Oral signs and symptoms and hyperglycemic status of type II diabetic patients in relation to cytomorphometric findings of gingival and buccal cytobrush smears

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ABSTRACT

Background: Brush cytology is an accepted technique that gets renewed interest. It is now used as an aid for the diagnosis and observation of possible epithelial changes that could be associated with oral mucosal diseases. This study aimed to evaluate the cytomorphometric changes in gingiva and buccal mucosa of type II diabetics and to assess their relation to oral symptoms and glycemic status.

Materials and methods: Cytological Papanicolaou stained smear were prepared from cheek and gingiva of 20 non treated cases, 20 treated diabetics and 20 healthy persons of both sex after measuring their HbA_{1c} and recording their oral symptoms. Hundred unfolded epithelial cells were evaluated qualitatively using MCID software to measure nuclear and cytoplasmic areas, ratio, perimeters, and form factors. Different statistical analyses were used to determine relations between studied parameters.

Results: Diabetics smears showed large nucleus, small cytoplasm, and small cytoplasm/nucleus ratio compared with healthy persons with no sex variation. Xerostomia and burning sensation were significantly correlated with nuclear parameters, while HbA_{1c} was significantly correlated with both cytoplasmic and nuclear parameters. Well-controlled patients showed reduction in nuclear area, but nucleus and cytoplasm form factors were unlike normal.

Conclusions: Oral cytology from type II diabetics is associated with detectable cytomorphometric changes that is better demonstrated in buccal than gingival mucosa and tend to return partially to their normal values in well-controlled patients, with no sex variation. NA seems to be the main parameter that changed during hyperglycemia and xerostomia, while both NA and CA were related to burning sensation.

Key words: type II diabetes mellitus, cytomorphometry, gingiva, xerostomia. (J Bagh Coll Dentistry 2013; 25(2):59-65).

INTRODUCTION

Diabetes mellitus (DM) is the most common metabolic disorder that produces multiple systemic complications [1] with multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism [2]. Type II DM accounts for 90- 95% of persons with diabetes most of them are adults older than 40 years, but it becomes more common in obese adolescents and children [3] Poorly-controlled diabetics are associated with oral health complications include gingivitis, periodontitis, salivary gland dysfunction, dental caries, burning mouth sensation, taste disturbances, infections (oral candidiasis) and mucosa changes [4,5]. Thus dentist has a major role in 1) screening and diagnosis of undiagnosed patients, 2) proper dental management of oral manifestations and 3) prevention of systemic and local complications [6,7].

Several clinical and paraclinical techniques are available for screening of oral mucosal changes and oral cytology is one of the appropriate method in identifying high risk population or for clinical follow up [8].

Oral cytology is a relatively inexpensive, simple, noninvasive, and risk-free technique that is well accepted by the patient [9]. And with the application of cytobrush sampling, advance technology and immuno-/geno- cytochemistry there was much improves in the potential accuracy of oral cytology [10-12].

Concerning cytomorphology of oral mucosa in type II DM, few published literatures described changes in buccal mucosal collected by different methods without specification of patient's hyperglycemic status [13-15]. However, Prasad et al [16] in 2010, declared that point; but they neglected treatment and site variation. Later on in 2011, Hallikerimath et al. studied cytomorphological changes and glycogen content in exfoliated cell from buccal mucosa [17]. Therefore, the goal of our study was to identify the quantitative nuclear and cytoplasmic changes of both buccal and attached gingiva in type II DM at different hyperglycemic status (uncontrolled, well controlled and poorly controlled) whether they were treated or untreated and assess their relation with xerostomia, burning mouth sensation and oral ulceration.

MATERIALS AND METHODS

In a cross-sectional study, a total of 40 type II DM patients were collected from Ali Naji Dispensary Clinic in Sulaimani city from Feb. to Aug. 2009, so that 20 of them were newly

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undiagnosed cases and 20 were receiving metformin therapy 500mg (tid) for not less than 1 year. According to their HbA1c levels (which indicates the degree of glycemic control achieved), they were subdivided into 3 groups; uncontrolled (HbA1c >12%), well-controlled (HbA1c ≤8%) and poorly controlled (HbA1c >10% and ≤12) [16]. The control group included 20 non-diabetic healthy volunteers with no risk factor for diabetes and their HbA1c< 6.5%. All participants' age ranged between 40-50 years. The exclusion criteria were: 1) smoker (18-19) or alcoholic [20] patients, 2) systemic diseases or other medications that affect the assay [21], and 3) ladies who were pregnant or during menstrual period or taking contraceptives [22] .

The study was approved by the local ethical committee and all patients signed a written consent form. Patient's name, age, sex, medical history, presence of burning mouth sensation (oral mucosal pain related to DM and not to other medical or dental cause) and xerostomia (subjective feeling of oral dryness) as described by the patients were recorded. Oral ulceration (presence of mucosal discontinuity) was assessed clinically by specialist using mouth mirror and under good light vision).

The participants were asked to gargle with tap water. The oral mucosa was dried with gauze to remove surface debris and excess saliva. Two smears were collected, one from the buccal mucosa and the other from upper anterior attached gingiva of each individual using oral cytobrush (Rover Orcellex/ Netherlands) and transferred to labeled, clean, dry glass slides. They were then fixed at once by soaking in 95% ethanol and stained by Papanicolaou stain.

For each individual (without identify his group) 100 unfolded, clearly outlined, separated cells (50 from buccal and 50 from attached gingiva) were selected manually by moving the slide in a stepwise manner (from upper left corner to the right and then downwards and going back in reverse direction in order to avoid measuring the same cells again) and their images were captured: using digital microscope camera (Moticam 2000/China) attached to Olympus microscope at power 100X (figure-1A). These images were transferred to a computer by using Motic Images Plus 2.0ML software. They were changed to gray scale and from JPEG format to Tiff format using Adobe Photoshop CS2. The cytomorphometric measurements; the nuclear and cytoplasmic areas (NA, CA), perimeters (NP, CP), ratio (NA/CA) and form factor (a standard estimate of circularity that relates perimeter length to area; NF, CF) were determined by using auto image segmentation that identifies their boundaries pixels using MCID image analysis software (figure-1B).

Raw data were collected, tabulated, and analyzed using SPSS software (version 16.0). The cytomorphometric parameters were represented as mean ± SD, while the clinical parameters (sex, xerostomia, burning sensation, oral ulceration and glycemic status) were presented as frequency and percentages distribution. One way ANOVA-test followed by multiple comparisons test (Post Hoc test) were applied to identify the significance among different studied group. Independent Student's t-test was applied to compare between sites as well as sex variations. Bivariate Person's correlation was used to determine the relationship between studied parameters. The level of significance was set at $P \leq 0.05$.

RESULTS

The number of subjects in each treated and non treated groups according to their HbA1c were distributed in relation to sex and oral symptoms are illustrated in table-1. The well controlled group included 20 patients (12 treated and 8 non-treated) and 50% of them were suffering from xerostomia, while the poorly controlled group included 14 patients (8 treated and 6 non-treated) and xerostomia was more frequent in non-treated patients (5 out of 6; 83.3%). Furthermore, the uncontrolled group included only 6 non-treated undiagnosed patients and all of them complain from xerostomia. Lastly treated patients described more frequently the feeling of burring mouth sensation than non-treated group regardless of their glycemic status, (table 1). Statistical analysis showed that HbA_{1c} correlated significantly with both xerostomia (r=0.63, P=0.000) and burning sensation (r=0.38, P=0.002).

Concerning cytomorphometric measurements in relation to site variation, buccal mucosa had significantly larger CA, CP, CF and CA/NA than gingival cells, both in healthy and well control treated patients (table 2 and 3). The remaining DM groups maintain only the significant large CF and CA/NA in buccal mucosa. On the other hand, NA and NP of healthy persons did not show site differences, nevertheless, in buccal smear of uncontrolled and poorly control treated patients nuclear areas were slightly larger ($p>0.05$) than that of gingiva, and a reverse findings is reported in well-controlled groups (in both treated and non treated groups; i:e nuclear areas of buccal mucosa were smaller than gingiva) (table 3).

The cytological smear obtained from oral mucosa of all DM subgroups in comparison to control group, at both sites, had significant larger

NA, NP and significant smaller CA/NA. Furthermore, the uncontrolled non-treated patients had significantly small CA and CP in gingival smears. Thus, the CA/NA reaches half the ratio of that of control subjects (P=0.000) (25.15 vs. 50.84). While all treated DM patients showed significantly larger CF at the buccal mucosa (0.75 in well controlled and 0.76 in poorly controlled) (table 2 and 4). No sex variation was observed in all measured parameters (data not shown).

Regarding the differences among DM subgroups in relation to their hyperglycemic control and therapy status, statistical analysis indicated no significant variation in nuclear measurements, despite that NA was reduced in relation to HbA1c level, especially in buccal mucosa. Thus DM patients with HbA1c ≤8 had smaller NA (58.42) in comparison to both poorly controlled (64.66) and uncontrolled (64.46) groups, and it became even smaller with therapy (i.e. in well controlled treated group 55.95) (table-2 and 4).

The buccal smears from poorly treated patients had larger cellular form factor (CF) measurements than non treated patients; both poorly controlled and uncontrolled (0.76 vs 0.73 and 0.74 respectively). Thus, changes in CF in diabetic patients vary from polygonal or oval or elliptical to circular in the following sequence healthy person > undiagnosed DM patients > treated patients, especially poorly controlled treated patients at buccal cells. Beside that uncontrolled patients had smaller CA/NA ration (29.14) than poor-controlled non-treated patients (37.69), well controlled treated (43.2) and well controlled non-treated patients (40.09), while the NP was smaller in well controlled treated patients than poorly controlled treated. On the other hand, cells collected from gingival mucosa demonstrated that well treated patients had smaller NF (0.879) than non treated patients (both well- controlled 0.898 and poor-controlled 0.895). However these variations did not reach statistical significant level (table 2). All the studied variables did not show sex variation (data not shown).

The correlation between cytomorphological parameters on one hand, the HbA_{1c} and oral symptoms on the other hand, results showed that the levels of HbA1c was significantly correlated with cytoplasmic and nuclear parameters (area and perimeter); it had highly significant negative correlation with CA/NA ratio in both studied sites (gingival and buccal mucosa, r=- 0.7, r= - 0.62 respectively) (table-4). On the other hand, xerostomia and burning sensation correlated significantly with NA, NP and CA/NA ratio. Furthermore, the CA and CP of the buccal mucosa were related significantly to the burning sensation. Finally, ulceration had significant week correlation only with CA and CP at buccal mucosa (table-5).

DISCUSSION

Oral conditions that are possibly seen in individuals with diabetes may include burning mouth, altered wound healing, an increased incidence of infection, and xerostomia [4,7] . In this study the frequency of xerostomia was increased as glycate hemoglobin level increased, especially in non treated patients. However, half of our patients who keep HbA1c level at ≤8 were suffering from xerostomia irrespective to the stat of therapy. On the other hand, burning mouth had weak correlation with HbA1c level and is more frequently reported in treated DM patients. Thus any observed changes in oral cytomorophology of well controlled DM may possibly attribute to therapy effect. Therefore, we further subdivided our non treated patients according to their HbA1c level into 3 groups to be compared with those corresponding treated groups.

From histological point of view, the oral cavity is lined by different types of stratified squamous epithelia. The regional differences in the patterns of epithelial maturation appear to be associated with different turnover rates; thus non-keratinized buccal epithelium turns over faster than keratinized gingival epithelium. Since such variations are clinically reflected by both; in the more rapid appearance of changes and in the prevalence of damage to non-keratinized rather than to keratinized surfaces [23], we compared buccal mucosa that had been frequently studied in previous researches with the gingiva to be studied for the first time in DM patients.

Previous study using cytobrush smear indicated that normal non-keratinized buccal mucosa had larger cytoplasmic and nuclear areas than floor of the mouth and the dorsum of the tongue [13]. In this study, normal gingival mucosa also had smaller cytoplasmic measurements than buccal mucosa, but they had nearly equal nuclear measurements. In fact cytoplasmic variation could be related to differences in cell turnover rate and maturation stage [23], beside the effect of existence of local inflammation [24]. Accordingly one expects to see the herein the reported greater reduction in NA at non-keratinized mucosa of well-controlled treated DM in response to therapy.

The present study showed that cytomorphometric measurements were not related to sex variation. This is in line with Prasad et al [16] and Cowpe et al [25]. Nevertheless, Patel et al [22] mentioned that hormonal changes during

menstruation, pregnancy and taking contraceptive pills can affect the results; anyhow we exclude such patient from our studied sample.

Oral mucosal cells from DM patients in general had significantly large nucleus (NA, NP) and small CA/NA ratio and had a tendency to be small in size (CA, CP) when compared with control, this agrees with most previous studies [13,14,16,17] and only contradicts Jajarm et al findings for CA [15] . This variation could be attributed to the difference in the procedure of tissue sample collection, as they collected cells by lancet that scribed more superficial cells. Such procedure is considered to be inferior in the cytology of oral mucosa in comparison to brush technique [26] which is specially designed to access all epithelial layers.

Our findings demonstrate that there is a real increase in nuclear measurements related to hyperglycemic status in type II DM that; it was greatest in poorly controlled group and smallest in well controlled treated group, and this was better demonstrated in buccal mucosal cells than gingival. This result bridges Prasad's et al findings [16] concerning glycemic condition and Alberti's et al. results [13] concerning site variation.

Although morphological changes in oral mucosa may be related to many variables [18-22], here in diabetes it may be related to the metabolic control of the diabetic state and medication beside the previous reported factors that related to the reduction in; epithelial nourishment [2], proliferation and turnover secondarily to microvascular and metabolic disorders [27] that may accompanied with reduction in the stimulatory effect of insulin and IGF-I on keratinocytes [28]. Furthermore diabetics are commonly suffering from xerostomia that may alter oral mucosa and predisposing them to microbial colonization with critical reduction in salivary lubricant effect. This lead to atrophic oral mucosa or ulceration [2,7] that showed cells with large NA which may indicate more basal and parabasal cells. However such finding need to be related to cellular morphological features as Prasad et al [16] suggested that an increase in nuclear size with nuclear pleomorphism, bilobed nuclei, and cytoplasmic vacuolizations in DM may related to cellular ageing, which resulted from reduction in cellular turnover and persistence of more number of mature cells. In addition to that, oral cytomorphologic findings from DM patients showed evidence of buccal mucosa keratinization [29].

Concerning the glycemic status and treatment condition, the uncontrolled patients showed reduction in CA and CP in both site and it was significant in gingival smears. This is in line with Prasad's et al [16] finding who reported similar reduction in cytoplasmic diameter of buccal mucosa smear. However, Alberti et al did not find significant differences in CA among tongue, floor of the mouth and buccal mucosa [13].

Oral anti-diabetic drug, metformin had been shown to stimulate apoptosis in addition to its anti-proliferative action [30-31] . In this study it had noticable effect on cytoplasm and nuclear form factors of buccal and gingival mucosa respectively. Thus well-controlled treated diabetics expressed more irregular nuclear shape and circular cell shape in comparison with other DM subgroups. This could be attributed, to some extent, to the well known side effect of metformin in producing lacto acidosis [32] that is believed to cause cellular swollen and coarsen of the nuclear chromatin [33]. In this context NF is a suggested quantitative parameter for nuclear functions, aging or death.

It is interesting to report strong correlations between HbA_{1c} and oral symptoms on one hand and cytomorphometric parameters on the other hand. Accordingly, the level of HbA1c was highest in patient who their oral smears showed largest nuclear measurements and smallest cell size. On the other hand, NA seems to be the main parameter that changed during xerostomia. While both NA and CA were related to burning sensation, that associated with small cells and large nucleus, which is a predominant finding in gingival smears. Finally, mucosal ulceration was only related to large CA which observed more in buccal mucosa.

Although some of the above findings are not unique for DM and larger sample may provide better results, still this work provides us with another profile about oral mucosal changes and their response to therapy in DM that may have clinical implications in public health. It is extremely beneficial to determine the severity of the DM and the degree of control of glycemia, but the glycated hemoglobin assay is not currently recommended as a screening tool or as an initial test for the diagnosis of diabetes. It is used to monitor glycemic control in patients with previously diagnosed diabetes. Therefore, dentist can use cytology as additional tool in the clinic for screening and referral for diagnosis of previously undiagnosed patients after thorough review of the patient's health history and oral examination, or uncontrolled DM patients and explains the associated oral manifestations to them as well as to seek possible measurements to prevent local complications.

As a conclusion cytomorphometric results of healthy gingival mucosa had smaller cytoplasmic but nearly equal nuclear measurements in comparison with buccal mucosa. However, this picture is altered in DM patients. They showed detectable cytomorphometric changes that not related to sex variation. Such changes were better demonstrated in buccal than gingival mucosa. The altered cytoplasmic and nuclear measurements tend to return partially to their normal values in well-controlled patients. NA seems to be the main parameter that changed during hyperglycemia and xerostomia, while both NA and CA were related to burning sensation. Lastly, CF and NF are suggested as quantitative parameters to be assessed in DM oral smears.

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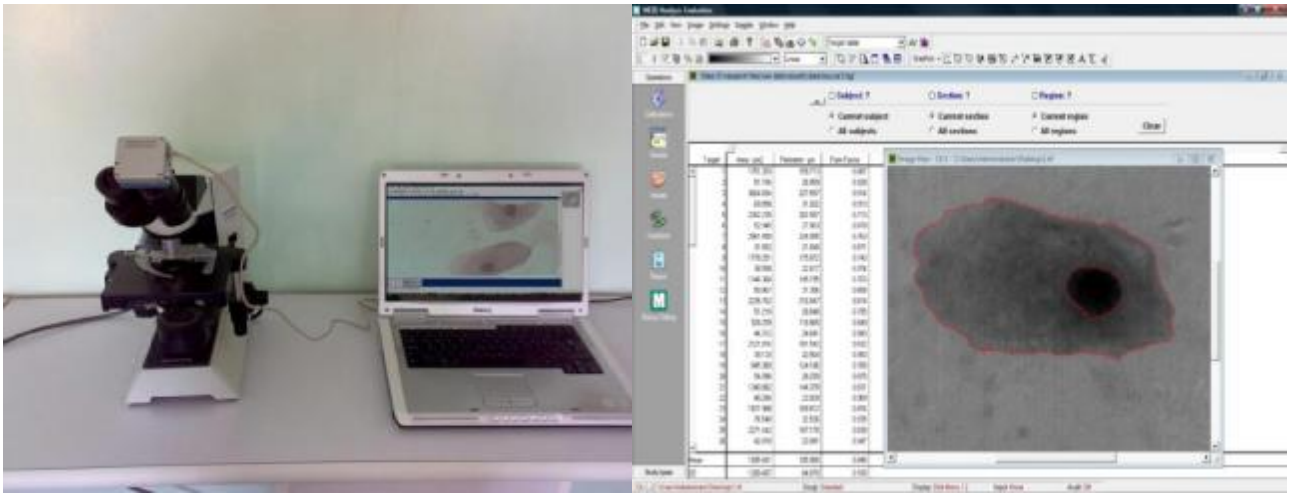

A **B**

Figure 1: A -Digital camera mounted on light microscope and connected to personal computer. B- Picture of MCID software during segmentation of a cell and its nucleus (the measurements appear at the left side).

Table 1 Frequency and percentage distributions of healthy subjects and type II DM patients according to their HbA1c in relation to sex and oral symptoms

Group	HbA1c	Subgroup	Total	Male	Female	Xerostomia		Burning		Ulcer	
						No	%	No	%	No	%
Healthy	<6.5	Total	20	6	14	0	0	1	5	2	10
DM	≤8	Non treated	8	5	3	4	50	1	12.5	2	25
		Treated	12	4	8	6	50	3	25	3	25
	>10≤12	Non treated	6	3	3	5	83.3	1	16.7	1	16.7
		Treated	8	6	2	5	62.5	3	37.5	2	25
	>12	Non treated	6	4	2	6	100	4	66.7	1	16.7

Table 2: The mean± sd values of cytomorphometric parameters in control and type II DM patients according to their HbA1c in relation to treatment at buccal and gingival mucosa

Parameter	Group / HbA1c / Site	Control	Non treated DM			Treated DM	
		<6.5	>12	≤8	>10 ≤12	≤8	>10 ≤12
CA (µm^2)	Buccal	2346.9±501.06	1796.6± 359.09	2174.2 ± 540.30	2272.7 ± 641.49	2235.9 ± 439.84	2157.9 ± 578.83
	Gingiva	1843.6±368.26	1420.6± 282.48	172.45 ± 266.75	1708.7 ± 322.58	1618.2 ± 244.77	1748.0 ± 310.03
CP (µrn)	Buccal	200±22.4	174.23± 15.79	192.23 ± 26.049	196.61 ± 29.31	1917.7 ± 19.61	187.18 ± 25.26
	Gingiva	182.28±16.57	159.30± 15.17	174.48 ± 14.21	174.43 ± 16.30	168.26 ± 14.39	175.23 ± 14.23
CF	Buccal	0.729±0.032	0.741 ± .021	0.741 ± .032	0.734 ± .019	0.757 ± .026	0.765 ± .0164
	Gingiva	0.698±0.043	0.703 ± .013	0.714 ± .021	0.706 ± .022	0.723 ± .030	0.719 ± .0267
NA (µm^2)	Buccal	39.58±8.28	64.46 ± 15.05	58.42 ± 7.41	64.88 ± 12.15	55.95 ± 7.98	64.66 ± 11.00
	Gingiva	39.7±10.83	58.28 ± 7.12	61.32 ± 9.62	66.52 ± 10.13	59.58 ± 8.16	62.72 ± 6.12
NP (µrn)	Buccal	23.59±2.53	30.27 ± 4.05	28.76 ± 1.91	30.41 ± 2.81	28.15 ± 2.20	30.47 ± 2.70
	Gingiva	23.63±3.11	28.85 ± 2.15	29.39 ± 2.44	30.65 ± 2.45	29.31 ± 2.12	29.95 ± 1.52
NF	Buccal	0.891±0.018	0.891 ± .034	0.892 ± .012	0.884 ± .021	0.89 0± .015	0.879 ± .0169
	Gingiva	0.887±0.026	0.891 ± .029	0.898 ± .015	0.895 ± .012	0.879 ± .016	0.886 ± .0192
CA/NA	Buccal	63.81±13.38	29.14 ± 3.37	40.09 ± 10.22	37.69 ± 7.56	43.21 ± 10.60	34.98 ± 6.241
	Gingiva	50.84±9.12	25.15 ± 3.12	29.65 ± 4.79	27.03 ± 3.95	28.18 ± 3.349	28.95 ± 4.430

Abbreviations: Diabetes mellitus(DM), hemoglobin A1c (HbA1c), nuclear area (NA) cytoplasmic area (CA), nuclear perimeter (NP), cytoplasmic perimeter (CP), nuclear form factor (NF), cytoplasmic form factor (CF), ratio of cytoplasmic to nuclear areas (CA/NA)

Table 3. The results of significant independent t- test for the differences in cytomorphometric parameters between buccal mucosa and gingiva in all MD groups and control.

	Control		Well-controlled				Poor-controlled				Uncontrolled	
No.	20		6		12		6		8		8	
	df=38		Non-treated df=14		Treated df=22		Non-treated df=10		Treated df=14		Non-treated df=10	
	t	Sig	t	Sig	t	Sig	t	Sig	t	Sig	t	Sig
CA	-3.619	.001	-2.11	.053	-4.251	.000						
CP	-2.87	.007			-3.348	.003						
CF	-2.622	.012			-2.954	.007	-2.296	.045	-4.121	.001	-3.607	.005
CA/NA	-3.583	.001	-2.615	.020	-4.679	.000	-3.057	.012	-2.229	.043	-2.128	.059

Table 4: multiple comparison for the mean values of cytomorphometric parameters among all studied groups at both sites using one-way ANOVA and Post Hoc test /Bonferroni procedure

parameter	Site	one-way ANOVA		Post Hoc test									
				Well-controlled				Poor-controlled				Uncontrolled	
				Non-treated		Treated		Non-treated		Treated		Non-treated	
		F	Sig.	Mean Difference	Sig.	Mean Difference	Sig.	Mean Difference	Sig.	Mean Difference	Sig.	Mean Difference	Sig.
CA	Gingiva	1.97	0.08									423.11	.05
CP	Gingiva	2.58	.036									22.98	.026
CF	Buccal	2.68	.031			-.0274	.009			-0.0354	.04		
NA	Gingiva	15.23	.000	-21.62	.000	-19.88	.000	-26.81	.000	-23.01	.000	-18.58	.001
	Buccal	14.02	.000	-18.84	.000	-16.37	.000	-25.30	.000	-25.08	.000	-24.88	.000
NP	Gingiva	15.14	.000	-5.75	.000	-5.68	.000	-7.01	.000	-6.32	.000	-5.22	.001
	Buccal	14.16	.000	-5.17	.000	-4.56	.000	-6.81	.000	-6.87	.000	-6.68	.000
CA/NA	Gingiva	35.67	.000	21.19	.000	22.65	.000	23.80	.000	21.88	.000	25.69	.000
	Buccal	17.66	.000	23.72	.000	20.60	.000	26.12	.000	28.83	.000	34.67	.000

Table 5: The significant values of the bivariate Pearson's correlation (r) for clinical symptoms and HbA1c with different cytomorphometric parameters for both buccal mucosa and gingiva.

Site	Parameters		CA	CP	NA	NP	CA/CN
Gingiva	HbA_{1c}	Pearson Correlation	-.330*	-.365**	.433**	.430**	-.601**
		Sig. (2-tailed)	.010	.004	.001	.001	.000
	Burning	Pearson Correlation			.334**	.331**	-.319*
		Sig. (2-tailed)			.009	.010	.013
	Xerostomia	Pearson Correlation		-.272*	.489**	.498**	-.587**
		Sig. (2-tailed)		.036	.000	.000	.000
Buccal	HbA_{1c}	Pearson Correlation			.615**	.609**	-.639**
		Sig. (2-tailed)			.000	.000	.000
	Burning	Pearson Correlation	-.315*	-.336**	.330*	.339**	-.464**
		Sig. (2-tailed)	.014	.009	.010	.008	.000
	Xerostomia	Pearson Correlation			.640**	.642**	-.577**
		Sig. (2-tailed)			.000	.000	.000
	Ulcer	Pearson Correlation	-.260*	-.279*			-.319*
		Sig. (2-tailed)	.045	.031			.013
*. Correlation is significant at the 0.05 level (2-tailed).							
**. Correlation is significant at the 0.01 level (2-tailed).							

Abbreviations: Hemoglobin A1c (HbA1c), nuclear area (NA) cytoplasmic area (CA), nuclear perimeter (NP), cytoplasmic perimeter (CP), ratio of cytoplasmic to nuclear areas (CA/NA)