ORIGINAL ARTICLE

Cytomorphometrical Analysis of Exfoliated Buccal Mucosal Cells: Effect of Smoking

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Introduction: Exfoliative cytology is a non-aggressive, non-invasive procedure with higher patient compliance and is therefore, an attractive technique for the early diagnosis of oral lesions. The purpose of this study is to evaluate and compare cytological changes using morphometric analysis of the exfoliated buccal mucosal cells in smokers, with results obtained for non-smokers. **Methods:** Smears were collected from the clinically normal buccal mucosa of 120 individuals. Age range of subjects taken was 40-60 years. Smears were then stained with Papanicolaou stain. **Results:** Mean NA for smokers was significantly elevated compared with the mean NA for non-smokers. Mean CA in smokers was decreased as compared to non-smokers but the difference was not significant. Also, N/C ratio was significantly elevated in smokers group. With increasing heavy exposure in duration of years, Cytomorphometric changes show significant altered values for all three measured parameters (NA, CA and N/C ratio). **Conclusion:** Increase in NA and decreased CA as well as altered N/C ratio would appear to be due to smoking tobacco. Cytomorphometric analysis can be used regularly to detect these cell alterations. This method can also aid in motivating individuals to withdraw from adverse effects of tobacco smoking. Currently, use of exfoliative cytology has increased as an adjunct to screening of precancerous lesions and malignancies of the oral cavity.

Keywords: Cytomorphology, Exfoliative cytology, Oral mucosa, Tobacco smoking

INTRODUCTION

Geographic variations exist among different countries of the world for the incidence of cancer of the head and neck, also among different regions within a country. It indicates that environmental factors may play a vital role in the pathogenesis of these malignancies. Tobacco smoking and alcohol intake have been attributed to as major risk factors. Strong association between cancers of the oral cavity and pharynx with the use of tobacco in any form is well established. Epidemiological studies show that the risk of developing oral cancer is five to nine times greater for smokers than for non-smokers.[1,2]

However incidence of head and neck cancers in some communities are decreasing, the incidence of oral cavity cancers has not fallen in recent years, one reason of which is the increased use of cigarettes and tobacco in those communities.[3] Despite the implementation of cancer prevention programs in some countries and the fact that the oral cavity is an accessible area (so the patients can easily examine their oral cavities), the majority of oral cavity cancers are diagnosed at advanced stages, resulting in poor prognosis and survival rate among patients.[4,5] In addition, the morbidity and mortality rates of oral cancer have risen despite advances in therapeutic techniques, leading to increased treatment costs and complications. Hence, the early diagnosis of oral cavity cancers is of immense value in successful treatment of patients.[1] In some apparently healthy smokers, changes are observed in the frequency of epithelial cell proliferation, the size of nucleus and the size of nucleus in relation to cytoplasm. In addition, an increase number of keratinized cells are also observed.[6-8]

Exfoliative cytology is the microscopic examination and measurements of shed or desquamated cells from the surface epithelium usually the mucous membrane. Those cells that have been collected by scraping the tissue surface or collected from body fluids such as sputum, saliva, etc. are also studied. Continuous exfoliation of epithelial cells is a part of physiological turnover. Deeper cells which are strongly adhered in normal conditions become loose in the cases of malignancies and tends to exfoliate or shed along with superficial cells.[9]

The role of exfoliative cytology in the detection of oral neoplasms has created various controversies. Few researchers say that oral exfoliative cytology is a simple, non invasive, less time consuming procedure with sensitivity of 89% and specificity of 89.5% while

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others strongly criticize that there is no role for exfoliative cytology in early cancer detection since it is not 100% sensitive. Even though exfoliative cytology is not 100% accurate, it has its own potential value in cases where biopsy is contraindicated like in systemically compromised patients, inaccessible areas, recurrent malignancies and in mass screening. 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. Of these parameters, the nuclear size, cytoplasmic size and their ratio have been shown to be significant in the evaluation of oral lesions.[10-13]

Exfoliative cytology is a non-aggressive, non-invasive technique with higher patient compliance and is therefore, an attractive technique for the early diagnosis of oral malignancies, including epithelial atypia and squamous cell carcinoma. However it has limited usage so far due to poor sensitivity and specificity in diagnosing oral malignancies. The purpose of this study is to evaluate and compare cytological changes using morphometric analysis of the exfoliated buccal mucosal cells in smokers, with results obtained for non-smokers. This technique might yield important information about the influence of tobacco upon nuclear area (NA) and cytoplasmic area (CA), particularly since these latter two variables are known to alter within dysplastic tissue for which smoking is a potential etiologic factor.

METHODS

This is Hospital based case-control analytical type of observational study to observe cytomorphological changes in exfoliated buccal mucosal cells of smokers. Smears were collected from the clinically normal buccal mucosa of 120 individuals. Age range of subjects taken was 40-60 years. These patients were attending the outpatient department of SMS medical college and associated group of hospitals, Jaipur, Rajasthan, India. Name, age, occupation and relevant medical history (including whether they smoked) were recorded. In addition hemoglobin and full blood counts were carried out for each patient, to exclude anemia.

Ninety subjects were placed in the smoker group and thirty others in the non-smokers group. Women were not included in the study due to cellular changes that occurs during menstruation, after menopause and also due to the possibility of pregnancy and other hormonal changes.[14] Furthermore, only those greater than 40 years of age were included in the study, since this is the age group in which cancer of the buccal mucosa is associated.[15]

Patients for the study group were selected because they fulfilled the following criteria:

1. Smoked at least 20 cigarettes, 3 cigars or 3 pipes per day for at least 5 years.
2. Did not suffer from systemic diseases such as anemia or diabetes.
3. Had not received radiotherapy and/or chemotherapy in the last 6 months.
4. Did not consume alcoholic drinks or using any drugs affecting the oral epithelium.

Neither the smokers nor non-smokers had any oral lesions, systemic disease or even any histopathological dysplasia in microscopic evaluation. The smears were taken from clinically normal buccal mucosa. Non-smokers were defined by no use of cigarette or any addictive material and smoke-producing substances during the preceding year. Sampling was carried out from 9 to 11 a.m to exclude possibility of diurnal variations. Informed consent was obtained from all the patients in the study. All the patients filled out a form and specified their age, the frequency and duration of their smoking, and diseases, if any or any other relevant medical history.

90 patients of smokers group were categorised in three subgroups based upon duration of history of smoking habit. Each group consists of 30 patients.

Group A: 5-10 years history of exposure to smoking but not less than 5 years.
Group B: 11-20 years.
Group C: More than 20 years of exposure history.

Oral examinations were performed using a mouth mirror and artificial light. Subjects were asked to rinse their mouth with water and a pre moistened wooden spatula was then scraped firmly across the mucosa and the cells transferred to a dry glass slide fixed in 95% ethanol, followed by washing in running tap water for a further hour. Smears were then stained with Papanicolaou stain.

Then stained slides were subjected to research microscopy. Fifty randomly selected cells were measured in a stepwise manner moving the slide from the right upper corner to left and then down to avoid measuring the same cell twice. Only clearly defined cells were measured, excluding the clumped or folded cells and unusually distorted nuclei and cells.

Cytomorphometric analysis was done by using Image J v 1.45 image analysissoftware. The nuclear (NA) and cytoplastmic (CA) areas were obtained by drawing round the nuclear and cell boundaries using the digital cursor (Figures 1-4).

Outcome variables are
(a) Mean Nuclear area/50 cells
(b) Mean cytoplasmic area/50 cells
(c) Mean nuclear and cytoplasmic ratio.

Statistical Analysis

All data were tabulated and statistical tests were performed using SPSS. Significant statistical differences between groups were examined using t-test for equality of means. Differences were considered statistically significant when $P < 0.05$.

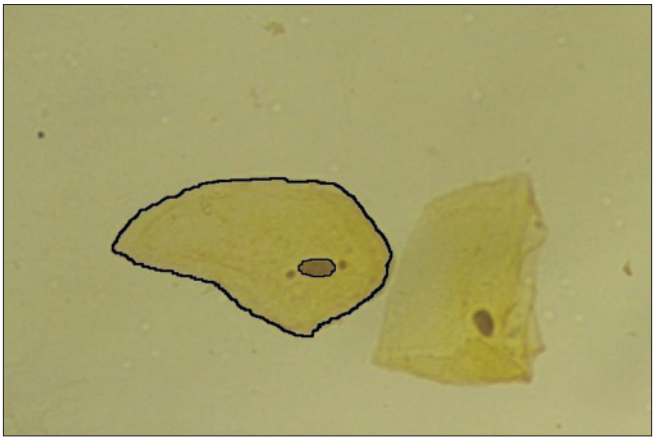

Figure 1: Cellular and Nuclear morphometric analysis ofexfoliated squamous epithelial cell in buccal smears of smokers using Image J v 1.45 image analysis software

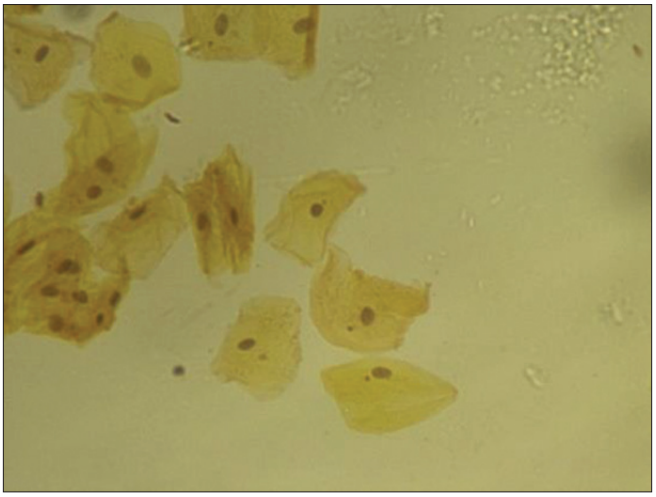

Figure 2: Only clearly defined cells were measured, excluding the clumped or folded cells and unusually distorted nuclei and cells

Figure 3: Armamentarium for PAP staining

RESULTS

The age range of subjects taken was 40-60 years with a mean age for smokers was 46.4 ± 4.9 years; the mean of non-smokers was 48.1± 5.7 years.

Table 1 contains the mean values for nuclear area (NA) and cytoplasmic area (CA) and N/C ratio in smoker and control group. Using a two sample "t-test" for independent samples the mean NA for smokers was significantly elevated compared with the mean NA for non-smokers. Mean CA in smokers was decreased as compared to non-smokers but the difference was not significant. Also, N/C ratio was significantly elevated in smokers group.

Table 2 depicts cytomorphometric analysis of smokers based upon duration of exposure. Table shows that with increasing

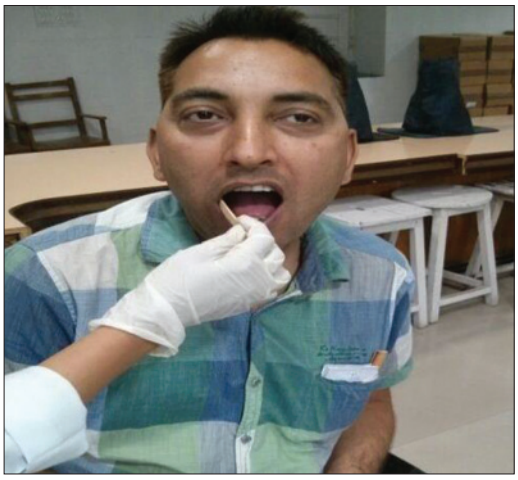

Figure 4: Procedure for taking buccal mucosa smear by scraping

Table 1: Cytomorphometric analysis of the buccal mucosa of smokers and controls

Variable	Smokers	Non-smokers
Mean nuclear area	72.15	66.43
Mean cytoplasmic area	2366.21	2492.51
Mean nuclear/ mean cytoplasmic ratio (N/C)	0.030	0.027

Table 2: Cytomorphometric analysis of smokers based upon duration of exposure

Variable	Duration of exposure to smoking		
	Group A 5-10 years	Group B 11-20 years	Group C >20 years
Mean nuclear area	69.47	72.43	74.56
Mean cytoplasmic area	2455.54	2389.46	2253.64
Meannuclear/ mean cytoplasmic ratio (N/C)	0.028	0.030	0.033

heavy exposure in duration of years, Cytomorphometric changes shows significant altered values for all three measured parameters (NA, CA and N/C ratio).

DISCUSSION

Few studies in the past appear to have investigated at the effects of smoking tobacco on the oral mucosa, with regard to the use of exfoliative cytology technique. Application of quantitative techniques has largely improved the potential accuracy of exfoliative cytology. Exfoliative cytology is considered a moderate, easy and non-invasive technique compared to conventional anatomopathological examination.

Wrubel & Scopp studied the keratinization of the hard palate and buccal mucosa, following smoking cessation, and found no definite changes. The karyopyknotic index (KI) for smokers was no different from that for non-smokers.[16] In contrast, Brown & Young, who investigated 100 cells from the hard palate and buccal mucosa, found that the Kl for smokers was increased, as compared to non-smokers.[17] Baric et al. who studied the prevalence of oral leukoplakia, found an increasing number of such lesions in those individuals who smoked tobacco. They observed that cigarette and cigar smokers had higher percentage buccal mucosal lesions whereas palatal lesions were more common in pipe smokers.[18] Hirayama in an extensive investigation of oropharyngeal cancers in Central and South East Asia, found a definite relationship between non chewing smokers of tobacco and cancer of the buccal mucosa.[19] In present study, majority of our patients smoked cigarettes. Hence according to the observations of Baric et al.[18] & Hirayama[19] if one were looking for changes in the oral mucosal cells of smokers, one would expect to find them in cells removed from the buccal mucosa. Since present study focuses on normal buccal mucosa, patients with lesions such as epithelial dysplasia, leukoplakia, erythroplakia, and squamous cell carcinoma were not included.

The effect of smoking, as a risk factor for oral malignancies, depends on the number of cigarettes smoked per day and the duration of exposure to smoking. Individuals who have been smoking for 10 years or more, and/or over 2 packs a day are defined as heavy smokers.[20,21] In this study, individuals comprising the study group smoked at least 2 pack a day and had been smoking for at least 5 years.

In present study, samples were taken from those patients, who were all greater than 40 years of age. Cowpe JG et al. showed that there were not any significant variations in NA and CA, after the age of 40 yr.[12] In present study, increase in NA and decreased CA as well as altered N/C ratio would appear to be due to smoking tobacco.

Ogden et al. investigated the effect of smoking on the oral mucosa in individuals over 40 years of age using cytomorphology. They reported a 5% average increase in the NA values of smokers when compared to non-smokers.[22] Goregen M et al. found 16.5% increase in the NA.[23] Similar findings were also reported by Seifi S et al.[24] Our findings are consistent with these studies; however, we observed a 8.6% increase in the NA value of smokers over non-smokers. Also decreased CA was found in our study. This increase in NA and decrease in CA can be attributed to a cellular adaptation that depends on smoking. Decrease in the cellular diameter and increase in the nuclear size are two significant morphological changes that occur in actively proliferating cells.[25]

Various other researchers also studied different parameters by cytomorphometrical analysis. Ramaesh et al. investigated that the nuclear diameter of the oral mucosa cells in cigarette smokers, chewed betel quid, or practiced both these habits, was significantly greater than control group individuals. They also found that the cytoplasmic diameter was significantly smaller than that of the control group individuals.[26] Similarly, Einstein and Sivapathasundraham also analyzed the effect of smoking and betel quid chewing on the oral mucosa and determined an increase in the average value of ND, and a decrease in cytoplasmic diameter values of smokers and individuals with both these habits.

In our study we found that with increasing heavy exposure in duration of years, Cytomorphometric changes shows significant altered values for all three measured parameters (NA, CA and N/C ratio). Hashemipour et al. found similar results.[27] Zimmermann and Zimmermann[14] and Ogden et al.[22] acknowledge the presence of cell alterations related to the number of cigarettes smoked per day and mentions that the number of cigarettes smoked must be considered as a factor. In present study it was found that there is a significant relationship between the duration of smoking and the NA, CA & N/C ratio. A decrease in cellular size and an increase in nuclear size are two important morphologic changes which are attributed to precancerous and cancerous changes. During the transition from the normal tissue to precancerous and cancerous lesions, some cellular changes take place at the molecular level, which can be determined.[28] Franklin and Smite reported that increased nucleus/cytoplasm ratio might be due to changes in the size of the nucleus relative to the size of the cytoplasm and is possibly a reflection of significant changes in the cell at the morphologic level.[29]

Cytological preparations are of established value in the diagnosis of a variety of disorders – local and systemic, neoplastic, infectious, endocrine, genetic, etc. In this study,

all the smears were obtained by liquid-based cytology, a new method of preparing oral and cervical samples for cytological examination. This technique results in slides with high cellularity dispersed in a homogeneous thin layer. Blood, inflammatory cells and mucus are reduced and distributed randomly throughout the slide. The clear background obtained enhances sensitivity and quality of the results.[28,30]

Diagnostic aids in the evaluation of oral mucosal lesions can serve an important role by identifying lesions that need to be biopsied in spite of a "benign" appearance. Early oral cancers and precancerous lesions are often subtle and asymptomatic. In addition, histopathological changes may be present in areas in which there is no clinical evidence of an oral lesion on visual examination alone. Therefore, it is important for the clinician to maintain a high index of suspicion, especially if risk factors such as tobacco use or alcohol abuse is present.[31,32] Consequently, there is an imperative need to develop early diagnostic tests to evaluate the cellular/genotoxic damage caused by smoking tobacco. Exfoliative cytology may aid in this goal.

CONCLUSION

The basic pathogenesis of any cell alteration begins at molecular level and initiates a cascade of reactions that affect entire cell system. That culminates in altered cell morphology. Cytomorphometric analysis can be used regularly to detect these cell alterations. Further, as the acceptance in reliability of measurable value increases, this method can also aid in motivating individuals to withdraw from adverse effects of tobacco smoking. Early diagnosis of oral lesions is an important aspect of health care. It has been shown that smoking may cause various changes in the cells of the oral mucosa, which can be determined by exfoliative cytology. Diagnosis of the underlying pathology is an important step in management of any disease. In early stages, malignancies of oral cavity sometimes demonstrate slow growth and may not be noticed by the patient. In this context, exfoliative cytology can be applied because it is simple, fast, inexpensive, and non-invasive and carries little risk. Currently, use of exfoliative cytology has increased as an adjunct to screening of precancerous lesions and malignancies of the oral cavity.

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