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Cytomorphometric analysis of buccal mucosa of tobacco chewers

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Abstract

Objective: The objective of the present study was to assess the effect of tobacco chewing on buccal mucosa by using cytomorphometry. *Study design*: We compared the cellular diameter (CD), nuclear diameter (ND) and the ratio of nuclear diameter to cellular diameter (N/C) of buccal mucosa squames of normal subjects (N) with buccal mucosa squames of tobacco users without lesion (A), with tobacco-lime lesion (B), leukoplakia (C), and oral squamous cell carcinoma (D). The study group consisted of 125 patients divided into five groups (N, A, B, C and D) between the ages of 21 and 75 years. *Results*: The mean of the cellular diameter (in micrometers) of group N, A, B, C, and D was 72.86±5.26, 68.30±3.02, 62.13±3.29, 57.75±4.66, 54.51±4.66 respectively ($p<0.01$). The mean of the nuclear diameter (in micrometers) of group N, A, B, C, and D was 8.70±1.30, 8.98±1.08, 9.06±0.83, 9.12±1.06, and 11.04±1.46 respectively ($p<0.01$). The mean of the ratio of nuclear diameter to cellular diameter of group N, A, B, C, and D was 0.11±2.00, 0.13±1.82, 0.14±1.35, 0.16±3.11, 0.21±4.51 respectively ($p<0.01$). Univariate analysis of variance (ANOVA) showed a significant group effect for cellular diameter, nuclear diameter and ratio of nuclear diameter to cellular diameter. Multiple comparison tests by Tukey–HSD procedure revealed a significant decrease in the mean cellular diameter, increase in the nuclear diameter and ratio of nuclear diameter to cellular diameter. *Conclusions*: Cytomorphometric changes could be the earliest indicators of cellular alterations. There is progressive decrease in cellular diameter, increase in nuclear diameter and increase in ratio of nuclear diameter to cellular diameter in smears from all tobacco users, as compared to normal subjects. This indicates that there could be cause–effect relationship between tobacco and quantitative alterations.

Keywords: exfoliative cytology, cytomorphometry, oral cancer, tobacco.

⊟ Introduction

There is a geographic variation in the incidence of cancer of the head and neck among different countries of the world and among different regions within a country. This indicates that environmental factors may play an important role in the pathogenesis of cancer of the head and neck. Tobacco smoking and alcohol intake have been attributed to as major risk factors. In Asia, chewing tobacco causes a high incidence of oral cancers and in the US there have been reports of oral snuff as risk factor in oral cancer [1].

In the early stages, oral cancer may disguise itself and appear as a benign and asymptomatic lesion. Patients usually report to the clinician at a time when the tumor is at an advanced stage. Although a visible lesion precedes the development of majority of oral cancers, it may be possible for a tumor to develop within apparently normal appearing mucosa [2].

Exfoliative cytology, which is a simple, noninvasive diagnostic technique, could increase the chances of earlier detection of premalignant and malignant lesions [3]. In exfoliative cytology, the quantitative parameters are objective and reproducible; they may be important aids in the making of a cytopathologic diagnosis in such situations. One such quantitative parameter is morphometry. The smear obtained by exfoliative cytology can be analyzed quantitatively and qualitatively. With advancements in the field of quantitative oral exfoliative cytology, various parameters such as nuclear size, cell size, nuclear-to-cytoplasmic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to confirm the diagnosis [4]. Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions [5, 6]. The variations obtained in these parameters have been attributed to exposure to carcinogenic agents like tobacco. The concept of cellular or nuclear alteration on exposure to varying forms of tobacco can be best explained by reviewing the nature of cellular response to stimuli from the end products of different types of tobacco usage. Decrease in the cellular diameter and increase in the nuclear size are two significant morphologic changes that occur in actively proliferating cells [7].

Different forms of tobacco usage are prevalent in India, and many of them are specific to certain areas. The habit of placing tobacco mixed with lime; usually in the canine–premolar region of the mandibular sulcus is widespread in the rural population of Central Maharashtra, India. Taking this into consideration the present study has been carried out to assess the effect of tobacco chewing on buccal mucosa and compare the cytomorphology of cells collected from buccal mucosa of tobacco chewers with those of tobacco non-users.

⊟ Patients and Methods

The study group consisted of 125 patients divided into five groups:

▪ Group N: 25 subjects without tobacco chewing habit and without any lesion;

▪ Group A: 25 subjects with tobacco chewing habit but without any lesion;

▪ Group B: 25 subjects with tobacco chewing habit and tobacco-lime lesion;

▪ Group C: 25 subjects with tobacco chewing habit and leukoplakia;

▪ Group D: 25 subjects with tobacco chewing habit and oral squamous cell carcinoma.

The detailed information about the habit of tobacco chewing with lime (duration and frequency of the tobacco chewing habit) was recorded for each individual. Tobacco chewing was defined as the consumption of tobacco with lime for a minimum of 3–5 times per day for minimum period of 10 years.

Twenty-five individuals without tobacco chewing habits (group N) served as controls. In group A, 25 subjects with tobacco chewing habit but without any lesion were included. In group B, 25 subjects with tobacco chewing habit and tobacco-lime lesion were included. Tobacco-lime lesion is a yellowish white to brown lesion, which unlike leukoplakia could be scraped off (Figure 1) [8].

Figure 1 – *Clinical photograph of tobacco-lime lesion on buccal mucosa.*

In group C, 25 subjects with tobacco chewing habit and leukoplakia were included. Leukoplakia is defined by *WHO Classification* as predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion (Pindborg JJ *et al.*, 1997) (Figure 2) [9].

Figure 2 – *Clinical photograph of leukoplakia on buccal mucosa*

In group D, 25 subjects with tobacco chewing habit and oral squamous cell carcinoma were included. Squamous cell carcinoma is defined as "a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges" (Pindborg JJ *et al.*, 1997) (Figure 3) [9].

Figure 3 – *Clinical photograph of oral squamous cell carcinoma on buccal mucosa.*

The differentiation degrees were categorized according to Broders [10]. Among these 25 subjects, 14 were histopathologically diagnosed as well-differentiated squamous cell carcinoma and nine were diagnosed as moderately differentiated squamous cell carcinoma. (Clinical staging was not considered). All patients used in the study were with an age of 21 to 75 years and of both genders. Informed consent was obtained from all patients to obtain a cytological smear and blood sample. Each patient underwent routine venepuncture to determine the hemoglobin levels. The patients found to be anemic (i.e., female patients with the hemoglobin concentration of less than 11 mg/dL and male patients with the hemoglobin concentration of less than 12 mg/dL) were excluded from this study.

Scrapings were obtained by using a cytobrush moistened with normal saline. Using a gentle scraping motion, exerting little pressure, cells were scraped from the clinically normal appearing buccal mucosa of the study group N and A. In the B group, the entire lesion was scraped if possible. If not, a representative area was scraped. In the C group, in cases where a heavy keratinized surface was present, fissured or reddish areas were scraped to obtain the sample. In the D group, smears were obtained from the ulcerated or erythematous areas. The scrapings were smeared on to the center of glass slide, over an area of approximately 2.5×2.5 cm. The slides were immediately sprayed with commercially available spray fixative to ensure proper fixation. All cytological smears were stained by Papanicolaou staining technique using a commercially available staining kit–RapidPap™ (Biolab Diagnostics, Tarapur, Maharashtra).

Procedure

In order to measure an object under a microscope, two types of micrometers are required, Ocular Micrometer (OM) and Stage Micrometer (SM). A 10× eyepiece containing an ocular micrometer disc (Carl Zeiss) and 40× objective were calibrated with a stage micrometer (Carl Zeiss). (The projected values of eyepiece micrometer graduations vary with the optical combination used and consequently should be pre-calibrated before accurate measurements can be made).

In calibration, the stage micrometer was brought into focus and moved until one of the graduations corresponded exactly with one of the divisions of the eyepiece micrometer. The true distance (A) seen on the stage micrometer, which corresponded to the number of divisions (B) of the eyepiece micrometer disc, was then

read, and this true distance was divided by the number of divisions of the eyepiece micrometer giving the distance each division subtended (C = A/B × 10, 10 refers to the value of 1 SM division). The number of divisions covered by the specimen multiplied by the calibration constant (C) gave the diameter of the specimen.

In the present study, the cellular diameter (CD) and the nuclear diameter (ND) of the cells were measured using calibrated eyepiece (Ocular) micrometer. Measurements were obtained in both axes of the cells and the nuclei by superimposition of the eyepiece micrometer on the smear (Figures 4 and 5).
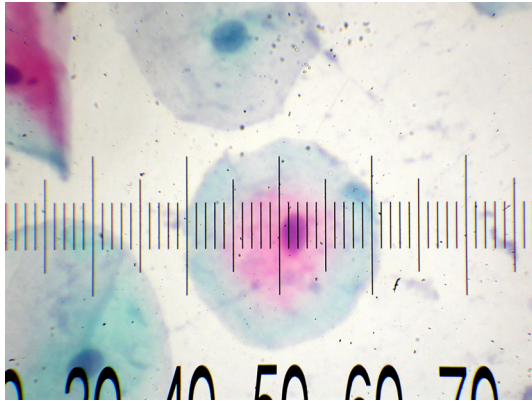

Figure 4 – *Photograph showing measurement of cellular diameter and nuclear diameter in X-axis (Papanicolaou staining, 400×).*
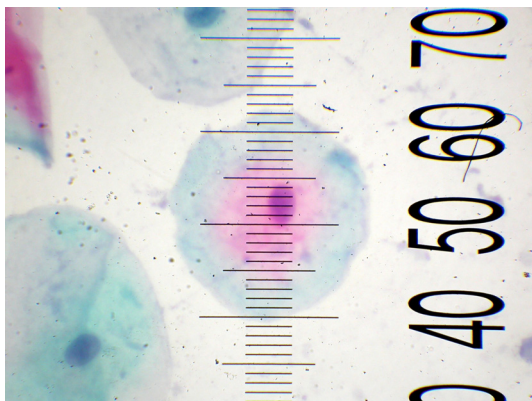

Figure 5 – *Photograph showing measurement of cellular diameter and nuclear diameter in Y-axis (Papanicolaou staining, 400×).*

The average of the values from axes was considered as the diameter of that cell and nucleus and was recorded. One hundred cells were selected from each slide and measured for cellular diameter and nuclear diameter, and recorded. The mean values of cellular diameter and nuclear diameter of all 100 cells were obtained and recorded. Only cells that were fully included in the field of vision and with clearly defined cellular and nuclear outlines were selected. Cells that were clumped or folded and cells with unusually distorted outline or nuclei were not considered for the analysis.

Analysis of variance (one-way ANOVA) was performed for the five groups to compare the mean of cellular diameter, nuclear diameter and ratio of nuclear diameter to cellular diameter. Comparison of the mean

values between groups was made using multiple comparison test by Tukey–HSD procedure, using the statistics package SPSS 10.0 for Windows.

Results

All the subjects in the study groups except group N practiced tobacco chewing habits for 10 years or more and minimum of 5–10 times/day (Table 1).

Table 1 – *Samples used for the study*

Group	Sample size	Sex		Age [years]		
		Male	*Female*	*<30*	*30–49*	*≥50*
N	25	12 (48%)	13 (52%)	5 (20%)	15 (60%)	5 (20%)
A	25	20 (80%)	5 (20%)	2 (8%)	15 (60%)	8 (32%)
B	25	25 (100%)	–	3 (12%)	16 (64%)	6 (24%)
C	25	21 (84%)	4 (16%)	6 (24%)	11 (44%)	8 (32%)
D	25	18 (72%)	7 (28%)	–	8 (32%)	17 (68%)

The cellular diameter, nuclear diameter and ratio of nuclear diameter to cellular diameter values plotted separately in the form of box and whisker plots are shown in Figures 6–8 respectively.
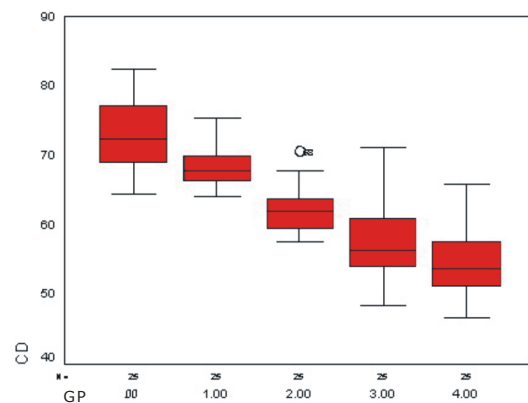

Figure 6 – *The box and whisker plots showing mean values of cellular diameter: 0.00 = Normal group (N); 1.00 = Without lesion group (A); 2.00 = Tobacco-lime group (B); 3.00 = Leukoplakia group (C); 4.00 = Oral squamous cell carcinoma group (D).*

Figure 7 – *The box and whisker plots showing mean values of nuclear diameter: 0.00 = Normal group (N); 1.00 = Without lesion group (A); 2.00 = Tobacco-lime group (B); 3.00 = Leukoplakia group (C); 4.00 = Oral squamous cell carcinoma group (D).*
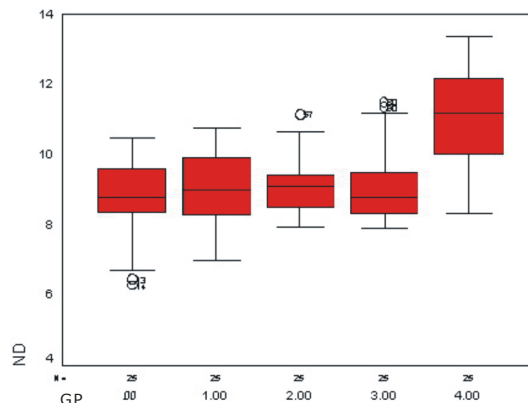
Figure 8 – *The box and whisker plots showing mean values of ratio of nuclear diameter to cellular diameter: 0.00 = Normal group (N); 1.00 = Without lesion group (A); 2.00 = Tobacco-lime group (B); 3.00 = Leukoplakia group (C); 4.00 = Oral squamous cell carcinoma group (D).*
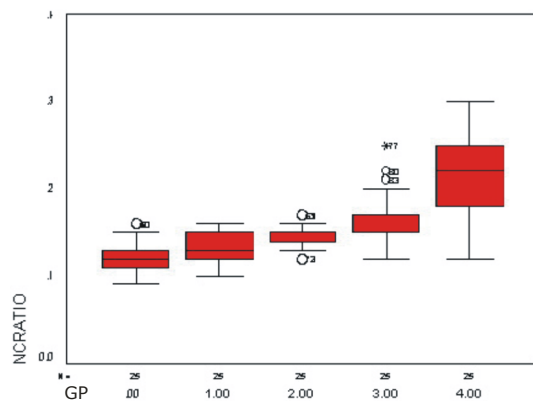
The cellular diameter was progressively reduced from normal (group N), through history of tobacco chewing but without lesion (group A), tobacco-lime lesion (group B) and leukoplakia (group C) to squamous cell carcinoma (group D) (Table 2).

Table 2 – *Mean of cellular diameter, nuclear diameter and ratio of nuclear diameter to cellular diameter*

Group	Total no. of cases	Cellular diameter ± SD [µm]	Nuclear diameter ± SD [µm]	Ratio of nuclear diameter to cellular diameter ± SD [µm]
N	25	72.86±5.26	8.70±1.30	0.11±2.00
A	25	68.30±3.02	8.98±1.08	0.13±1.82
B	25	62.13±3.29	9.06±0.83	0.14±1.35
C	25	57.75±6.02	9.12±1.06	0.16±3.11
D	25	54.51±4.66	11.04±1.46	0.21±4.51

When multiple comparisons of cellular diameter using Tukey–HSD test was done, the different groups showed statistically significant difference from each other except for group C and D, which did not show any statistically significant difference between the cellular diameter (Table 3).

Table 3 – *Multiple comparisons of cellular diameter using Tukey–HSD test*

| Group | Mean difference (*p*-value) | | | | |
	N	A	B	C	D
N					
A	4.55 (<0.01)				
B	10.73 (<0.01)	6.17 (<0.01)			
C	15.11 (<0.01)	10.55 (<0.01)	4.38 (<0.01)		
D	18.34 (<0.01)	13.79 (<0.01)	7.61 (<0.01)	3.23 (>0.05)	

The nuclear diameter showed a progressive increase in the mean nuclear diameter from normal (group N), through history of tobacco chewing but without lesion (group A), tobacco-lime lesion (group B) and leuko-plakia (group C) to squamous cell carcinoma (group D) (Table 2). When mean nuclear diameter was compared amongst different groups, the mean difference of group D and group N, A, B, and C was found to be statistically

significant (*p*<0.01). Whereas the mean difference between group D and group A, group B, group C, was not found statistically significant (*p*>0.05) (Table 4).

Table 4 – *Multiple comparisons of nuclear diameter using Tukey–HSD test*

| Group | Mean difference (*p*-value) | | | | |
	N	A	B	C	D
N					
A	0.27 (>0.05)				
B	0.35 (>0.05)	7.76 (>0.05)			
C	0.41 (>0.05)	0.14 (>0.05)	6.40 (>0.05)		
D	2.33 (<0.01)	2.05 (<0.01)	1.98 (<0.01)	1.91 (>0.01)	

The ratio of nuclear diameter to cellular diameter showed a progressive increase in the mean from normal (group N), through history of tobacco chewing, but without lesion (group A), tobacco-lime lesion (group B) and leukoplakia (group C) to squamous cell carcinoma (group D) (Table 2). When mean ratio of nuclear diameter to cellular diameter of group N (0.11± 2.00) was compared with group A, B, C and D, the mean difference of group N and group B, group C, group D was found to be statistically significant (*p*<0.01). No statistical significance was found between group N and group A, between group B and group C and between group A and group B (Table 5).

Table 5 – *Multiple comparisons of ratio of nuclear diameter to cellular diameter using Tukey–HSD test*

| Group | Mean difference (*p*-value) | | | | |
	N	A	B	C	D
N					
A	1.44 (>0.05)				
B	2.84 (<0.01)	1.40 (>0.05)			
C	4.68 (<0.01)	3.24 (<0.01)	1.84 (>0.05)		
D	9.72 (<0.01)	8.24 (<0.01)	6.88 (<0.01)	5.04 (<0.01)	

Univariate analysis of variance (ANOVA) showed a significant group effect for cellular diameter, nuclear diameter and ratio of nuclear diameter to cellular diameter. Multiple comparison tests by Tukey–HSD procedure revealed a significant decrease in the mean cellular diameter, increase in the nuclear diameter and ratio of nuclear diameter to cellular diameter.

⊟ Discussion

All of the major forms of tobacco use like cigarettes, cigars, pipes and smokeless tobacco (chewing tobacco and snuff) are known to cause oral cancer. This is evidenced by the magnitude of the risks associated with greater amounts or longer duration of tobacco usage and the consistency of the findings for oral cancer across numerous cultures [11]. Different forms of tobacco usage are prevalent in India, and many of them are specific to certain areas [8].

Users of smokeless tobacco exhibit oral cancer preferentially in areas where the quid is held, that is the

buccal or alveolar surfaces. The habit of placing tobacco mixed with lime (Khaini) in the mandibular sulcus, usually in the canine premolar region is widespread in the rural population of Central Maharashtra, India (Bhonsle RB *et al.*, 1979) [8]. It is a thick, yellowish white intra oral lesion, occasionally with loose tags of tissue that occurs at the site where tobacco-lime is placed in combination as a quid and was seen more often among men especially in premolar region. Bhonsle RB *et al.* (1979) suggested that the tendency for the superficial layers of the lesion to be scraped off is probably due to the caustic action of the mixture (pH 8.3). They found the prevalence of tobacco-lime lesion among 101 761 Maharashtrian villagers to be 2.9%, which is four times more common than leukoplakia (0.67%). Therefore, they state that this particular lesion is a specific entity with consistent histological features among the tobacco-lime users [8].

During transformation of normal tissue to premalignancy or malignancy, cellular changes occur at the molecular level before they are seen under the microscope and much before clinical changes become evident. Identification of high-risk oral premalignant lesions and intervention at premalignant stages could constitute one of the keys in reducing the mortality, morbidity and cost of treatment associated with oral squamous cell carcinoma. In addition, certain patients are known to be at high risk for head and neck cancer, specifically those who use tobacco or alcohol and those over 45-year-old. Such patients can be screened by clinical examination, as early-stage disease, if diagnosed, is curable [12].

Tobacco induced mucosal changes have been identified in exfoliated cells. The morphology of the exfoliated cells depends on the nature of the changes taking place in the epithelial layer; conversely, alteration in cytological pattern may be attributed to the changes occurring in the epithelial layer. Applying this possibility, exfoliative cytological techniques have been applied to examine the effect of tobacco on the oral mucosa [13–16].

The smear obtained by exfoliative cytology can be analyzed quantitatively and qualitatively [4]. With advancements in the field of quantitative oral exfoliative cytology, various parameters such as nuclear size, cell size, nuclear-to-cytoplasmic ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to confirm the diagnosis accurately [17]. Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions [5, 6]. In addition, it has been demonstrated that exfoliative cytology is valuable for monitoring clinically suspect lesions and malignant lesions after definitive treatment [3, 18]. Cowpe JG *et al.* (1985) demonstrated that exfoliative cytology is capable of detecting malignant changes through estimation of ratio of nuclear size to cytoplasmic size, using planimeter method in Papanicolaou-stained smears [5, 12, 19, 20]. Since then a number of studies had been carried out using the quantitative cytomorphometric techniques to evaluate the influence of diverse systemic and external factors on cellular size, nuclear size and ratio of nuclear size to

cellular size. Ramaesh T *et al.* (1998) used cytomorphometric techniques to assess nuclear diameter and cellular diameter in normal oral mucosa, in dysplastic lesions, and in oral squamous cell carcinoma. They found that cellular diameter was highest in normal mucosa, lower in dysplastic lesions and lowest in oral squamous cell carcinoma. By contrast, nuclear diameter was lowest in normal mucosa, higher in dysplastic lesions, and highest in oral squamous cell carcinoma. These studies suggested that reduced cell size and increased nuclear size are useful early indicators of malignant transformation, and thus exfoliative cytology is of value for monitoring clinically suspect lesions and for early detection of malignancy [21]. Recently, in 2005, Einstein TB and Sivapathasundharam B reported cytomorphologic alterations in the form of reduction in cellular diameter and increase in nuclear diameter in buccal squames of tobacco users in the south Indian population [4].

Our study showed significant quantitative alterations in the form of decreased cellular diameter, increased nuclear diameter and increase in ratio of nuclear diameter to cellular diameter in the A, B, C, and D groups, compared to N group. This significant progressive reduction in cellular diameter shows that the reduction in cell size could be an early indication of malignant change, as suggested by Cowpe JG *et al.* [6].

Increase in the nuclear diameter could be due to increased DNA content of the nucleus. Increase in ratio of nuclear diameter to cellular diameter is due to the changes in nuclear size and cytoplasm, same as reported by Cowpe JG *et al.* [6]. Franklin CD and Smith CJ (1980) reported that the N:C ratio has the advantage of relating nuclear volume to cytoplasmic volume and possibly represents the significant changes that occur in the cell, more accurately at a morphological level [22].

These observations suggest that tobacco chewing is responsible for the significant cellular and nuclear alteration in the A, B, C, and D groups. In group A, that is tobacco users but without any lesion, though the oral mucosa appears clinically normal, the mean difference of cellular diameter shows statistically significant difference as compared to group N, B, C, and D. This indicates that the alterations are probably due to changes at the molecular level, which is not apparently appreciable at the clinical level.

This study has assessed only the quantitative changes associated with effect of tobacco chewing and the results show that the alterations similar to those occurring in histopathological sections of premalignant and malignant lesions are observed in the exfoliated buccal squames of tobacco chewers. Such alterations would have resulted from an increased cellular activity. Although there is decrease in cellular diameter and increase in nuclear diameter and ratio of nuclear diameter to cellular diameter in buccal mucosa squames of tobacco users without lesion and tobacco–lime lesions, it is inappropriate to state that the cytological alterations seen in the squames of the tobacco-chewing patients indicate an impeding premalignant or malignant lesion, unless specific markers are employed to demonstrate them. This study, therefore, confirms only the cause–effect

relationship between tobacco chewing and quantitative cellular and nuclear alterations. In study groups N, A, B, and C the mean values of cellular diameter and nuclear diameter differed significantly from the values obtained for the oral squamous cell carcinoma group (D).

⊟ Conclusions

Our study thus elucidates the importance of early recognition of cellular alterations for identification of individuals who require early intervention even in the absence of visible changes of mucosal surface. Application of quantitative techniques to smears obtained from oral premalignant and malignant lesions and suspicious area with clinical lesion, can possibly improve the diagnostic value of oral exfoliative cytology. Hence we emphasize that cytomorphology is an invaluable parameter to assess the influence of tobacco on buccal mucosa. Our study restricts itself to performing linear measurements on exfoliative cytology and hence, we propose that further studies using automated image analysis need to be carried out.

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