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Could oral cytomorphometry be of value in distinguishing diabetes mellitus?

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Abstract

The present study refers to a quantitative, morphometric analysis of exfoliative cytology smears collected from diabetes mellitus (DM) patients, in order to distinguish subtle changes in cellular and nuclear parameters. The study was carried out on 30 adult subjects: a control group of 10 healthy subjects and a study group of 20 diabetic subjects (type 1 and type 2 DM). Another factor that was taken into consideration was the abundance of the microbial flora. The oral smears were stained using Hematoxylin and Eosin and several parameters were measured (nuclear diameter, perimeter and area, cell large diameter and area), and calculated: nuclear/cytoplasmic ratio and nuclear roundness factor. We found out that the cells collected from DM patients had higher values of the nuclear parameters (the nuclei were larger) and lower cell dimensions. The nuclear to cytoplasmic ratio was increased in these patients, but the nuclear roundness factor was closer to one in the study group. Also, an increased number of bacteria, often seen in DM patients, decreased the nuclear parameters. Our findings complete recently descriptive cytology studies with the morphological measurements in case of bacterial abundance and sustain the possible value as screening method for morphometry.

Keywords: diabetes mellitus, bacterial abundance, oral exfoliative cytology, morphometry.

Introduction

Diabetes mellitus (DM) is a frequent metabolic disorder of complex and multiple etiologies, characterized by chronic hyperglycemia, with disturbances of sugar, fat and protein metabolic pathways, which lead to great morbidity and mortality [1, 2].

The clinical signs and symptoms of DM involving the oral cavity are numerous: xerostomia, burning mouth syndrome, halitosis, dental cavities and various inflammatory processes (of non-infectious or infectious etiology), as chronic periodontopathies (periodontitis and gingivitis) [3], periapical abscesses and candidiasis [4–7].

Exfoliative cytology of the oral mucosa is a rapid and low-cost method and can be an efficient diagnostic tool in some pathological conditions with oral manifestations, including DM [8]. In order to assess the DM oral changes, several descriptive cytology studies have been performed [4, 7, 9, 10]. In DM, oral cytology can detect altered cells, at both nuclear (enlargement, dyschromasia, binucleated cells) and cytoplasmic level (cytolysis, fatty degenerescence, erratic glycogen accumulation) [8, 9]. Nevertheless, in spite of abundant morphological information, descriptive cytology was not statistically proved as being a useful screening tool in the diagnostic and management of DM and its complications [4, 7, 9].

From a pathologists' angle, descriptive cytology is an important screening and diagnostic tool in cervical neoplastic and preneoplastic changes [11, 12], or even in some transformations of the oral mucosa in tumors and systemic diseases [13, 14]. However, when it comes to discriminating changes induced by the clinical type of DM in the oral mucosa, descriptive cytology is not enough. Therefore, a more precise and statistically significant method is needed, in order to objectify these alterations. After designing and accomplishing a previous descriptive cytology study on diabetic patients' oral mucosa changes and illustrating the cellular alterations (regretfully without clinical staging and prognostic utility) [9], we turned to computerized cytomorphometry, a domain which offers the promise to emphasize cytological alterations that cannot be observed by microscopic examinations of the cells, even under high power magnification [15].

Computerized cell morphometry is a relatively new instrument in evaluating subtle changes at microscopic level. Although cell measurements have been performed since the invention of the graded eyepiece, real progresses in cell morphometry have been achieved with the development of dedicated software, facilitating measurements and mathematical processing of the acquired images in different pathologies [1, 15].

Therefore, the purpose of our present study was to evaluate the cytomorphometrical changes of the squamous cells of oral mucosa in patients with DM, to test the hypothesis that these findings correlate with DM's oral bacterial loading, and consecutively, to emphasize the utility of oral exfoliative cytomorphometry as a diagnostic tool for DM patients.

Patients, Materials and Methods

The study group was selected from the Clinic of Diabetes, Nutrition and Metabolic Diseases, Emergency County Hospital, Cluj-Napoca, Romania; it was composed of 20 diabetic adult patients (10 with type 1 DM and 10 with type 2 DM). Ten non-diabetic adult subjects represented the control group. Each patient consented to a protocol approved by the Medical Ethics Committee of "Iuliu Haţieganu" University of Medicine and Pharmacy, Cluj-Napoca. Relevant biographical and personal pathological antecedents were recorded. The age in studied groups ranged between 20 and 35 years old.

In order to prevent all other significant modifications of oral mucosa, we investigated the selected patients through a clinical examination of oral cavity and biochemical and hematological measurements. Patients with frank oral lesions, gingivitis, periodontal disease, or with other systemic diseases, smokers, and alcoholics, were excluded from the study.

For realizing exfoliative cytology smears, after cleaning the oral cavity with mouthwash and gauze swab, the cells were collected using 10 gently Cytobrush® rotations from the jugal and lingual mucosa. Immediately after prelevation, the cells were spread on a glass slide and fixed with 96% ethyl alcohol solution. After drying, all slides were labeled with a code identifying each investigated group and subject and were Hematoxylin–Eosin (HE) stained (Mayer's hemalum and Eosin Y 0.2% solution, ORSAtec GmbH, Germany).

For maintaining uniformity of each assessed case, the smears were firstly examined at ×400 (for testing the quality of staining), followed by examination at ×1000. Each of the two observers counted about 40 unfolded cells with clear outline / each smear / at least 20 separate fields and the results were tabulated.

Considering the total cell number/smear and the cytology Bethesda System reporting [16], all specimens (from our study) were accepted as satisfactory for interpretation, and were clinically normal, without any neoplastic modifications.

In mostly oral smears, we found mainly cocci, bacilli, and no fungal mycelia. We considered smears with less than 30 microbes colonized epithelial cells and less than 50 neutrophils associated epithelial cells, as with scarce/ normal bacterial flora. Because an abundant bacterial flora can alter cell shape and nuclear to cytoplasm ratio [9], the diabetic subjects were rearranged for more accurate statistical interpretation in two subgroups: a subgroup of 11 patients with abundant bacterial flora (DM B+ group) (Figure 1), and a subgroup of nine patients with scarce microorganisms on the oral smear (DM B- group).

Image acquisition was performed with a Leica DM 750 microscope, equipped with a 5-megapixel digital camera mounted on a trinocular head. No software adjustments on the images (concerning exposure, light or white balance) were carried out.

Morphometry was manually performed, using ImageJ® software, a multiplatform valuable analysis tool, used for various types of biological computerized investigations. For the cell contouring, an 800 dpi computer mouse was used. ImageJ is an open source image-processing program, which has a cell counter plug-in, any time being possible to add or remove count features; the desired feature is selected, and the count is done by clicking on the feature in the image.

We considered two categories of parameters: measured parameters and calculated parameters (Figure 2).

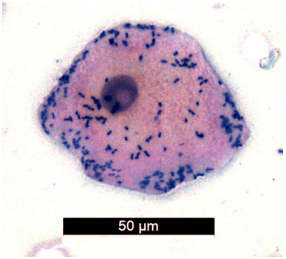

Figure 1 – ***Exemplification of abundant bacterial flora (DM B+ group).***

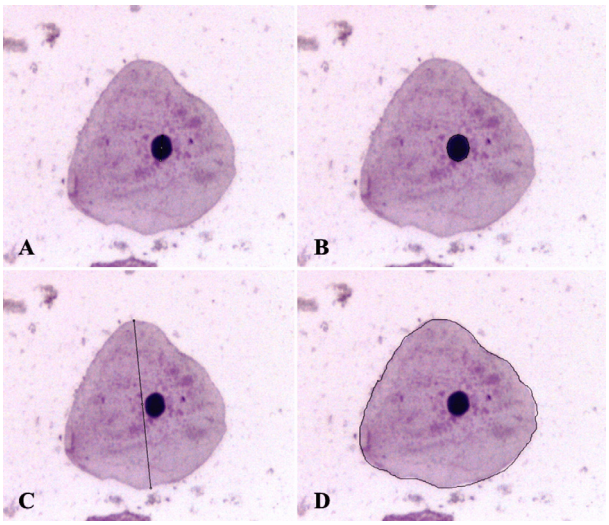

Figure 2 – ***Exemplification of acquisition of morphometric parameters: (A) Nuclear diameter; (B) Nuclear area; (C) Cell larger diameter; (D) Cell area.***

The measured parameters were represented by the nuclear diameter (ND), the nuclear area (NA), the nuclear perimeter (NP), the cell larger diameter (CD), and the cell area (CA); the calculated parameters were the nuclear to cytoplasmic ratio (NCR) and the nuclear roundness factor (NRF), derived according to the following formulas:

$$NCR = \frac{NA}{CA - NA} \qquad (1)$$

$$NRF = \frac{NP^2}{4 \times \pi \times NA} \qquad (2)$$

The data was statistically analyzed using Microsoft Office Excel 2016. Descriptive statistics parameters were calculated (mean, standard deviation, standard error of the mean and 95% confidence interval for mean). One-way analysis of variance (ANOVA) was used for multiple groups' comparison, followed by the Student's *t*-test (two-sample assuming equal variances). A *p*-value less than 0.05 was considered of statistical significance.

Results

Measured parameters

According to the digital measurements, all the nuclear dimensions (diameter, perimeter and area) had higher

values in DM patients, in both subgroups (with and without bacterial abundancy) compared to control (Table 1), but the differences were strongly statistically significant (p=0.006) only for NA, in patients with low bacterial abundancy, compared to control (Figure 3). There were no statistically significant differences regarding the nuclear dimensions obtained from DM B- and DM B+ patients (Table 1).

Table 1 – ***Parameter's values for control group and DM patients, with low or high bacterial colonization and statistical significance***

Parameter	Group	Mean ± SD	SEM	*P*-value, Student's *t*-test: two-sample equal variances
Nuclear diameter [µm]	Control	5.17±0.7	0.013401	C – DM B- = 0.064994 C – DM B+ = 0.237439 DM B- – DM B+ = 0.724206
	DM B-	5.47±0.8	0.02475	
	DM B+	5.2±0.6	0.02793	
Nuclear perimeter [µm]	Control	15.3±2.5	0.043706	C – DM B- = 0.0774 C – DM B+ = 0.526916 DM B- – DM B+ = 0.20444
	DM B-	16.5±2.5	0.0736	
	DM B+	16.2±2.3	0.19396	
Nuclear area [µm^2]	Control	45.9±12.8	0.00116	C – DM B- = ***0.006014*** C – DM B+ = 0.266917 DM B- – DM B+ = 0.356199
	DM B-	55.8±16.6	0.00259	
	DM B+	50.4±16.0	0.00313	
Cell larger diameter [µm]	Control	32.3±5.2	0.0899	C – DM B- = ***0.009742*** C – DM B+ = ***0.018973*** DM B- – DM B+ = 0.872111
	DM B-	28.6±6.5	0.19275	
	DM B+	23.2±3.5	0.26429	
Cell area [µm^2]	Control	172.81±53.74	0.048494	C – DM B- = ***0.006014*** C – DM B+ = 0.266917 DM B- – DM B+ = 0.356199
	DM B-	152.37±70.55	0.10937	
	DM B+	169.67±64.56	0.12544	
Nuclear/cytoplasmic ratio	Control	0.2±0.08	0.01029	C – DM B- = ***0.000854*** C – DM B+ = ***0.044109*** DM B- – DM B+ = 0.384597
	DM B-	0.3±0.02	0.04847	
	DM B+	0.3±0.02	0.04249	
Nuclear roundness factor	Control	10.8±1.3	0.168229	C – DM B- = ***0.025654*** C – DM B+ = ***0.030586*** DM B- – DM B+ = 0.259971
	DM B-	10.1±0.2	0.04412	
	DM B+	10.2±0.1	0.12469	

SD: Standard deviation; SEM: Standard error of the mean; C: Control; DM: Diabetes mellitus; B-: Low bacterial density; B+: High bacterial density.

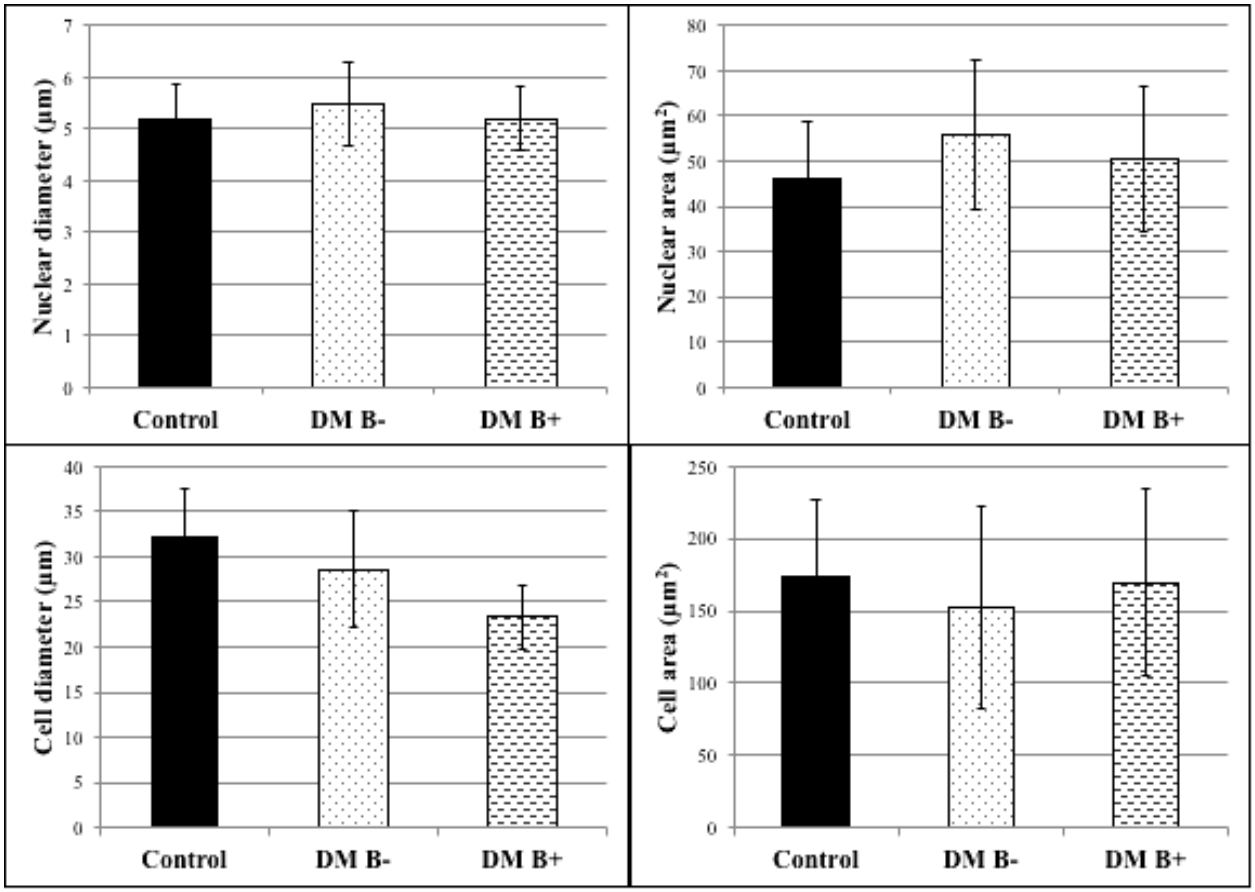

Figure 3 – ***Measured morphometric parameters. DM: Diabetes mellitus; B: Bacterial abundancy.***

Cell size parameters (CD, CA) measurements, showed decreased values for both diabetic patients types, in both B- and B+ subgroups, compared to control (Table 1). The CD values in DM groups compared to control were statistically significant for both subgroups (p=0.009 for B- subgroup and p=0.019 for B+ subgroup). The CA values in DM groups showed statistically significant difference regarding the control group, only for DM B- patients (p=0.006) (Table 1).

Calculated parameters

The NCR was higher in both subgroups of diabetic subjects compared to control, statistically significant for DM B+ group (p<0.05), and highly significant for DM B- group (p<0.001) (Table 1).

The NRF was significantly lower in DM patients (p<0.05), regardless of their microbiological status (Table 1). In other words, the nucleus was closer to a perfect circle in control subjects, and farther from a perfect circle in diabetic subjects (Figure 4).

For both calculated parameters, the group with bacterial abundancy had lower values than the group without bacterial loading, without significant difference (Table 1).

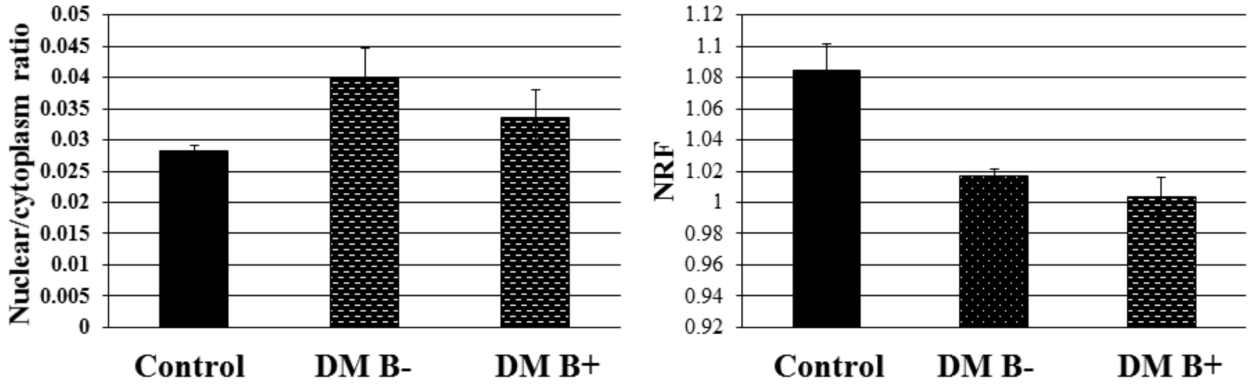

Figure 4 – ***Derived morphometric parameters. DM: Diabetes mellitus; B: Bacterial abundancy; NRF: Nuclear roundness factor.***

Discussion

Exfoliative oral cytology is a simple, fast, and non-invasive technique, widely accepted by patients, low-cost and easily reproducible, which allows a reliable assessment of suspicious lesions. It can detect early changes of diseases even in the absence of clinical manifestations and has the potential to be developed as an early diagnostic procedure in DM, in order to separate the population at risk and evaluate the complications [4, 17–20].

From the three stainings (Papanicolaou, APT–Drăgan and HE) used in our previous study [9], several reasons lead to HE's choice for morphometric purposes. HE provided clear cellular and nuclear edges, perfectly suitable for tracing the contours and intranuclear details [21]. It is also the main staining widely used for medical diagnosis, large accessible, rapid and low-cost, technically the easiest one.

Cell morphometry offers a unique insight on cytological alterations; therefore, the modern methods using computerized morphometry software have been widely used in various studies, in order to assess subtle changes [1, 19, 22–24], or/and, also, for an early and accurate (92–95%) detection of the oral preneoplastic and neoplastic lesions [25, 26]. Our research focused on two categories of classical morphometric parameters: measured parameters and calculated (derived) parameters.

One of the most significant and relevant finding was the increase of nuclear size in cells of DM patients, which was also observed in other studies [9, 19]; in our study, it was more significant for diabetic patients with low bacterial loading. In DM, this increase could be due to different factors: cellular adaptation to ischemia, higher supply of glucose to tissues, metabolic processes favoring depletion of adenosine triphosphate (ATP) and activating phosphorylase and binding of enzymes to their regulatory proteins in the nucleus; hence, the nucleus evolve with chromatin decondensation and it is hypertrophied with a much higher DNA [19, 27, 28].

The findings concerning the NCR were not surprising: the ratio is increased in diabetic patients, the differences being statistically significant in both subgroups, compared to the control and highly significant for DM patients without low bacterial loading. Moreover, the NRF was lower and closer to 1 in DM subjects, being an indication of an increased metabolic activity in these cells, and correlating with the increase of the NCR.

For interpretation of the nuclear changes, we have taken into consideration the high probability of a neoplastic process (almost 90% of human cancers have epithelial origin [29] and the oral squamous cell carcinoma is the most frequent malignancy of the oral mucosa [30]). It is well known that abnormalities of nucleus are hallmarks of preneoplastic or neoplastic changes of cells on oral smears, but even in malignant squamous cells, nuclear size and NCR also increase [30, 31], NCR alone cannot be used as an accurate criterion for separating neoplastic from normal cells [32].

Several descriptive cytology studies revealed that the nuclear changes (hyperchromasia, intranuclear clear inclusions, naked nuclei, or fading of the nuclear membrane, peri-nuclear halo, binucleation, nuclear vacuoles [4, 9, 17, 18, 33–35]) have limited clinical relevance. We interpreted the changes in nuclear shape and the increase in size of the nucleus only of reactive and not, neoplastic origin: the chromatin was regularly scattered (no coarse areas) and the nucleus was mainly of regular (round)

shape. Another argument for the nucleus reactive changes was the close to 1 NRF observed in diabetic patients, which signifies that those nuclear changes appeared exclusively in the context of DM, and they are not dysplastic [9]; in this latter situation, the nuclei must be irregular in shape and chromatin distribution, which was not the case in our study.

Our results showed a decreased cell size in DM patients, with statistical significance only for CD. The decrease in cell size is sustained by other studies [19, 22, 24, 33], but the biological explanation is controversial. One possible mechanism could be the ischemia induced by vessel deterioration secondary to DM [4, 7]. Therefore, the cells lack proper oxygenation and shrink in size [22]. Another mechanism involves the accelerated glycosylation of extracellular matrix proteoglycans, resulting in the alteration of cell bonding and a consecutive decrease in cell size [9]. Nevertheless, these alterations do not seem to be pathological or to predispose to any pathological consequence. Concerning clinical correlations, xerostomia, a typical feature of DM (due to hyperglycemia, dysfunctionalities in the mechanism of saliva secretion and general dehydration), can also generate significant oral mucosa abnormalities, which can be revealed by morphometry [18]. The decrease in cell size might be due to a lack of cytoplasmic hydration, with subsequent cell shrinking and partial collapse of the plasma membrane around the cytoplasm [9, 18].

According to our results, it looks like the abundance of the microbial flora has an attenuating effect on the NA, CD, NCR, and also on the NRF; the values for those parameters were lower in patients with abundant microbial flora, but the differences between the two B- and B+ subgroups, for every measured or calculated parameter were not statistically significant. This is consistent with the fact that only for the DM patients without low bacterial loading (compared to control group) the NA was significantly increased and the CA was significantly decreased. The calculated parameters sustain the previous findings and are consistent with other morphometric studies [22, 24, 33]. The fact that the statistical significance was higher (lower p values) in case of diabetic patients with low oral bacteria density sustain also a reactive effect of high bacterial density onto the nucleus of oral mucosa squamous cells.

The results of the present paper complete our previous inquiry of qualitative cytology in DM, and are consistent with other recently morphometric studies [8, 19, 35, 36], sustaining morphometry's possible value as diagnostic method. The morphological transformations induced by the bacterial abundance on the exfoliated oral squamous cells represent the new idea brought in by this study.

Conclusions

HE staining proved to be a good choice for squamous cells morphometry and can be recommended for further morphometry studies. DM induces reactive, non-neoplastic modifications of exfoliated squamous cells from oral mucosa: decrease of cell area, increase of nuclear area. In DM subjects, cell size significantly decreases and nuclear size significantly increases; consecutively, the NCR is higher. The NRF is significantly lower in DM subjects. The abundance of microbial flora does not have a statistically significant effect on morphometric parameters. Establishing new, precise intervals for morphological parameters can lead to a correlation with DM's clinical staging and to implementation of oral exfoliative cytology as an early diagnostic procedure in DM.

Conflict of interests

The authors declare that they have no conflict of interests.

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