional condition of the cell. The purpose of this study was to determine the values of normality of the optical densities of the cytoplasm (LDC, RDC, BDC, GDC) and nucleus (LDN) in the oral mucosa free of pathology and not exposed to any toxic factors (alcohol, tobacco, medication) or irritants (local trauma, poor hygiene). These values may be used in future studies as a control with which to compare pathological oral mucosa values or the values found in subjects exposed to toxic or irritant factors. As the oral mucosa presents morphofunctionally differentiated zones with clear characteristics, we were prompted to see the densitometric values of five zones in the oral cavity to determine whether or not there were significant differences among the zones as regards the different parameters studied.

Material and Methods

Smears were collected from thirty three individuals ranging between five and seventy five years of age from five sites of the oral cavity (ventral tongue, dorsum of tongue, hard palate, buccal mucosa immediately above the right white line and lower vestibule at the same level as the right premolars). The individuals tested came to the dental clinic for a check-up. They showed no signs of pathology on the oral mucosa or any systemic pathology and not were they taking any medication. None of them smoked or consumed alcohol; there were no local irritants in the mucosa and they all displayed adequate oral hygiene. Excess saliva was wiped from the sample zone using a plastic spatula, sliding it gently over the study area four times. Samples were collected using a Cytobrush®. The smear was immediately fixed with Labofix®. The samples were stained with Hematoxylin-Eosin (Harris Hematoxylin Papanicolaou 1ª during five minutes, 0.5% Eosin B in 70% alcohol during three minutes). The samples were analysed on a work station consisting of an Olympus CX40 microscope connected to a video camera (TK-C621-EG colour JVC) which was connected to an image analyser (Microimage 3.0, Foster Finley Model Pc-Image) using an image card from Matrox, model Comet: 720(H) x 580(U). The following parameters were measured: luminic density of the cytoplasm, luminic density of the nucleus, red density of the cytoplasm, blue density of the cytoplasm and green density of the cytoplasm. Approximately fifty cells from each site were measured (two hundred fifty from each individual).

The light in the microscope and camera was adjusted. The slide area was located using 4x and 10x lenses, which allowed us to carry out an appropriate study, according to cell extension. A series of cells was selected based only on their distribution. The cells were required to be as close as possible in order to have the greatest number in the same field, with the least possible amount of overlap and contact. The cells to be studied were centred in the field using the 20x lens. Brightness and contrast were adjusted on the screen and the images were obtained. The cytoplasm surfaces to be measured were selected using a colour. The process consisted of clicking with the mouse on the colour similar to that of the preparation until complete selection of cytoplasms was achieved. The program was set up so that very large or very small objects (which we were sure of owing to their size - in this case less than 400µm or more than 20.000µm) that did not refer to a cytoplasm were not selected or measured. Objects touching the edge of the field were automatically rejected by the program (Fig. 1). The following tasks were completed manually: finish defining the contours, separate two cytoplasms that were touching, eliminate objects whose measurements held no interest for us, and so on. After the cytoplasms had been defined, they were measured (Fig. 2). The data obtained were exported to a Microsoft Excel table. Once we had finished measuring the cell and cytoplasm, we selected the measurements to be taken from the nucleus (Fig. 3). The surface corresponding to the nuclei was selected in the same way as for the cytoplasm. In this case, area limits were established (between 20µm and 300µm) within which we were sure that all the nuclei would be found. In this way all the remaining objects as well as objects that touched the edge of the field under study were automatically rejected (Fig. 4). After making the necessary changes by hand, such as eliminating objects that did not correspond to nuclei, we took the measurements and exported these data to Excel (Microsoft Office 97). The program measures the objects from top to bottom and from left to right so that each line in Excel will have the exact data from a single cell.

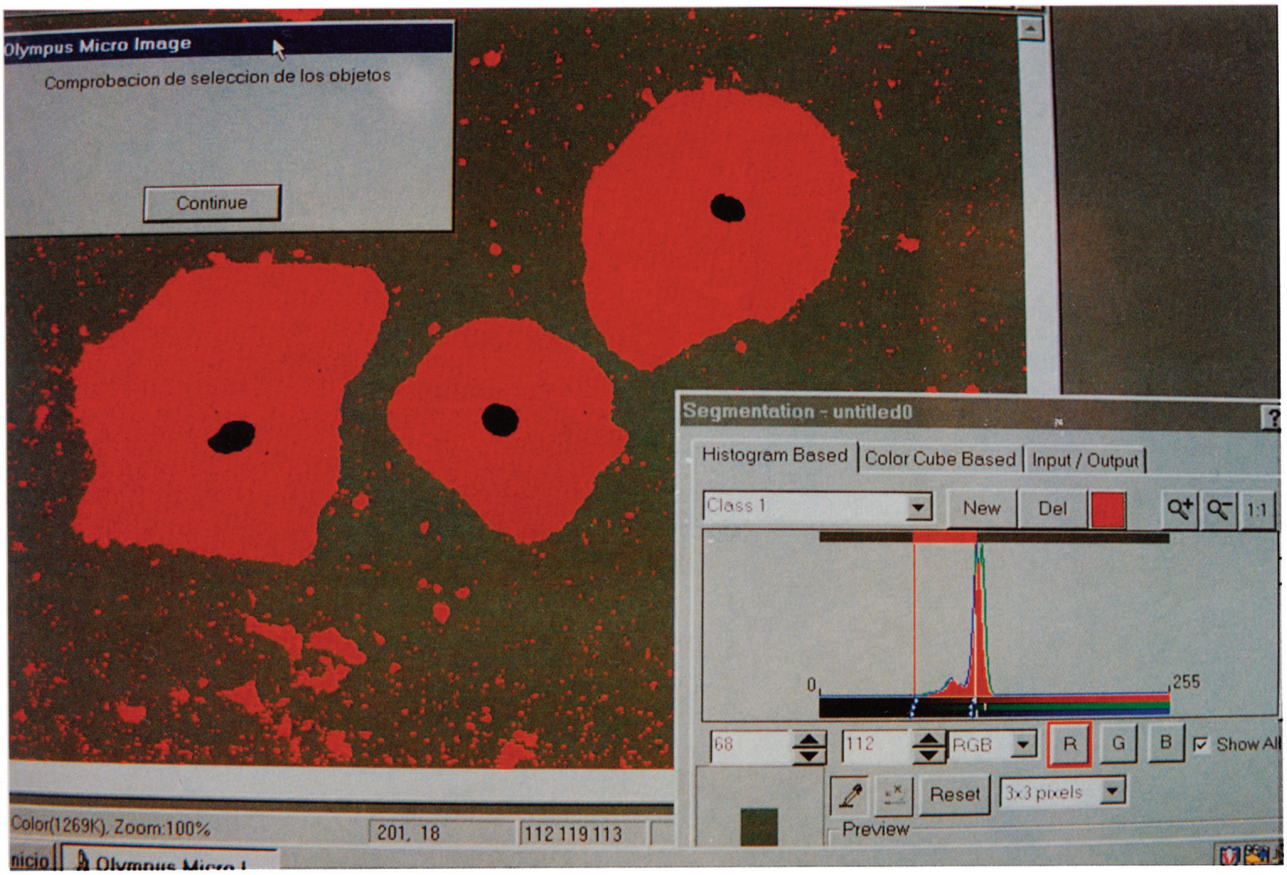


Figure 1. Semiautomatic selection of cytoplasms. Hematoxylin-Eosin. 20X. Digitized image.

Similarly, each column contained the data for one parameter. Other columns of data were added regarding the age of the patient sampled and site of the oral mucosa where the sample was taken from. In the process described, three or five cells were analysed (in most cases with the 20x lens). The process was repeated as many times as necessary in order to analyse fifty cells from different parts of the smear pertaining to a zone.

Densities were studied under standard conditions (equal amounts of light fixed to the videocamera and microscope) using the same lens (20x). The consistency of the measurements was verified using several measurements under the same conditions. Several groups of these measurements were carried out in different optical and stain conditions. The readings of our work station ranged between maximum values (slide free of cell and stain) of LD=198,88; RD=197,47; BD=197,98; GD=201,17, and minimum values (opaque zone of slide) of LD=2,10; RD=0,98; BD=1,16; GD=4,15. Figure 5 shows the correspondence between the intensity value (image analyser) and optical density.

The next equations shows the relation between the intensity or grey level (image analizer lecturre) and optical density.

$$\text{Optical Density} = -\log_{10}\left(\frac{\text{Transmited intensity}}{\text{Incident intensity}}\right)$$

$$\text{Optical Density} = -\log_{10}\left(\frac{\text{Maximum grey level (255)}}{\text{Grey level}}\right)$$

We carried out a descriptive statistical analysis of all the variables included in the study, estimating a 95% confidence interval. For multiple comparisons of the means, we carried out variance analysis with the Bonferroni correction, which considered $p<0.01$ to be statistically significant. SPSS 7.5 was used for the statistical analysis.

Although it is not the objective of the study, we have kept in mind the age and the sex. Using a correlation study (Pearson or Spearman), correlation doesn't exist among densitometric parameters and the age ($p<0.001$). Using an analysis of the variance (ANOVA), significant differences of means exist ($p<0.000$) among densitometric parameters of oral mucousa of men and women.

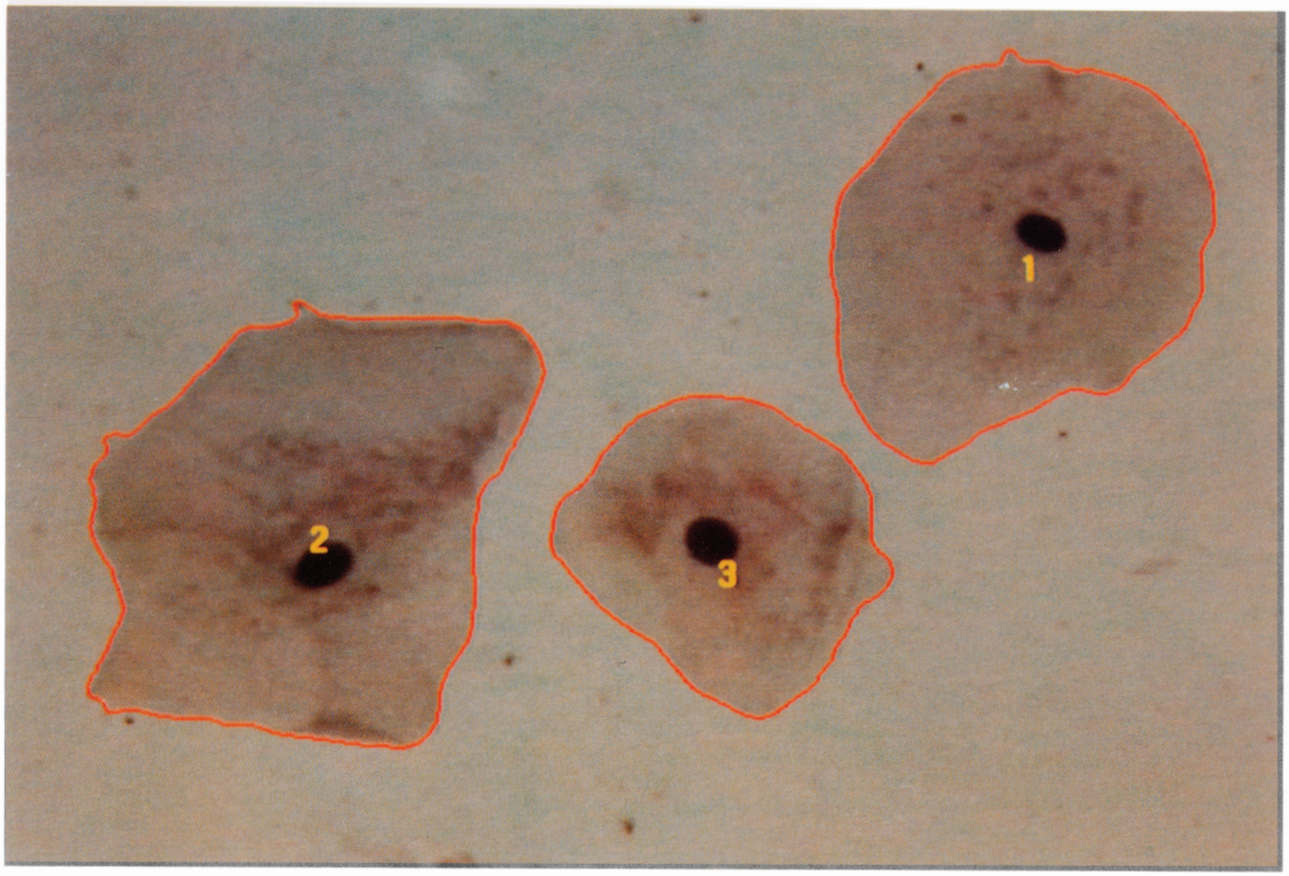


Figure 2. Measurement of cytoplasms. Hematoxylin-Eosin. 20X. Digitized image.

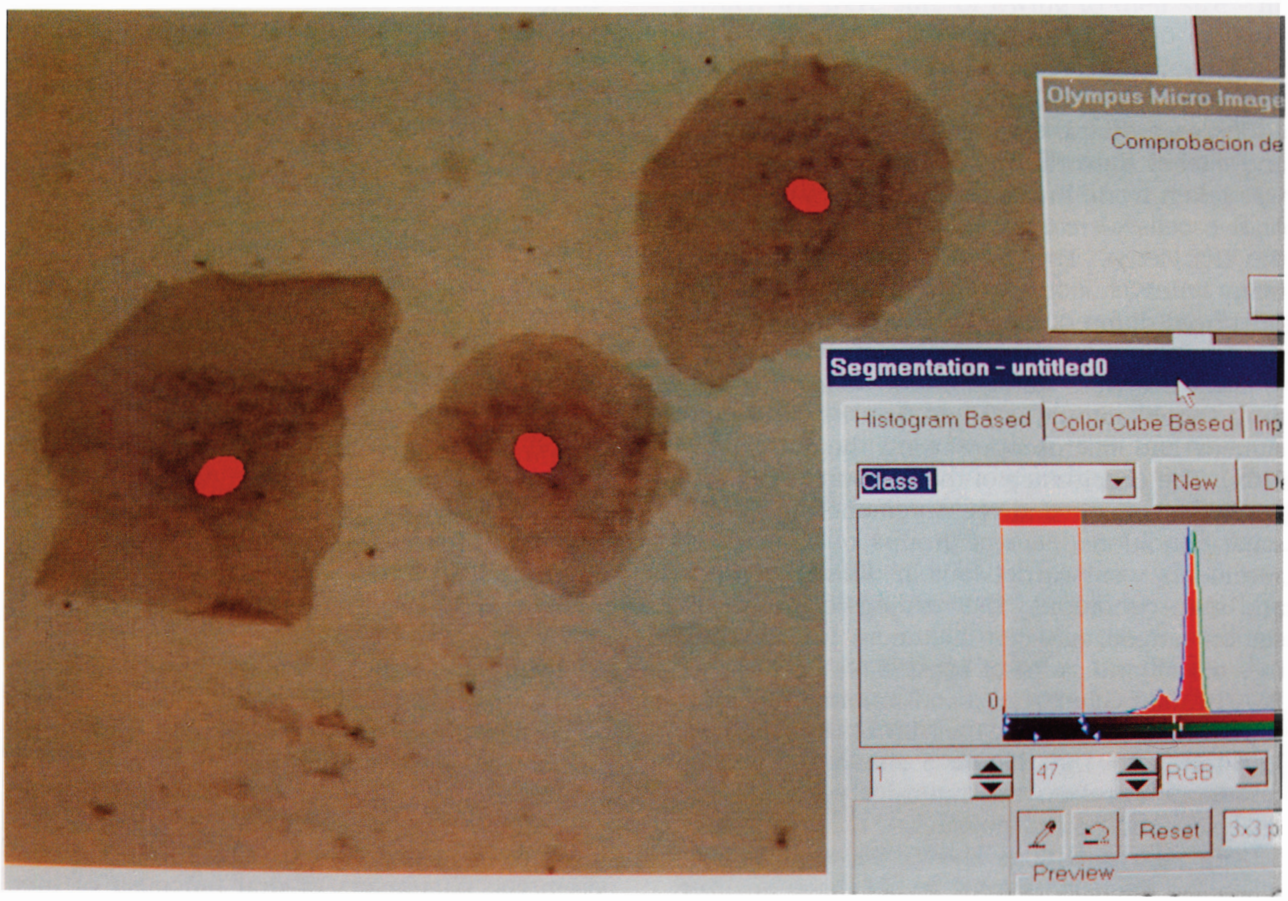


Figure 3. Semiautomatic selection of nucleus. Hematoxylin-Eosin. 20X. Digitized image.

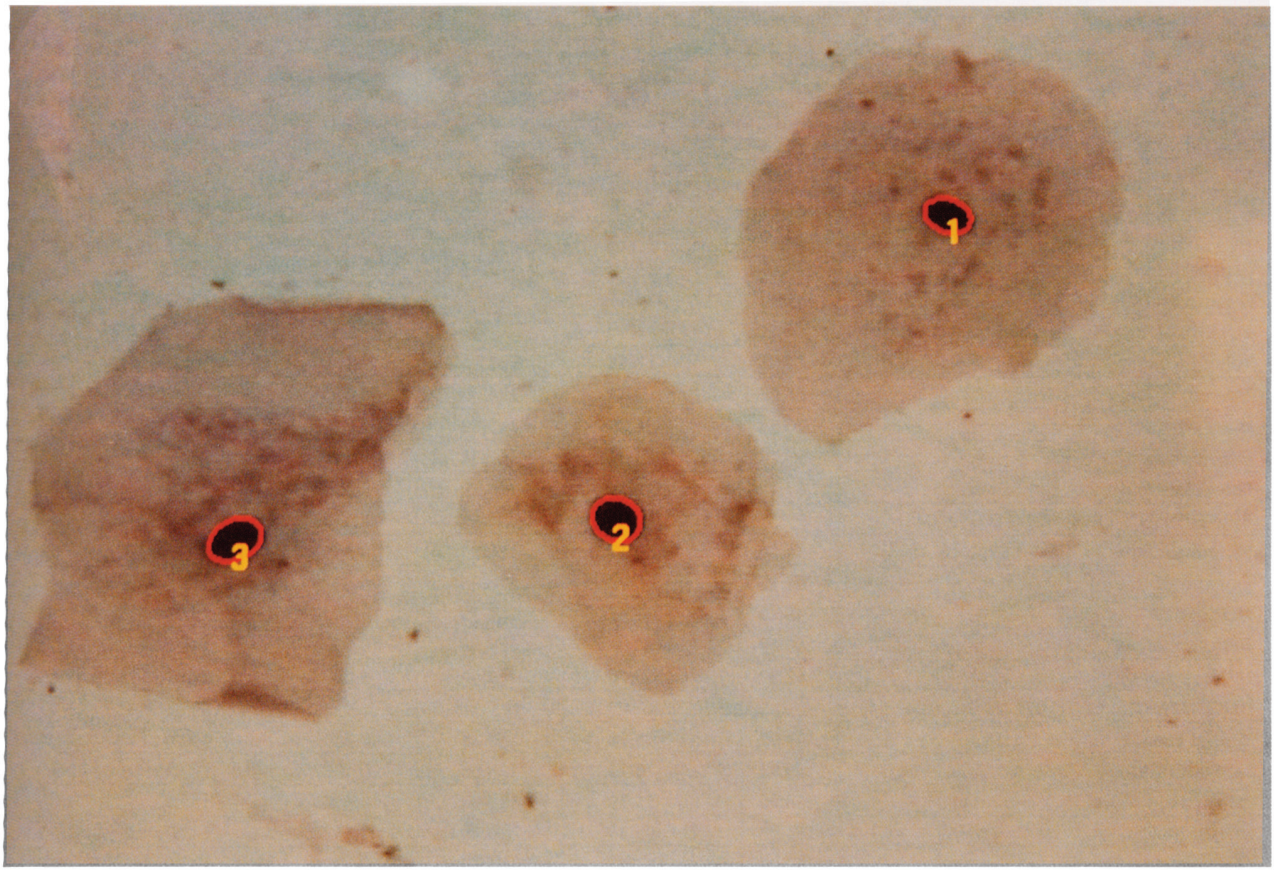


Figure 4. Measurement of nucleus. Hematoxylin-Eosin. 20X. Digitized image.

Results

Table 3 offers the descriptive statistics of the different optical densities of the five zones of the oral mucosa under investigation. The high values of luminic density in the cytoplasm contrast with the low values of luminic density in the nucleus. The values of the red, blue and green densities were very similar for the cytoplasm. The nucleus absorbed a large quantity of light while the cytoplasm was less opaque. The cytoplasms are similar in shape as regards the luminic density and the three colours studied.

The luminic density of the cytoplasm and nucleus was reduced in the dorsum of the tongue compared with the other zones. The same pattern was followed by the red, blue and green densities (table 3).

Table 4 shows the results of the variance analysis with the Bonferroni correction for the multiple comparison of means between the different zones of the oral cavity. There were

Table 1: Use of oral exfoliative cytology. (Jones AC et al., 1995; Jones AC et al., 1994, Cowpe JG et al., 1995):

- Red or erithematous lesions.
- Control of patients who have been treated for oral cancer.
- Orientation of the appropriate biopsy site in extensive mucosa lesions or at several locations.
- Study of suspect lesions when the patient chooses not to undergo a biopsy.
- Study of suspect lesions in patients of high surgical risk, medically compromised or who have received radiotherapy.
- Patients who want to be investigated although they have been informed of the innocuousness of the lesion.
- Periodic check-ups of patients with a high risk of contracting oral cancer.
- White lesions that come off by scraping them, leaving a bleeding surface.
- Lesions that are suspected of having viral origin (Herpes).

Table 2: Characteristics of cellular atypia in oral cytology.
(Jones AC et al., 1994; Cowpe JG et al., 1995; Ligthelm AJ et al., 1989; Silverman S, 1987):

- Increased keratinization (red, orange, brown).
- Increased nuclear area.
- Increase of nuclear area/cytoplasmic area.
- Nuclear hyperchromatism.
- Nuclear pleomorphism.
- Chromatin clumping.

Table 3: Descriptive statistics of the optical densitometric study in different zones of the oral mucosa.

	Mean	Minimum value	Maximum value	Standard error	Standard deviation	n (sample size)
LUMINIC DENSITY OF THE CYTOPLASM						
Ventral tongue	141,93	88,73	179,74	0,3527	14,65	1725
Dorsum of tongue	136,85	75,16	176,35	0,4133	16,84	1660
Hard palate	142,27	74,45	180,87	0,3605	15,92	1950
Buccal mucosa	148,08	97,59	177,63	0,3685	15,08	1675
Lower vestibule	149,94	115,30	182,50	0,3218	12,67	1550
LUMINIC DENSITY OF THE NUCLEUS						
Ventral tongue	72,58	12,52	132,96	0,4729	19,64	1725
Dorsum of tongue	62,05	9,43	124,50	0,7104	24,61	1200
Hard palate	70,55	24,85	105,82	1,8828	22,67	145
Buccal mucosa	75,78	23,08	162,81	0,5919	24,15	1665
Lower vestibule	87,17	42,29	138,79	0,5228	20,38	1520
RED DENSITY OF THE CYTOPLASM						
Ventral tongue	144,76	89,31	180,93	0,3407	14,15	1725
Dorsum of tongue	141,82	76,72	178,88	0,3976	16,20	1660
Hard palate	148,26	80,57	183,79	0,3451	15,24	1950
Buccal mucosa	150,59	102,66	178,42	0,3505	14,36	1675
Lower vestibule	152,52	118,76	182,53	0,3053	12,02	1550
BLUE DENSITY OF THE CYTOPLASM						
Ventral tongue	144,97	93,98	180,18	0,3361	13,96	1725
Dorsum of tongue	139,92	76,72	178,88	0,4077	16,61	1660
Hard palate	144,65	75,79	184,92	0,3583	15,82	1950
Buccal mucosa	151,03	99,34	180,36	0,3577	14,67	1675
Lower vestibule	152,02	117,41	184,70	0,3096	12,19	1550
GREEN DENSITY OF THE CYTOPLASM						
Ventral tongue	136,05	82,91	178,11	0,3867	16,06	1725
Dorsum of tongue	128,82	68,64	172,46	0,4420	18,01	1660
Hard palate	133,83	66,98	173,90	0,3884	17,15	1950
Buccal mucosa	142,63	89,28	174,55	0,4005	16,39	1675
Lower vestibule	145,29	107,37	180,26	0,3548	13,97	1550

highly significant differences between nearly all the zones of the oral mucosa for the different luminic densities ($p<0.01$).

A significant difference was found in the means of the luminic density of the cytoplasm amongst almost all the zones ($p=0.000$), except for the ventral part of the tongue-hard palate and buccal mucosa-lower vestible. These differences are shown in figure 6.

Significant variations in the means of the red density of the cytoplasm were observed amongst all the zones ($p=0.000$), except for the ventral part of the tongue-dorsum of tongue, buccal mucosa-lower vestibule and buccal mucosa-hard palate. A significant variation was seen between the hard palate and the ventral part of tongue ($p=0.011$). These differences are shown in figure 7.

A significant variation in the means of the blue density of the cytoplasm was observed amongst almost all the zones ($p=0.000$), except for the ventral tongue-hard palate, the buccal mucosa- lower vestibule and the ventral part of the tongue-hard palate. These differences are shown in figure 8.

There was a significant difference in the means of the green density of the cytoplasm amongst almost all the zones ($p=0.000$), except for the ventral part of the tongue-hard palate and buccal mucosa-lower vestible. These differences are shown in figure 9.

There was a significant difference in the means of the luminic density of the nucleus amongst almost all the zones ($p=0.000$), except for the dorsum of tongue-hard palate, buccal mucosa-hard palate, hard palate-ventral part of the tongue and buccal mucosa-ventral of tongue. These differences are shown in figure 10.

Discussion

Hematoxylin has affinity for nuclear components, whilst eosin is an acid colouring used by the cytoplasm, and is constituting a good contrast for the dyes with hematoxylin (Andrei, 1995). The affinity for the colouring and the intensity of the stain varies according to the stratum that the cell belongs to (Sugerman and

Table 4: Analysis of variance with the Bonferroni correction for the multiple comparison of means between zones.

		Means difference	Standard error	p*	95% confidence interval	
LUMINIC DENSTY OF THE CYTOPLASM					Inferior	Superior
Ventral tongue	Dorsum of tongue	5,072	1,165	0,000	1,797	8,347
	Hard palate	-3,480	1,120	1	-3,496	2,800
	Buccal mucosa	-6,156	1,163	0,000	-9,423	-2,888
	Lower vestibule	-8,018	1,186	0,000	-11,352	-4,684
Dorsum of tongue	Hard palate	-5,420	1,132	0,000	-8,601	-2,2399
	Buccal mucosa	-11,229	1,174	0,000	-14,520	-7,930
	Lower vestibule	-13,091	1,197	0,000	-16,455	-9,726
Hard palate	Buccal mucosa	-5,802	1,129	0,000	-8.981	-2,634
	Lower vestibule	-7,670	1,153	0,000	-10,911	-4,429
Buccal mucosa	Lower vestibule	-1,862	1,194	1	-5,219	1,495
LUMINIC DENSITY OF THE NUCLEUS					Inferior	Superior
Ventral tongue	Dorsum of tongue	10,531	1,862	0,000	5,294	15,767
	Hard palate	2,029	4,283	1	-10,015	14,0735
	Buccal mucosa	-3,195	1,702	0,606	-7,981	1,589
	Lower vestibule	-14,586	1,743	0,000	-19,487	-9,686
Dorsum of tongue	Hard palate	-8,502	4,355	0,511	-20,749	3,744
	Buccal mucosa	-13,727	1,876	0,000	-19,001	-8,452
	Lower vestibule	-25,118	1,913	0,000	-30,497	-19,738
Hard palate	Buccal mucosa	-5,224	4,289	1	-17,286	6,836
	Lower vestibule	-16,615	4,306	0,001	-28,723	-4,5086
Buccal mucosa	Lower vestibule	-11,391	1,757	0,000	-16,332	-6,449
RED DENSITY OF THE CYTOPLASM					Inferior	Superior
Ventral tongue	Dorsum of tongue	2,943	1,116	0,085	-0,194	6,081
	Hard palate	-3,497	1,073	0,011	-6,514	-0,480
	Buccal mucosa	-5,827	1,114	0,000	-8,958	-2,696
	Lower vestibule	-7,764	1,136	0,000	-10,958	-4,570
Dorsum of tongue	Hard palate	-6,441	1,084	0,000	-9,488	-3,393
	Buccal mucosa	-8,771	1,125	0,000	-11,932	-5,610
	Lower vestibule	-10,708	1,147	0,000	-13,931	-7,484
Hard palate	Buccal mucosa	-2,330	1,082	0,314	-5,370	0,710
	Lower vestibule	-4,267	1,105	0,001	-7,372	-1,161
Buccal mucosa	Lower vestibule	-1,936	1,144	0,908	-5,153	1,280
BLUE DENSITY OF THE CYTOPLASM					Inferior	Superior
Ventral tongue	Dorsum of tongue	5,048	1,138	0,000	1,851	8,246
	Hard palate	0,322	1,094	1	-2,751	3,396
	Buccal mucosa	-6,061	1,135	0,000	-9,252	-2,871
	Lower vestibule	-7,049	1,158	0,000	-10,304	-3,794
Dorsum of tongue	Hard palate	-4,726	1,105	0,000	-7,832	-1,620
	Buccal mucosa	-11,110	1,146	0,000	-14,331	-7,889
	Lower vestibule	-12,098	1,169	0,000	-15,383	-8,813
Hard palate	Buccal mucosa	-6,384	1,102	0,000	-9,483	-3,286
	Lower vestibule	-7,372	1,126	0,000	-10,537	-4,207
Buccal mucosa	Lower vestibule	-0,987	1,166	1	-4,265	2,290
GREEN DENSITY OF THE CYTOPLASM					Inferior	Superior
Ventral tongue	Dorsum of tongue	7,226	1,263	0,000	3,675	10,776
	Hard palate	2,220	1,214	0,677	-1,193	5,633
	Buccal mucosa	-6,579	1,260	0,000	-10,121	-3,036
	Lower vestibule	-9,241	1,286	0,000	-12,855	-5,627
Dorsum of tongue	Hard palate	-5,006	1,227	0,000	-8,454	-1,557
	Buccal mucosa	-13,805	1,272	0,000	-17,381	-10,228
	Lower vestibule	-16,467	1,298	0,000	-20,114	-12,819
Hard palate	Buccal mucosa	-8,799	1,224	0,000	-12,239	-5,359
	Lower vestibule	-11,461	1,250	0,000	-14,975	-7,943
Buccal mucosa	Lower vestibule	-2,662	1,295	0,400	-6,301	0,977

p*: p value (statistical significance p<0.01).

Savage, 1995). In exfoliative oral cytology cells are obtained from the stratum corneum and stratum granulosum. An image analysis system consisting of a microscope-video camera-image analyser, used to measure densitometric parameters, provides sufficiently accurate and reproducible results (Sánchez et al., 1990). An optical densitometric cellular study, along with the study of the volume of DNA and morphometric parameters has proven effective in detecting atypical and malignant cells (Tucker and Shipey, 1983; Van der Poel et al., 1991; Jin et al., 1995).

A study carried out with a wooden spatula and Cytobrush® showed that there were no significant differences in the degree of discomfort and the number of cells sampled. Both methods provide an accurate sample for the cytopathologist, but the Cytobrush® offers the advantage of

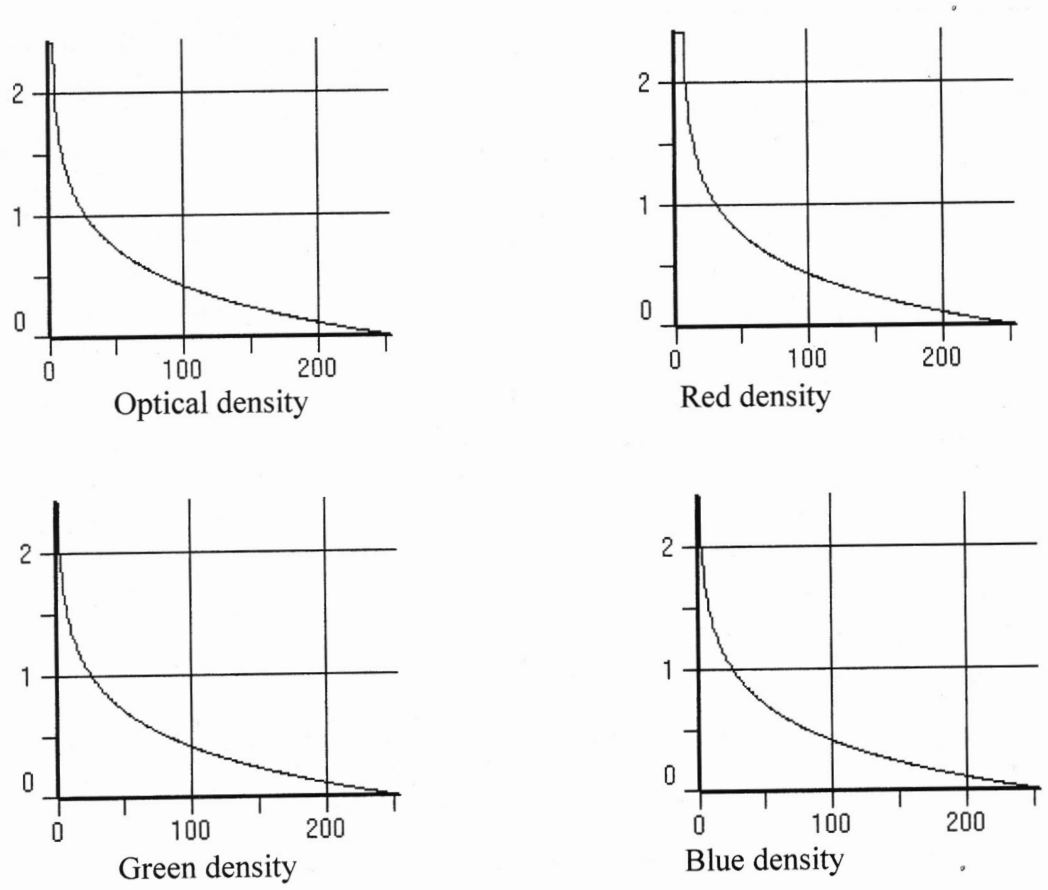
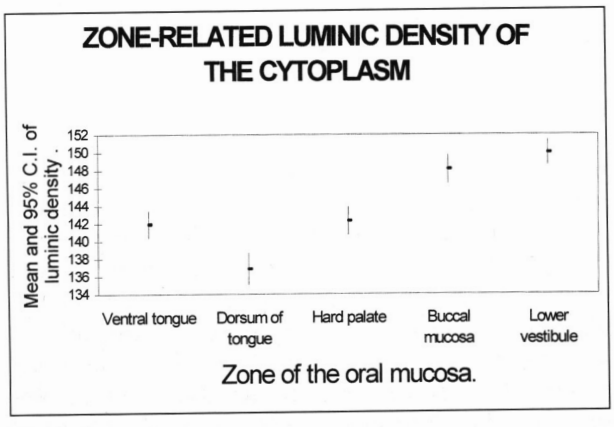


Figure 5: The graphs shows the image analizer lecture on the X axis (intensity). The intensity values varies between 0 (opaque area) at 250 (transparent area). The Y axis show the optical density values that varies between 0 (opaque area) at 2.5 (transparent area).

an improved distribution of the cells on the slide (Jones et al., 1994). Therefore, we chose to use the Cytobrush®. An alcohol base fixer (Labofix®) was used. Using a semiautomatic analysis of the nucleus and cytoplasm area, Ogden et al. (1989) found no significant differences using different methods of fixation such as diethyl ether-ethanol at 1:1, a spray fixer (Vale Smear Fix), isopropyl alcohol in polyethylene glycol and then air drying.

The sample was fixed immediately to avoid handling errors. It was transported (slide totally protected in boxes) and stained during the first forty eight hours after collection. The staining technique was carried out adhering strictly to times and concentrations. As indicated earlier, we eliminated the possibility of errors occurring during the measurement procedures by repeatedly checking consistency.

As shown by the variance analysis and the graphic representation of the descriptive analysis, we found significant differences in the optical density of the different zones of the oral mucosa.



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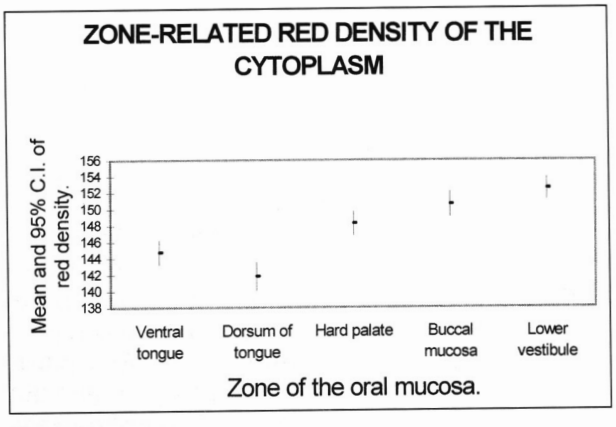

Figure 7.

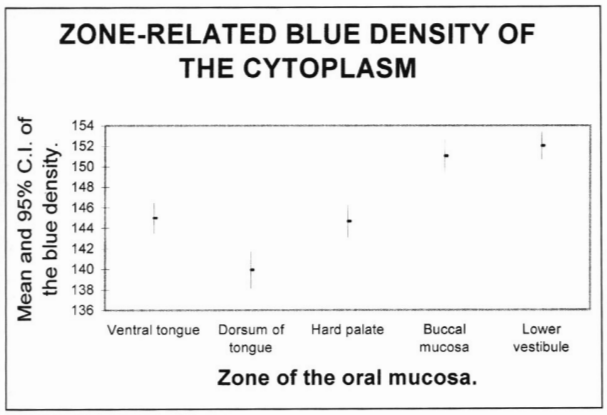

Figure 8.

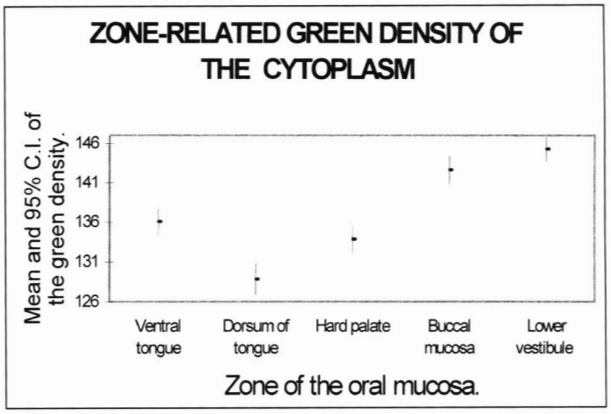

Figure 9.

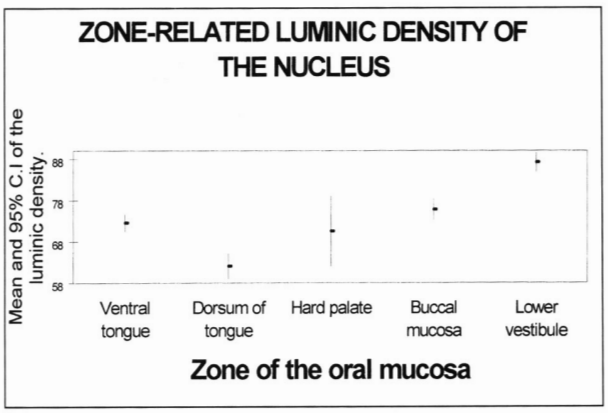

Figure 10.

The fact that a high statistical significance was found between the different zones of the oral cavity, although this difference was quantitatively small, could be linked to the large sample size. Morphometric studies in oral exfoliative cytology (Cowpe et al., 1985) have revealed significant differences in morphometric variables in different zones of the oral cavity. The variability of the morphometric and optical parameters may be related, although this must be verified in future studies.

The results show the densitometric parameters of healthy oral mucosa to compare them in future studies with parameters of oral mucosa with pathology.

Our results suggest that the densitometric values, studied with the same stain and analysis method, varies in function of the area where we collected the smears. Therefore we can compare different patients' samples collected in the same area. We cannot compare smears of different areas to study the oral mucosa using optic densitometry.

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