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Original Article

The role of tobacco as an etiological agent for oral cancer: Cytomorphometrical analysis of the buccal mucosa in tobacco users

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ABSTRACT

Background: Histopathological diagnosis of lesions arising from the intake of tobacco is based on subjective evaluation of morphological alterations within the lesional tissue. Oral exfoliative cytology is a non-invasive diagnostic technique for early detection of oral premalignant and malignant lesions. Morphometric techniques have been advocated as objective and reproducible methods of detecting changes before they are visible by routine microscopy and can facilitate differentiation of normal and abnormal epithelium. This study was conducted to assess the morphometric parameters (cell diameter, nuclear diameter and nuclear cytoplasmic ratio [N:C ratio]) in tobacco smokers and chewers and to evaluate the variations, if any.

Materials and Methods: The study was conducted on cytological smears obtained from oral lesions of patients with habit of tobacco smoking (Group B) and tobacco chewing (Group C). Group A comprised of subjects free from oral lesions and not using tobacco in any form. Patients with both the habits were excluded. The smears were stained using Papanicoloaou staining method. For morphometric analysis, Microimage 3.0 image analysis software was employed. The statistical test employed was an analysis of variance and $P < 0.05$ was considered as significant.

Results: The results of this study showed that the cellular diameter was progressively reduced and nuclear diameter progressively increased from Group A to Group B to Group C. The N:C ratio also showed a progressive increase from Group A to Group C.

Conclusion: The results confirmed that tobacco chewing and smoking influenced the cytomorphology of normal appearing buccal mucosa and the degree of these changes were found to be greater in chewers as compared to smokers.

Key Words: Morphometry, papanicoloaou stain, tobacco chewers, tobacco smokers

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INTRODUCTION

Oral cancer, a tobacco related disease,[1] represents 2-4% of malignancies in the western population and accounts for almost 40% of all cancers in Indian subcontinent.[2] Although a visible lesion precedes the development of majority of oral cancers, a tumor may develop directly from within an apparently normal appearing mucosa.[3] Routine cytology could increase the chances of earlier detection of such lesions. In addition, the collection of multiple smears from large areas of clinically abnormal mucosa could provide a valuable diagnostic indicator, as an adjunct to clinical judgment, each time patient attends for review; with no or minimum distress to the patient.[4]

Oral exfoliative cytology is simple but non-invasive diagnostic technique for early detection of oral premalignant and malignant lesions.[5] In addition, it has been demonstrated that exfoliative cytology is valuable for monitoring clinically suspect lesions and malignant lesions after definitive treatment.[6]

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Morphometry may be used to determine quantitative parameters such as nuclear size, cell size, nuclear to cytoplasmic ratio, nuclear shape, optical density and nuclear texture in order to establish the diagnosis of malignancy. Among these parameters, cellular diameter, increase in nuclear size and increased nuclear cytoplasmic ratio (N:C ratio) are the significant morphologic changes that occur in actively proliferating cells.[7] The Papanicoloaou technique has been widely used in exfoliative cytology as it imparts a different color to the cytoplasm of epithelial cells based on the degree of cellular differentiation.[8] The biological activity of any cell is best reflected in the nucleus and the functional activity in the cytoplasm.[9] The present study was thus planned to compare and to analyze the cytomorphometric changes in the exfoliated squamous epithelial cells of persons using tobacco in different forms and those not using tobacco in any form.

This study evaluated the cytomorphometric changes in the exfoliated cells of persons using different forms of tobacco irrespective of whether oral tobacco associated lesions were present or not. The presence of cytological changes in the absence of oral lesions would emphasize the need for screening tests to be carried out in all tobacco users to enable early detection of cellular changes which precede the development of oral malignancies.

MATERIALS AND METHODS

The sample for this study included patients attending the Outpatient Department of D J college of Dental Sciences and Research, Modinagar. Informed consent was obtained from each patient. All procedures followed were in accordance with the ethical standards of the institutional committee on human experimentation and with the Helsinki Declaration.

Patients with a habit of both smoking and chewing tobacco were excluded from the study. Patients under chemotherapy, radiotherapy, recent viral infection, diabetes mellitus, with history of alcohol consumption were also excluded from this study. The study groups were divided into 3 subgroups as given below:

1. Group A (control group): 25 subjects with mean age of 45 years (range: 30-60), free from oral lesions and not using tobacco in any form.
2. Group B (smoking tobacco group): 25 subjects with a mean age 45 years (range: 30-60), with or without oral lesions and history of smoking tobacco (up to 10-15/day).
3. Group C (Chewing tobacco group): 25 subjects with a mean age 45 years (range: 30-60) with or without oral lesions and history of chewing tobacco (up to 10-15/day).

The area to be smeared was first wiped clean of excessive saliva and surface debris. Scraping of surface cells from the selected area was obtained with a moistened wooden spatula and the material obtained was immediately spread on a fresh, clean and dry slide. The slides were fixed with alcohol and stained using the Rapid Pap kit (Biolab Diagnostics, Tarapur, Maharashtra, India), following the manufacturer's instructions.

All samples were analyzed by using Motic BA 400 microscope (magnification ×40) connected to image analysis system (Microimage 3.0) running on a HP pavilion dv2000 laptop, Intel(R) CPU, T2050 @ 1.60 Ghz, 798 MHz, 504 MB of RAM. Slides were scanned under ×4 magnification followed by observation under ×10 and then ×40 magnifications for cellular diameter and nuclear diameter. Measurements were obtained from 100 cells in both axes for the cell and for the nucleus. The average of the values from both axes was considered as the diameter of the cell and the nucleus and was recorded. The mean values of cell diameter and nuclear diameter of all 100 cells were calculated and recorded. Only cells with clearly defined cellular and nuclear outlines were included for morphometric analysis. Cells that were clumped or folded or distorted were not considered for the analysis. Analysis of variance (one-way ANOVA) was performed for three groups to compare the mean of cellular diameter, nuclear diameter and N:C ratio. Comparison of the mean values between groups was made using Student's *t*-test, employing the statistics package SPSS 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The study was conducted on 75 individuals between the ages of 30 and 60 years, out of which 52 were males and 23 were females. The results of this study showed that the cellular diameter was progressively reduced from Group A with mean 188.8 μ, to Group B with mean 155.78 μ to Group C with mean 143.41 μ. [Figure 1], whereas the nuclear diameter showed a progressive increase from Group A with mean 32.15 μ, to Group B with mean 36.66 μ, to Group C with mean 41.61 μ [Figure 2]. The

photomicrographs showing the measurements of nuclear and cellular diameters using image analysis in Group A, B and C have been depicted in Figures 3-5 respectively.

The N:C ratio also showed a progressive increase from Group A with mean 0.17 μ, to Group B with mean 0.23 μ to Group C with mean 0.29 μ [Table 1].

ANOVA showed a significant group effect for cellular diameter, nuclear diameter and N:C ratio. Multiple comparison tests by Students *t*-test revealed a significant decrease in the mean cellular diameter [Table 2], increase in the nuclear diameter [Table 3] and increase in N:C ratio [Table 4] from Group A, to Group B to Group C ($P < 0.05$).

Table 1: Comparison of ratio of nuclear to cellular diameter of all patients among various groups (μ)

S. No.	A	B	C
1	0.1529	0.2028	0.2483
2	0.1965	0.2686	0.2823
3	0.1952	0.2383	0.1922
4	0.1888	0.2711	0.2935
5	0.1878	0.2069	0.2675
6	0.1656	0.2658	0.2627
7	0.1898	0.2689	0.3030
8	0.1892	0.1938	0.2679
9	0.1823	0.2639	0.2912
10	0.1922	0.2316	0.2950
11	0.1839	0.2400	0.3925
12	0.1833	0.2639	0.3557
13	0.1575	0.2399	0.3289
14	0.1505	0.2331	0.3514
15	0.1529	0.2192	0.1998
16	0.1528	0.1984	0.2845
17	0.1782	0.2221	0.2950
18	0.1657	0.2279	0.2486
19	0.1559	0.2290	0.3196
20	0.1703	0.2294	0.3557
21	0.1724	0.2074	0.2823
22	0.1566	0.2380	0.2669
23	0.1325	0.2639	0.3031
24	0.1764	0.2641	0.2894
25	0.1629	0.2383	0.3030
Mean	0.1717	0.2371	0.2911
Standard deviation	0.0172	0.0242	0.0454

Table 2: Multiple comparisons of cellular diameter using student's *t*-test

t-test	Group A and B	Group A and C	Group B and C
t value	6.138580	8.483812	2.848307
P value	0.000000	0.000000	0.000000
Significance	$P < 0.05$	$P < 0.05$	$P < 0.05$

DISCUSSION

Oral cancer, a tobacco related disease,[1] represents 2-4 % of malignancies in the western population

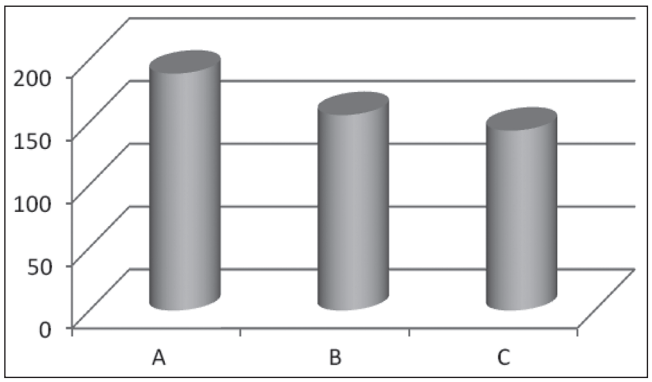

Figure 1: A comparative distribution of mean cellular diameter among various groups

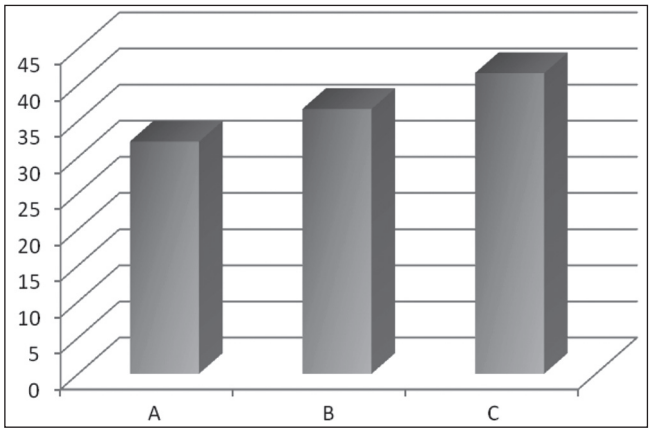

Figure 2: A comparative distribution of mean nuclear diameter among various groups

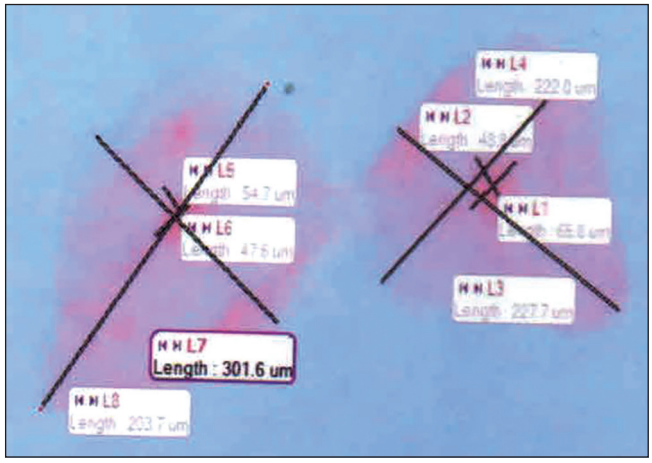

Figure 3: Cytomorphometry: Measurements of nuclear and cytoplasmic diameters of oral epithelial cells from control group (Group A)

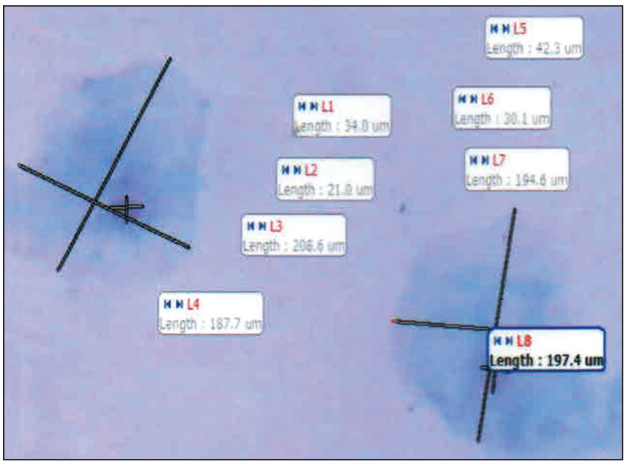

Figure 4: Cytomorphometry: Measurements of nuclear and cytoplasmic diameters of oral epithelial cells from tobacco smoking group (Group B)

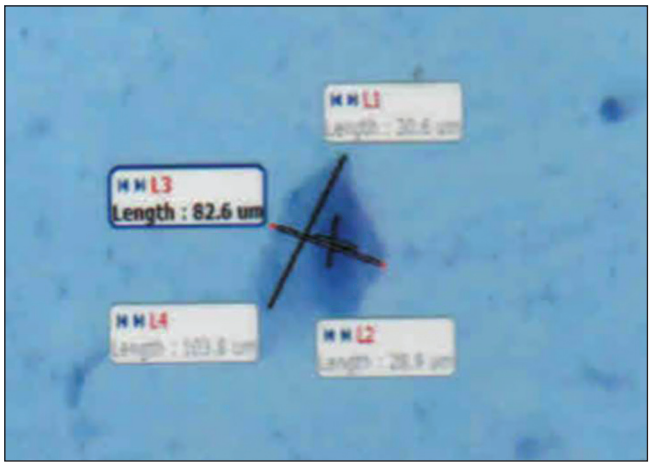

Figure 5: Cytomorphometry: Measurements of nuclear and cytoplasmic diameters of oral epithelial cells from tobacco chewing group (Group C)

Table 3: Multiple comparisons of nuclear diameter using student's *t*-test

t-test	Group A and B	Group A and C	Group B and C
t value	6.020355	6.012638	3.131352
P value	0.000000	0.000000	0.000000
Significance	$P < 0.05$	$P < 0.05$	$P < 0.05$

Table 4: Multiple comparisons of ratio of nuclear to cell diameter using student's *t*-test

t-test	Group A and B	Group A and C	Group B and C
t value	11.006866	12.287647	5.251223
P value	0.000000	0.000000	0.000000
Significance	$P < 0.05$	$P < 0.05$	$P < 0.05$

and accounts for almost 40% of all cancers in Indian subcontinent.

During the transformation of normal tissue to premalignant or malignant tissue, cellular changes occur at the molecular level, much before any clinical or even histological changes are evident. Identification of high risk individuals even before any potentially malignant lesions are present in the oral mucosa could constitute one of the keys in reducing the mortality, morbidity and cost of treatment associated with oral squamous cell carcinoma.[2]

Increased cellular activity is characterized by morphologic changes such as nuclear membrane undulation, hyperchromatism, enlarged and prominent nucleoli, increased mitosis and multinucleation. Decrease in the cellular diameter and increase in the nuclear diameter are two significant morphologic changes that occur in actively proliferating cells. The amount of cytoplasm that a cell makes decreases relative to the amount of nucleoplasm, so that when there is an actual increase in the nuclear size, the cell diameter decreases. The increase in the size of the nucleus may be related to the increased amount of deoxyribonucleic acid (DNA) required for cell replication. As a result, the nuclear to cytopalsmic ratio increases at times to an extreme degree.[10] Tobacco induced mucosal changes have also been identified in exfoliated cells, which are indicative of the changes that have taken place in the epithelial layer.

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, N:C ratio, nuclear shape, nuclear discontinuity, optical density and nuclear texture can be evaluated collectively in order to confirm the diagnosis. Of all the parameters, the nuclear size, cytoplasmic size and the N:C ratio have been shown to be statistically significant in the evaluation of oral lesions.[7,11]

The present study was carried out to assess the effect of tobacco on cells of buccal mucosa by comparing cytomorphology of cells collected from buccal mucosa of those smoking tobacco or chewing tobacco with those not using tobacco in any form. Smears obtained from the groups showed variation in the size of the cells and nuclei. Such a variation in the size of the cells and nuclei is in agreement with that reported for normal buccal squames.[12]

The reliability of the different instruments used in oral exfoliative cytology has been analyzed in different studies. The instrument used for making cytologies should be easy to use in any location, cause little discomfort to the patient and provide an adequate number of epithelial cells.[13] In this study, we used the wooden spatula and found it to be an adequate instrument due to the ease in sampling and the good quality of oral cytologic sample obtained as evidenced by the significant number of basal cells in the smears obtained.

This study showed significant quantitative alterations in the form of decreased cellular diameter, increased nuclear diameter and increase in N:C ratio in the tobacco smokers group as compared to the control group. The cellular diameter values showed a progressive reduction from the control group to the tobacco chewing habit. This reduction was found to be statistically significant. This is in agreement with studies, which state that the use of tobacco influences the cytomorphology of the normal buccal mucosa.[11] They observed a significant reduction in cellular diameter in tobacco users when compared with non-users.[11] This significant progressive reduction in cellular diameter shows that the reduction in cell size could be an early indication of malignant change.[14]

The nuclear diameter values showed a progressive increase from the control group to the tobacco smoking habit, to the tobacco chewing habit, which was found to be statistically significant. Cowpe *et al*.[11] in their study also reported an increase in nuclear diameter in tobacco users as compared to nonusers. This increase in the nuclear diameter could be due to increased DNA content of the nucleus.[12]

Various studies also suggest that increased nuclear diameter and reduced cellular diameter are useful early indicators of malignant transformation and thus suggest that exfoliative cytology is of value for monitoring clinically suspect lesions and for early detection of malignancy.[15,16] However, cytopathologists sometimes find the distinction between reactive and malignant cells extremely difficult. Thus, there has been a steady decline in popularity of exfoliative cytology as a method of choice due to the preponderance of false negative results. This is because subjective assessment of size is less accurate than overall pattern recognition. Since quantitative parameters are objective and reproducible, they may be important aids in cytopathologic diagnosis in such situations.[17-19]

Cowpe *et al*. found that the nuclear size varied significantly with advancing age, but the variation was greater among the older age groups when compared to the younger age groups.[11] In this study, the correlation of age with various cytomorphological parameters could not be established due to a small number of patients in different age groups.

This study also evaluated N:C ratio in all the groups. We found a progressive increase from the control group, to the tobacco smoking group and to the tobacco chewing group. This increase was found to be statistically significant. These observations suggest that tobacco smoking and chewing is responsible for significant cellular and nuclear alterations.

The increase in N:C ratio is due to the changes in nuclear size and cytoplasm. The N:C ratio has the advantage of relating nuclear volume to cytoplasmic volume and possibly represents significant changes that occur within the cell at a morphological level.[11]

Remmerbach *et al*. analyzed the reliability of oral cytology and its cytometric analysis in the early detection of oral cancer. The results of the study showed that the sensitivity of the cytology was 94.6% and specificity 99.5% respectively.[20]

According to Center for Disease Control, chewing tobacco used 7-8 times a day may be equivalent to smoking 30-40 cigarettes/day. Though the nicotine content of chewing tobacco is lower than the smoking form, it is said to have an increased carcinogenic potential because it remains in contact with the oral mucosa for longer periods of time.[21]

This study showed statistically significant difference in the nuclear diameter, cellular diameter and N:C ratio of tobacco smoking, tobacco chewing and non-tobacco user patients. It was also observed that chewing tobacco group showed a significant increase in nuclear diameter, decrease in cell diameter and increase in N:C ratio as compared to other groups. These alterations are probably due to changes at the molecular level, which may be appreciable at the clinical level.

We also detected a variety of epithelial cells including basal, spinous and superficial cells in the smears. The cytomorphometric alterations appeared

to be generalized rather than restricted to a certain generation of cells.

Earlier studies have shown that there are various factors such as alcohol,[22] radiotherapy,[23] anemia,[24] chemotherapy and nutritional deficiencies, which influences the cytomorphology of cells. Therefore, this study only included patients who were not anemic and had not given any history of radiotherapy, chemotherapy or history of habitually drinking alcohol. Thus, any compounding effect of these parameters was ruled out. This study assessed only the quantitative cellular changes associated with tobacco smoking and chewing. The dysplastic changes seen in the histopathology of premalignant and malignant lesions are well defined.[25]

In this study, the mean values of cellular diameter and nuclear diameter in tobacco smoking and chewing groups differed significantly from the values obtained from the control group. However, no statistically significant baseline could be drawn that indicates the presence of premalignancy based on quantitative measurement from the results because of limitation of sample size. The nuclear diameter, cellular diameter and the N:C ratio can be regarded as early indicators of alterations occurring in cells due to tobacco usage.

This study evaluated only linear measurements and did not explore the ploidy status of the cells or the three dimensional changes in the cells. However, the cell diameter and nuclear diameter were found to be important objective parameters, which could be used as an important aid in making a cytopathologic distinction between cells from buccal mucosa and cells from dysplastic oral lesions.

The results of this study emphasize the importance of early recognition of cellular alterations even in the absence of visible changes of the mucosal surface.

CONCLUSION

This study assessed the effect of tobacco on the buccal mucosa by cytomorphometry. There were significant quantitative alterations like decreased cellular diameter, increased nuclear diameter and increase in N:C ratio in tobacco smoking group, tobacco chewing group when compared to the control group. This indicates that cytomorphometric changes could be the earliest indicators of cellular alterations. Hence, cytomorphometric analysis of oral smears can be carried out regularly in order to detect alterations in the cellular and nuclear dimensions especially among tobacco users. Furthermore, this method can also aid in motivating individuals to withdraw from tobacco use.

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